Analysis of influenza A virus reinfection in children in Japan during 1983–91

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SUMMARY

The epidemiology of influenza A in Japan was studied during 1979–91 and viruses isolated from reinfections during 1983–91 were analysed. Of 2963 influenza viruses isolated during this period, 922 and 1006 were influenza A(H1N1) and A(H3N2) viruses respectively; the others were influenza B viruses. Influenza A(H1N1) and A(H3N2) caused 5 and 6 epidemics respectively, most accompanied by antigenic drift. Seventeen reinfections with H1N1 and 17 with H3N2 were detected during our study. The primary and reinfection strains isolated from 7 H1N1 and 10 H3N2 cases were studied by haemagglutination-inhibition, and amino acid and nucleotide sequences of the HA1 region of the haemagglutinin. Most of the primary and reinfection strains were antigenically and genetically similar to the epidemic viruses circulating at that time. However, in 4 out of 10 cases of reinfection with influenza H3N2 virus, reinfection strains were genetically different from the epidemic viruses.

INTRODUCTION

Soon after the appearance of the influenza A/USSR/90/77 (H1N1)-like viruses in 1977, a drift strain, A/Brazil/11/78, appeared in 1978 [1]. Since then antigenic drift occurred continuously. The nucleotide sequences of the haemagglutinin (HA) gene of drift strains such as A/Taiwan/1/86 and A/Yamagata/120/86 which appeared in the spring of 1986 changed drastically, resulting in a change in antigenic structure of the HA [2, 3]. After that, antigenic variants closely related to the A/Yamagata/120/86 virus prevailed until 1991. Analysis of the HA gene revealed that this line of H1N1 virus was derived from an A/Hong Kong/32/83-like virus, a minor antigenic variant isolated in 1983 [4]. On the other hand, since the appearance of Hong Kong influenza A(H3N2) viruses in 1968, an extensive antigenic change occurred in the 1975/76 influenza season when A/Victoria/1/75-like viruses circulated, but thereafter moderate antigenic drift occurred continuously until 1991.

In a previous report, we studied reinfection of children with influenza B and analysed the viruses isolated from the same patients [5]. In the present study, we

compared influenza A viruses isolated from the same study population during the same period.

MATERIALS AND METHODS

Study population

As in our previous studies on influenza B, we studied only those patients who visited hospital with influenza-like illness and whose illness was confirmed by the isolation of influenza A virus. Epidemiological studies were carried out during 1979–91 and analysis of the viruses isolated during 1983–91.

Viruses

The strains and histories of the influenza A viruses analysed in this study are shown in Table 1. They were isolated during 1983–9 (H1N1) or 1985–91 (H3N2) from 7 and 10 children with influenza, respectively. The viruses were isolated in either primary monkey kidney cells or MDCK cells and then grown in MDCK cells at 37 °C.

Haemagglutination-inhibition tests

Haemagglutination-inhibition (HI) tests were performed with post-infection ferret sera treated with receptor-destroying enzyme. Ferret sera and reference strains of influenza A viruses were kindly provided by Dr M. Ishida, National Institute of Health, Japan.

Nucleotide sequencing of the HA genes

The HA genes of the viruses were sequenced by the reverse transcriptase polymerase chain reaction method (RT-PCR) to amplify cDNA and subsequently by an automatic sequencer (Applied Biosystems, Inc., ABI) after the second PCR with tagged primers as described previously [5]. The second PCR product was extracted and electrophoresed, and then the gel band was cut out and eluted. Sequencing was done with an ABI cycle sequencing kit using –21M13 and M13 reverse dye-labelled primers.

Oligonucleotide primers

Two sets of synthetic oligonucleotides covering the HA1 region of the HA gene for influenza A virus H1N1 or H3N2 were used as the primers for the first PCR reactions. The primers for the H1N1 viruses were: 5' > AGCAAAAAGCAGGG-GAAAATA < 3' (sense), 5' > GCTATTTCTGGGGTGAATCT < 3' (antisense), 5' > AAAATGCTTATGTCTCTGTA < 3' (sense), and 5' > TTAATCCGCAGCATAGCCAG < 3' (antisense). These correspond to nucleotide positions 1–20, 727–708, 664–683, and 1175–1156, respectively, numbered according to the positive strand sequence of the HA gene of the A/WSN/33 strain [6]. The primers for the H3N2 viruses were: 5' > AGCAAAAGCAGGGGATAATT < 3' (sense), 5' > TCACGGTTTTACTATTGTCC < 3' (antisense), 5' > GAACAAACCAACCT-ATATGT < 3' (sense), and 5' > GAATTCCTTTTCGATTTGAT < 3' (antisense). These correspond to nucleotide positions 1–20, 692–673, 645–664, and 1274–1255, numbered according to the positive strand sequence of the HA gene of the A/Aichi/2/68(H3N2) strain [7]. Tagged primers used for the second PCR had additional bases, 5' > TGTAACGACGGCCAGT < 3' or 5' > CAGGAAACA-

Table 1. *Influenza A virus strains analysed in the present study*

Case no.*	Strain	Month of isolation	Age of patient	Vaccination history† (vaccine strain)
H1N1 strains				
1 A	A/Kamata/495/83	December	7	No
1 B	A/Kamata/932/88	July	12	Yes (3)
2 A	A/Kamata/504/83	December	7	Yes (1)
2 B	A/Kamata/1034/88	December	12	Yes (3)
3 A	A/Kamata/523/83	December	5	Unknown
3 B	A/Kamata/987/88	December	10	No
4 A	A/Kamata/555/83	December	6	No
4 B	A/Kamata/31/87	January	10	No
5 A	A/Kamata/568/83	December	12	Yes (1)
5 B	A/Kamata/85/87	January	15	No
5 C	A/Kamata/1060/88	December	17	No
6 A	A/Kamata/40/84	February	7	Yes (1)
6 B	A/Kamata/82/87	January	10	Yes (2)
7 A	A/Nagano/984/86	February	10	Yes (2)
7 B	A/Nagano/1654/89	January	12	Yes (3)
H3N2 strains				
1 A	A/Kamata/547/85	November	11	No
1 B	A/Kamata/187/88	February	13	No
2 A	A/Kamata/577/85	November	8	No
2 B	A/Kamata/70/90	January	12	No
3 A	A/Kamata/664/85	December	15	No
3 B	A/Kamata/422/87	November	17	No
4 A	A/Kamata/539/85	December	7	No
4 B	A/Kamata/1224/88	March	9	Yes (4)
5 A	A/Kamata/503/87	December	2	No
5 B	A/Kamata/49/90	January	4	No
6 A	A/Nagano/1221/88	February	10	Yes (4)
6 B	A/Nagano/336/91	February	12	Yes (6)
7 A	A/Nagano/211/88	March	7	No
7 B	A/Nagano/643/90	February	9	Yes (5)
8 A	A/Kamata/316/88	March	9	Yes (4)
8 B	A/Kamata/29/90	January	11	No
9 A	A/Kamata/50/90	January	4	No
9 B	A/Kamata/152/91	February	5	No
10 A	A/Kamata/170/90	February	11	No
10 B	A/Kamata/14/91	January	12	No

* A, primary infection; B and C, reinfection.

† (1) A/Kumamoto/37/79(H1N1); (2) A/Bangkok/10/83(H1N1); (3) A/Yamagata/120/86(H1N1); (4) A/Fukuoka/C29/85(H3N2); (5) A/Sichuan/2/87(H3N2); (6) A/Guizhou/54/89(H3N2).

GCTATGACC < 3' (which correspond to the M13 universal primers), to the 5' end of each sense and antisense first PCR primer, respectively.

RESULTS

Surveillance of influenza A isolates in Japan during 1979–91

Figure 1 shows the detection of influenza A virus by public health laboratories throughout Japan during 1979–91 [8]. During this period, there were 5 epidemics

Fig. 1. Isolation of influenza A virus in Japan during 1979–91. The total number of H1N1 and H3N2 viruses isolated by public health laboratories throughout the country and reported to the National Institute of Health in Japan [8] is plotted. The main epidemic viruses detected in Japan during this period are shown above each epidemic peak.

and 3 sporadic outbreaks caused by influenza H1N1 viruses, and 6 epidemics and 3 sporadic outbreaks caused by H3N2 viruses. The main epidemic influenza H1N1 viruses in the 1979–81, 1983–4, 1986–9, and 1990–1 seasons in Japan were antigenically similar to A/Kumamoto/37/79, A/Bangkok/10/83, A/Yamagata/120/86, and A/Yamagata/32/89, respectively (Fig. 1). On the other hand, the main epidemic H3N2 viruses in the 1979–82, 1982–3, 1985–6, 1987–8, and 1990–1 influenza seasons in Japan were antigenically similar to A/Bangkok/1/79, A/Philippines/2/82, A/Fukuoka/C29/85, A/Sichuan/2/87, and A/Guizhou/54/89, respectively (Fig. 1).

Surveillance of reinfection with type A influenza

Patients who visited the children's hospitals with influenza-like symptoms were followed from 1979 to 1991. Influenza viruses were isolated from throat swabs in embryonated chicken eggs, primary monkey kidney cells, or MDCK cells. The patients were 1–15 years old with occasional exceptions. During this period, we

Table 2. *Haemagglutination-inhibition reactions of H1N1 viruses*

Case no.	Strain	Ferret antisera*			
		Kumamoto/ 79	Bangkok/ 83	Yamagata/ 86	Yamagata/ 89
	A/Kumamoto/37/79†	<u>80</u>	30	< 10	< 10
	A/Bangkok/10/83†	80	<u>160</u>	< 10	< 10
	A/Yamagata/120/86†	< 10	40	<u>640</u>	20
	A/Yamagata/32/89†	10	10	160	<u>120</u>
1 A	A/Kamata/495/83	40	160	< 10	< 10
1 B	A/Kamata/932/88	20	40	320	40
2 A	A/Kamata/504/83	30	160	< 10	< 10
2 B	A/Kamata/1034/88	10	20	160	20
3 A	A/Kamata/523/83	40	160	< 10	< 10
3 B	A/Kamata/987/88	< 10	< 10	120	25
4 A	A/Kamata/555/83	60	240	< 10	< 10
4 B	A/Kamata/31/87	< 10	< 10	80	20
5 A	A/Kamata/568/83	40	240	< 10	< 10
5 B	A/Kamata/85/87	20	20	160	40
5 C	A/Kamata/1060/88	10	80	320	80
6 A	A/Kamata/40/84	25	160	< 10	< 10
6 B	A/Kamata/82/87	10	20	100	25
7 A	A/Nagano/984/86	< 10	10	80	10
7 B	A/Nagano/1654/89	10	40	160	60

* All ferret sera were treated with receptor-destroying enzyme to inactivate non-specific inhibitors. Each titre is the mean of duplicate tests. Homologous titres are underlined.

† Egg-passaged vaccine strains. Other virus strains were isolated in either primary monkey kidney or MDCK cells and passaged in MDCK cells.

isolated a total of 2963 influenza viruses. Of these, 922 (31%) were influenza A(H1N1) and 1006 (34%) were A(H3N2) viruses, and 1035 (35%) were influenza B viruses. During this period, 17 children were each reinfected with influenza A(H1N1) or A(H3N2). Primary and reinfection viruses from these patients were isolated in either primary monkey kidney cells or MDCK cells. One patient was infected with influenza A (H1N1) virus three times during this period, and there were two patients reinfected with H3N2 virus during successive influenza seasons.

Antigenic analysis of H1N1 strains

Antigenic analysis of 15 H1N1 strains isolated from seven children during 1983–9, and which represented primary and reinfection isolates, was done by the HI test. In one child (case 5), reinfection occurred twice during the period studied (Table 1). Six primary strains were isolated in the 1983–4 season and one during the 1985–6. Reinfection strains were isolated in either the 1986–7 or 1988–9 influenza season. The patterns of the HI reaction of these H1N1 strains with ferret antisera raised against vaccine strains in Japan during 1979–89 are shown in Table 2. There was an extensive antigenic difference between A/Bangkok/10/83 and A/Yamagata/120/86 and a minor antigenic difference between A/Kumamoto/37/79 and A/Bangkok/10/83, and A/Yamagata/120/86 and A/Yamagata/32/89. All strains isolated from primary infections in 1983 were antigenically close to A/Bangkok/10/83. Primary and reinfection viruses isolated in the 1986 or

Table 3. *Haemagglutination-inhibition reactions of H3N2 viruses*

Case no.	Strain	Ferret antisera*					
		Philippines/82	Fukuoka/85	Sichuan/87	Guizhou/89	Beijing/89	Brazil/91
	A/Philippines/2/82†	160	10	< 10	40	—	—
	A/Fukuoka/C29/85†	20	<u>40</u>	80	80	—	—
	A/Sichuan/2/87†	< 10	< 10	<u>160</u>	40	—	—
	A/Guizhou/54/89†	< 10	< 10	80	<u>640</u>	10	40
	A/Beijing/352/89†	< 10	< 10	40	40	<u>80</u>	40
	A/Brazil/2/91†	10	< 10	40	40	40	<u>160</u>
1 A	A/Kamata/547/85	< 10	< 10	40	80	—	—
1 B	A/Kamata/187/88	20	40	320	160	—	—
2 A	A/Kamata/577/85	160	10	80	160	—	—
2 B	A/Kamata/70/90	< 10	< 10	80	160	40	20
3 A	A/Kamata/664/85	10	10	80	40	—	—
3 B	A/Kamata/422/87	< 10	< 10	80	80	—	—
4 A	A/Kamata/539/85	< 10	40	160	160	—	—
4 B	A/Kamata/1224/88	20	< 10	80	80	—	—
5 A	A/Kamata/503/87	10	< 10	160	320	—	—
5 B	A/Kamata/49/90	< 10	< 10	160	320	10	20
6 A	A/Nagano/1221/88	< 10	< 10	40	80	—	—
6 B	A/Nagano/336/91	10	< 10	160	80	10	10
7 A	A/Nagano/211/88	< 10	40	80	160	—	—
7 B	A/Nagano/643/90	10	< 10	80	80	10	10
8 A	A/Kamata/316/88	< 10	40	320	80	—	—
8 B	A/Kamata/29/90	< 10	< 10	80	80	10	10
9 A	A/Kamata/50/90	20	< 10	80	160	20	10
9 B	A/Kamata/152/91	< 10	< 10	40	40	80	40
10 A	A/Kamata/170/90	< 10	< 10	160	80	10	40
10 B	A/Kamata/14/91	< 10	< 10	40	40	80	80

* All ferret sera were treated with receptor-destroying enzyme to inactivate non-specific inhibitors. Each titre is the mean of duplicate tests. Homologous titres are underlined.

† Egg-passaged vaccine strains. Other virus strains were isolated and passaged in MDCK cells.

1987–8 influenza season were A/Yamagata/120/86-like except 5C(H1)/88, and 5C(H1)/88 and 7B(H1)/89 were A/Yamagata/32/89-like as judged from the HI results.

Antigenic analysis of H3N2 strains

Antigenic analysis of 20 H3N2 strains isolated during 1985–91, which corresponded to primary and reinfection viruses from ten children, was done by the HI test. In two children (cases 9 and 10), reinfection occurred during successive influenza seasons (Table 1). Of the primary isolates, 4 were detected in 1985–6, 4 in 1987–8, and 2 in 1989–90. Reinfection isolates were detected in the 1987–8, 1989–90, or 1990–1 influenza season. The patterns of the HI reaction of these strains with ferret antisera raised against vaccine strains in Japan during 1982–91 are shown in Table 3. There were considerable antigenic differences between A/Philippines/2/82 and A/Fukuoka/C29/85, and between A/Guizhou/54/89 and A/Beijing/352/89. Although the homologous titre of anti-A/Fukuoka/C29/85 serum was low, primary isolates from 1985 were antigenically different from A/Fukuoka/C29/85, but were close to A/Sichuan/2/87. The 1987–8 strains isolated from patients with either primary infection or reinfection were close to A/Sichuan/2/87. The 1990 strains were similar to either A/Sichuan/2/87 or A/Guizhou/54/89. Two of the 1991 strains, 9B(H3)/91 and 10B(H3)/91, were related to either A/Beijing/352/89 or A/Brazil/2/91, but one strain, 6B(H3)/91, was A/Sichuan/2/87-like.

Amino-acid changes in the 1983–92 HA1 polypeptides of the H1N1 strains

The viruses were placed on the evolutionary tree of the HA1 polypeptide of H1N1 viruses (Fig. 2). The viruses isolated in the 1983–4 influenza season [1A(H1)/83, 2A(H1)/83, 3A(H1)/83, 4A(H1)/83, 5A(H1)/83, and 6A(H1)/83], all of which were isolated from patient with primary infections, were located on the twigs derived from the same branch as A/Dunedin/6/83. On the other hand, the viruses isolated in the 1985/6 influenza season [7A(H1)/86] or thereafter and which were all reinfection viruses were located on branches derived directly from the main stem. These viruses, isolated from patients with either primary or reinfections formed three groups and were located on branches 1–5 mainstream changes away from A/Yamagata/120/86. All strains had a few strain-specific changes. There were no specific amino-acid changes shared by primary and reinfection viruses.

Amino-acid changes in the 1985–92 HA1 polypeptides of the H3N2 strains

H3N2 viruses were placed on the evolutionary tree of the HA1 polypeptide of the H3N2 viruses (Fig. 3). Three strains isolated in the 1985–6 influenza season [1A(H3)/85, 2A(H3)/85, and 3A(H3)/85] were located close together on a branch derived from the same point of the stem as A/Fukuoka/C29/85 or A/Yamanashi/497/85. One 1985–6 [4A(H3)/85] and two 1987–8 [5A(H3)/87 and 4B(H3)/88] strains were located on the stem two mainstream changes away from these viruses, from which the branches of three 1987–8 [3B(H3)/87, 7A(H3)/88, and 8A(H3)/88] and two 1989–90 [2B(H3)/90 and 7B(H3)/90] strains, and A/Sichuan/2/87 were derived. One 1987–8 strain [1B(H3)/88] was located on the stem three mainstream changes away from A/Sichuan/2/87. All viruses isolated

Fig. 2. The evolutionary relationships of the 1983–92 HA1 polypeptide of influenza A(H1N1) strains based on the amino-acid changes of A/USSR/90/77 HA1 polypeptide [9]. Numbers refer to the mainstream amino-acid changes (vertical lines), or strain-specific amino-acid changes on the side branches. The primary infection (⊕) and second (○), and third (●) reinfection viruses are shown together with the reference viruses. The sequences of the reference viruses were taken from references 4, 10, and our unpublished data.

in the 1989–90 and 1990–1 influenza seasons except 6B(H3)/91 were divided into two groups. 9A(H3)/90, 5B(H3)/90, 8B(H3)/90, and 9B(H3)/91 formed one group together with reference strains A/Aichi/1/89 and A/Guizhou/54/89. 10A(H3)/90 and 10B(H3)/91 formed another group together with reference strains A/Beijing/352/89, A/Brazil/2/91, and A/Aichi/2/92 and one 1987–8 strain, 6A(H3)/88. It is not clear at this point which changes in amino acids will be fixed as mainstream changes. In four cases, reinfection viruses [1B(H3)/88, 2B(H3)/90, 7B(H3)/90, and 6B(H3)/91] were genetically different from the epidemic viruses. In two cases, 4A(H3)/85 and 4B(H3)/88, and 7A(H3)/88 and 7B(H3)/90, primary infection and reinfection viruses had the same amino-acid

Fig. 3. The evolutionary relationships of the 1985-92 HA1 polypeptide of influenza A(H3N2) strains based on the amino-acid changes from A/Bangkok/1/79 HA1 polypeptide [11]. Numbers refer to the mainstream amino-acid changes (vertical lines), or strain specific amino-acid changes on the side branches. Primary (⊙) and reinfection (○) viruses are shown together with the reference viruses. The sequences of the reference viruses were taken from references 10, 11, 12, and unpublished data of ours and obtained from Dr Nancy J. Cox. * Denotes the amino acids which changed differently from the preceding ones. ** Denotes the amino acids which returned to those of the Bangkok/1/79 strain.

sequences. In two further cases, 9A(H3)/90 and 9B(H3)/91, and 10A(H3)/90 and 10B(H3)/91, they had closely related amino-acid sequences and were located on the same branches.

DISCUSSION

Reinfection with influenza A(H3N2) virus was investigated during 1975-8 in family studies in Houston, in which two epidemics of influenza A(H3N2) occurred [13]. The study showed that 7 out of 57 (12.3%) young children had second infections, and at least 5 of them (8.8%) accompanied respiratory illness. In our study, we followed only those children who visited hospital with influenza-like illness and from whom influenza A viruses were isolated. During 1979-91, five epidemics were caused by influenza A(H1N1) and six by A(H3N2) viruses.

Seventeen out of 904 (1·9%) and 17 out of 989 (1·7%) children had reinfections with influenza A(H1N1) and A(H3N2) viruses, respectively. These reinfection rates were lower than the 3·7% detected in our study of influenza B in the same population during the same period [5]. However, 16 out of 249 (6·4%) children initially infected with A/Kumamoto/37/79(H1N1)-like or A/Bangkok/10/83(H1N1)-like viruses had reinfections after 1986, and because our study detected the minimum clinical rate, more children would be reinfected. This may be attributed to extensive antigenic differences between these viruses and the viruses isolated in 1986 or later, although the frequency of reinfection with influenza B was similar regardless of antigenic differences [5]. Among seven children who had reinfections with H1N1 virus, five had been vaccinated (Table 1). Three of them were reinfected with virus antigenically close to that used for vaccination in 1989. Among 10 children reinfected with H3N2 virus, four had been vaccinated. In these cases, there were slight antigenic differences between vaccine and epidemic viruses. However, it is obvious that vaccination failed to prevent the illness in these children.

Surveillance of influenza A virus during 1979–91 showed that the size of epidemics did not necessarily relate to the extent of antigenic drift. For example, a large antigenic drift occurred in influenza H1N1 in 1986, but the size of subsequent epidemics was not noticeably affected.

In constructing the evolutionary tree based on the amino-acid changes in the influenza H3N2 viruses, amino acid substitutions occurred at positions 156, 186, and 193 are involved in egg adaptation of influenza A(H3N2) viruses [14, 15] and the tree shown in Figure 3 takes account of the changes caused by egg adaptation.

A/Yamanashi/497/85(H3N2), which co-circulated during the 1985–6 season in Japan at the same time as A/Fukuoka/C29/85(H3N2), had the same HI reaction pattern as A/Sichuan/2/87(H3N2) (unpublished data). The primary infection H3N2 viruses [1A(H3)/85, 2A(H3)/85, 3A(H3)/85, and 4A(H3)/85] which were isolated in 1985 and antigenically similar to A/Sichuan/2/87(H3N2) might be A/Yamanashi/497/85(H3N2)-like viruses. In the present study, primary and reinfection H1N1 viruses were closely related to the epidemic viruses at that time. On the other hand, the H3N2 viruses isolated in the same influenza season were heterogeneous. Furthermore, in 4 out of 10 cases (cases 4, 7, 9, and 10), primary and reinfection viruses had the same or closely related amino-acid sequences. In two patients who had primary and reinfection in the 1989–90 and 1990–1 influenza seasons (cases 9 and 10), primary infection viruses were antigenically A/Guizhou/54/89(H3N2)-like and reinfection viruses were either A/Beijing/352/89(H3N2) or A/Brazil/2/91(H3N2)-like which were antigenically different from A/Guizhou/54/89(H3N2). Therefore, it is reasonable to think that these patients were infected with the influenza A(H3N2) viruses epidemic at that time. With cases 4 and 7, in which primary and reinfection viruses had the same amino-acid sequences despite isolation in 3 or 2 separate years, repeat analysis gave the same results. One reinfection virus [6B(H3)/91] had the same amino-acid sequence as A/Yamanashi/497/85(H3N2). This was supported by the HI reaction pattern. These results suggested that the patients were infected with H3N2 virus which survived in some way for 2–5 years. Hope-Simpson proposed that outbreaks of influenza were initiated by the reactivation of latent virus [16]. Our result with

H3N2 isolates may support his hypothesis. However, our previous study showed that most epidemic strains of H1N1 virus were imported [17], or that an H3N2 strain which was not observed off-season in Japan became an epidemic [10], which also suggested the importation of epidemic strain.

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