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Reinfection with influenza B virus in children: analysis of the reinfection influenza B viruses

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SUMMARY

Influenza B virus reinfection in Japanese children was studied epidemiologically during 1979–91 and virologically during 1985–91. During this investigation, there were four epidemics caused by influenza B viruses, each of which accompanied antigenic drift. Between the epidemics in 1987/88 and 1989/90, the viruses changed drastically, both genetically and antigenically. The minimum rate of reinfection with influenza B virus during the whole period was 3–25% depending on the influenza seasons. The antigens of primary and reinfection strains of influenza B virus isolated from 18 children during 1985–90, which covered three epidemic periods, were studied by haemagglutination inhibition tests. The results showed that the viruses isolated in the 1984/85 and 1987/88 influenza seasons, which belonged to the same lineage, were antigenically close, and reinfection occurred with these viruses. The results of amino-acid analysis of the HA1 polypeptide of these viruses corresponded with those of antigenic analysis. There were no specific amino-acid changes shared by the primary infection and reinfection influenza B viruses; the patients were infected with the viruses epidemic at that time.

INTRODUCTION

Since the first isolation of human influenza A(H1N1) virus in 1933, three subtypes of type A influenza virus and also type B influenza virus have caused human epidemics. These viruses differ drastically in their immunological structure and so repeated influenza infections caused by different types or subtypes of influenza viruses may occur three or four times in one individual's lifetime. In addition to the major antigenic changes (antigenic shift) which occur only in type A influenza virus, influenza virus also undergoes less drastic antigenic changes (antigenic drift) which also enable the changed viruses to evade neutralizing antibody. Reinfection with the same type or subtype of influenza virus has been reported [1–3], although the definition of reinfection is not consistent. This causes

problems when immune response and efficacy of vaccine are assessed. Most reports of influenza reinfection were based on serological or clinical surveillance and little is known about the viruses responsible for reinfection. Recent advances in molecular genetics make it possible to combine the results of virology with classical epidemiology.

Influenza caused by influenza B viruses occurs every 2–3 years in Japan. Illness is determined by both host and virus, but the immune state of the patient is difficult for us to study because of difficulty in obtaining the sera systematically. The most important virus characteristic is the antigenic structure of the haemagglutinin (HA). Analysis of the HA genes of influenza B virus has revealed the existence of two co-circulating evolutionary lineages [4–7]. The epidemic viruses in Japan isolated during the 1980/81, 1989/90, and 1990/91 influenza seasons belonged to one lineage, while those isolated in the 1984/85 and 1987/88 influenza seasons belonged to another lineage [5, 7]. The influenza B viruses which were isolated between 1987 and 1991 and belonged to different lineages did not cross-react at all antigenically [7, 8]. Although the two evolutionary lineages diverged after 1973 [6, 7], antigenic crossing in haemagglutination inhibition (HI) test was observed between the viruses isolated in 1979–82 and 1984–8, which were located on different evolutionary lineages [7].

Here, we analysed antigenetically the influenza B viruses isolated from reinfected patients to determine the difference necessary for reinfection. We also studied the difference in amino acids in the HA1 polypeptide between reinfection influenza B viruses and influenza B viruses epidemic at that time.

MATERIALS AND METHODS

Viruses

The strains and passage histories of the influenza B viruses analysed in this study are shown in Table 1. They were isolated during 1985–90 from 18 children with influenza-like illness at three clinics in Tokyo and Nagano prefecture. The viruses were grown in MDCK cells at 34 °C.

Haemagglutination inhibition tests

Haemagglutination inhibition tests were performed with post-infection ferret sera treated with receptor-destroying enzyme according to the procedure described by Dowdle and colleagues [9]. Ferret sera and reference strains of influenza B virus 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 polymerase chain reaction (PCR) method to amplify the cDNA and subsequently by an automatic sequencer (Applied Biosystems, Inc., ABI) after the second PCR with tagged primers. Briefly, the second PCR was carried out in 100 μl of reaction mixture including 1 μl of the first PCR product, 5 units of Taq DNA polymerase, 10 μl of 10 × Taq buffer, and 5 μl each of 20 μM tagged primers. The second PCR product was extracted with 100 μl of phenol/chloroform (1 : 1) and electrophoresed with 1 %

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

Case no.*	Strain	Month of isolation	Age of patient	Vaccination history
1A	B/Nagano/314/85	January	12	No
1B	B/Nagano/1227/88	March	15	No
2A	B/Kamata/36/85	January	7	Yes
2B	B/Kamata/391/88	April	10	No
3A	B/Kamata/48/85	January	8	Yes
3B	B/Kamata/69/88	January	11	No
4A	B/Kamata/78/85	January	8	Yes
4B	B/Kamata/150/88	February	11	Yes
5A	B/Kamata/98/85	February	8	Yes
5B	B/Kamata/76/88	January	11	Yes
6A	B/Kamata/124/85	February	6	Yes
6B	B/Kamata/322/88	March	9	Yes
7A	B/Kamata/157/85	February	6	No
7B	B/Kamata/82/88	January	9	Yes
8A	B/Kamata/111/85	February	9	Yes
8B	B/Kamata/111/90	January	14	No
9A	B/Kamata/201/85	February	2	No
9B	B/Kamata/277/90	February	7	No
10A	B/Nagano/130/88	February	7	Yes
10B	B/Nagano/776/90	March	9	No
11A	B/Nagano/141/88	February	10	No
11B	B/Nagano/778/90	March	12	No
12A	B/Nagano/1211/88	February	8	No
12B	B/Nagano/117/90	March	10	No
13A	B/Kamata/107/88	February	7	No
13B	B/Kamata/223/90	February	9	No
14A	B/Kamata/131/88	February	7	Unknown
14B	B/Kamata/299/90	March	9	Unknown
15A	B/Kamata/218/88	February	3	No
15B	B/Kamata/209/90	February	5	No
16A	B/Kamata/236/88	February	5	No
16B	B/Kamata/162/90	February	7	No
17A	B/Kamata/280/88	March	3	No
17B	B/Kamata/193/90	February	5	No
18A	B/Kamata/317/88	March	8	No
18B	B/Kamata/105/90	January	9	No

* A, primary infection; B, reinfection.

agarose gel in Tris-acetate-buffer (40 mM, pH 8·1). The gel band was cut out and then eluted by using Easytrap (Takara Co., Japan). The DNA was suspended in 20 μl of 10 mM Tris HCl/1 mM EDTA, pH 8·0. 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 were used as the first PCR primers. These were: 5′ > CAGAAGCGTTGCATTTT-CTAA < 3′ (sense), 5′ > CAGTAATTCGGTCTTCTCCTTT < 3′ (antisense), 5′ > ACCATACATTTGTACAAAAG < 3′ (sense), and 5′ > TTAAGGTCTGCTGCCAC-TGC < 3′ (antisense), which correspond to nucleotide positions 3–23, 643–633, 606–625, and 1232–1213, respectively, numbered according to the positive strand

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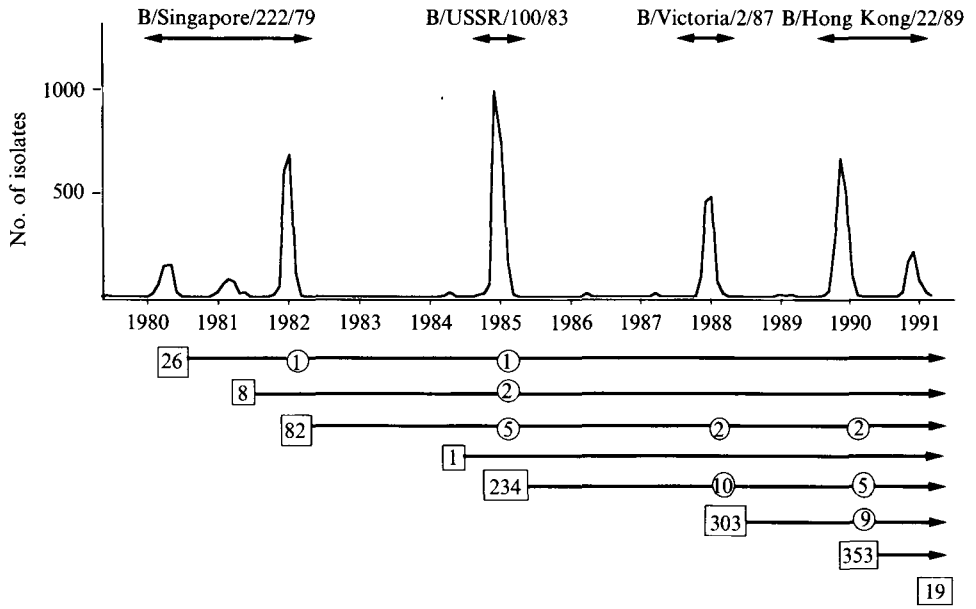

Fig. 1. Chronological sequence of isolation of influenza B viruses and the number of cases of reinfection with influenza B virus. The numbers of influenza B viruses isolated by public health laboratories throughout Japan and reported to the National Institute of Health in Japan [11] are shown in the upper part of the figure and the numbers of influenza B virus studied by us are shown in the lower part. The number of cases of primary infection are boxed, those of reinfection are circled. The main epidemic viruses detected during this period are shown above each epidemic peak.
Fig. 2. For legend see opposite.

sequence of the HA gene of the B/Lee/40 strain [10]. Tagged primers used for the second PCR had additional bases, 5′ > TGTAAAACGACGGCCAGT < 3′ and 5′ > CAGGAAACAGCTATGACC < 3′ to the 5′ end of each sense and antisense first PCR primer, respectively.

RESULTS

Influenza surveillance in Japan during 1979–91

Fig. 1 shows the chronological surveillance of influenza B viruses isolated by public health laboratories throughout Japan during 1979–91 [11]. In the 12 influenza seasons during this period, there were 4 epidemics and 3 sporadic outbreaks caused by influenza B viruses. The main epidemic influenza B viruses in the 1979–82, 1984/85, 1987/88, and 1989–91 influenza seasons in Japan were antigenically similar to B/Singapore/222/79, B/USSR/100/83, B/Victoria/2/87, and B/Hong Kong/22/89, respectively.

Surveillance of reinfection with influenza B viruses

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, or primary monkey kidney, or MDCK cells. The

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Table 2. *Haemagglutination inhibition reactions of influenza B viruses*

Case no.	Strain	Ferret antisera*			
		Ibaraki/85	Nagasaki/87	Yamagata/88	Hong Kong/89
	B/Ibaraki/2/85†	*160*‡	60	< 10	< 10
1A	B/Nagano/324/85	30	30	< 10	< 10
2A	B/Kamata/36/85	30	60	< 10	< 10
3A	B/Kamata/48/85	100	100	< 10	< 10
4A	B/Kamata/78/85	25	25	< 10	< 10
5A	B/Kamata/98/85	20	40	< 10	< 10
6A	B/Kamata/124/85	60	100	< 10	< 10
7A	B/Kamata/157/85	30	30	< 10	< 10
8A	B/Kamata/111/85	15	30	< 10	< 10
9A	B/Kamata/201/85	40	40	< 10	< 10
	B/Nagasaki/3/87†	20	*40*	< 10	< 10
1B	B/Nagano/227/88	15	50	< 10	< 10
2B	B/Kamata/391/88	25	50	< 10	< 10
3B	B/Kamata/69/88	20	40	< 10	< 10
4B	B/Kamata/150/88	20	45	< 10	< 10
5B	B/Kamata/76/88	20	40	< 10	< 10
6B	B/Kamata/322/88	10	30	< 10	< 10
7B	B/Kamata/82/88	20	30	< 10	< 10
10A	B/Kamata/130/88	80	160	< 10	< 10
11A	B/Nagano/141/88	80	90	< 10	< 10
12A	B/Nagano/211/88	45	60	< 10	< 10
13A	B/Kamata/107/88	30	80	< 10	< 10
14A	B/Kamata/131/88	20	40	< 10	< 10
15A	B/Kamata/218/88	20	60	< 10	< 10
16A	B/Kamata/236/88	30	80	< 10	< 10
17A	B/Kamata/280/88	20	60	< 10	< 10
18A	B/Kamata/317/88	25	50	< 10	< 10
	B/Yamagata/16/88†	30	20	*480*	80
	B/Hong Kong/22/89†	< 10	< 10	< 10	*80*
8B	B/Kamata/111/90	< 10	10	60	60
9B	B/Kamata/277/90	10	25	80	60
10B	B/Nagano/776/90	< 10	15	80	60
11B	B/Nagano/778/90	< 10	15	80	60
12B	B/Nagano/117/90	< 10	10	80	80
13B	B/Kamata/223/90	< 10	15	60	80
14B	B/Kamata/299/90	< 10	25	60	60
15B	B/Kamata/209/90	< 10	< 10	40	40
16B	B/Kamata/162/90	< 10	20	60	60
17B	B/Kamata/193/90	10	25	120	100
18B	B/Kamata/105/90	< 10	15	60	60

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

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

‡ Homologous titres are in italics.

patients were 1–15 years old with only occasional exceptions. During this period, we isolated 2963 influenza viruses; of these, 1034 (34·9%) were influenza B. The rate of reinfection with influenza B viruses in children differed with influenza seasons the patients had experienced, and ranged from 3 to 25%.

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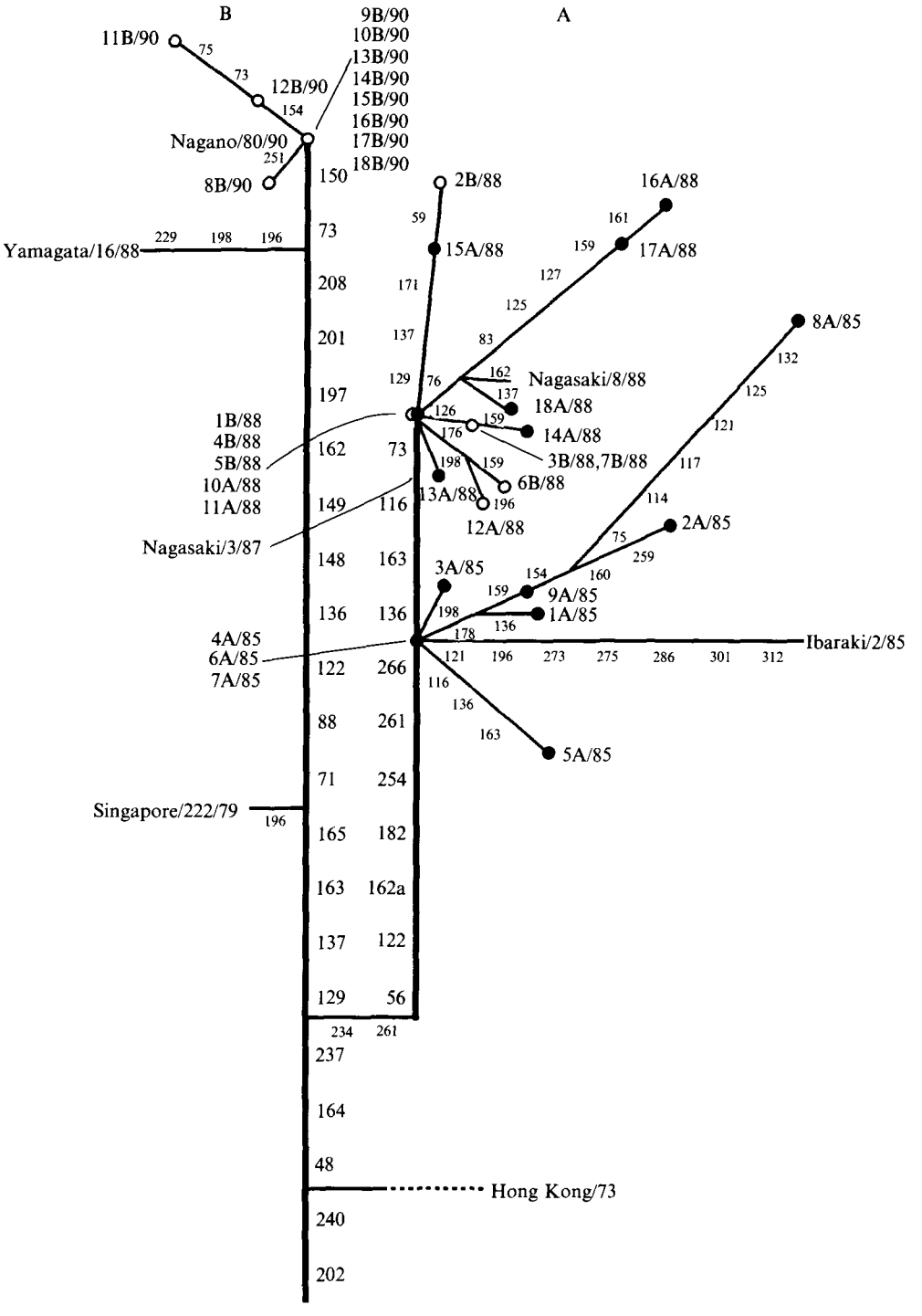

Fig. 2. For legend see opposite.

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Antigenic analysis of influenza B viruses

Antigenic analysis of 36 isolates of influenza B virus from 18 children and which corresponded to primary infection and reinfection viruses was done by HI tests. Among the primary infections, nine strains each were isolated either in the 1984/85 or 1987/88 influenza season. Seven and 11 reinfection strains were isolated in the 1987/88 and 1989/90 influenza seasons, respectively. The patterns of the HI reaction of these influenza B viruses to ferret antisera raised against vaccine strains in Japan during 1985–90 are shown in Table 2. Although the HI titres of anti-B/Ibaraki/2/85 and anti-B/Nagasaki/3/87 were low even with the homologous viruses, the 1984/85 and 1987/88 viruses were antigenically close. However, these viruses and the 1988/89 or 1989/90 viruses were distinct antigenically. All 1989/90 viruses gave HI titres more than four times lower than that of the homologous virus with anti-B/Yamagata/16/88, while they reacted with anti-B/Hong Kong/22/89 as well as the homologous virus. In this study, unlike the other 1989/90 viruses, B/Hong Kong/22/89 reacted only with the homologous ferret serum.

Amino-acid changes in the 1985–90 HA1 polypeptides of the influenza B strains

Amino-acid sequences of the HA1 polypeptide of the influenza B viruses were deduced from their nucleotide sequences. Influenza B viruses analysed in the present study were divided into three groups on the evolutionary tree of the HA1 polypeptide of influenza B virus in accordance with the influenza season they were isolated (Fig. 2). All viruses isolated in the 1984/85 and 1987/88 influenza seasons were located on one lineage (A), while the viruses isolated in the 1989/90 influenza season were located on another lineage (B). The viruses isolated in the same influenza season were located on the branches derived from the same point of the stems. The 1984/85 strains (group 1) had up to nine strain-specific changes. Among them, the amino-acid changes at residues 178, 159, and 154 were shared by a few strains. The 1987/88 strains had up to six strain-specific changes, and the amino-acid change at residue 76 was shared by several strains. Between the strains of 1984/85 and 1987/88 (group 2), there existed four mainstream changes (136, 163, 116, and 73). The 1989/90 strains (group 3) had two mainstream changes (73 and 150) from the B/Yamagata/16/88 virus and had a few strain-specific amino-acid changes. As deduced from the antigenic analysis, none of the 1987/88 viruses studied here was B/Yamagata/16/88-like. There were no specific amino-acid changes shared by the primary infection and reinfection viruses; the patients were infected with the viruses epidemic at that time.

Fig. 2. The evolutionary relationships of the 1985–90 HA1 polypeptide of influenza type B strains based on the amino-acid changes from B/Singapore/64 HA1 polypeptide [4]. Numbers refer to the mainstream amino-acid changes which have become fixed in most of the subsequent strains (vertical lines), or strain-specific amino-acid changes on the side-branches. Strain-specific changes are shown only after B/Singapore/222/79 in the figure. The primary infection (●) and reinfection (○) viruses are shown together with the reference viruses. The sequences of the reference viruses were taken from references 4, 5, and 7.

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DISCUSSION

Reinfection with influenza B virus has been investigated during 1976–84 in family studies in Houston [3]. The study showed that 25% of children but only 2% of adults had a second infection. Because ours was not a family study, there are several possible reasons why we detected a lower frequency of reinfection. For example, most of the patients who visit children's hospitals in Japan are up to 15 years old and after that we cannot follow them; we followed only those children who visited the hospitals with influenza-like illness and from whom influenza B virus was isolated; it is possible that the patients will move and become lost to follow-up. Furthermore, the rate will differ according to the influenza seasons the patients had experienced. In our study, attempts were made to isolate influenza viruses from all the patients who visited the hospitals. From 1979 to 1991, 1034 influenza B viruses were isolated in three hospitals. Although the total data in this study are shown (Fig. 1), there were considerable differences in the frequency of reinfection between the hospitals. In one hospital, the minimum rates of reinfection were 5·0–25·0%, while they were 1·6–4·6% in another hospital.

The evolutionary tree of the influenza B viruses showed two lineages which diverged after 1973 [6, 7]. Among the representative drift strains during this period, B/Singapore/222/79, B/Yamagata/16/88, and B/Hong Kong/22/89 belonged to lineage B, while B/Ibaraki/2/85 and B/Victoria/2/87 belonged to lineage A [5]. However, these two lineages had several amino-acid changes in common [7]. The antigenic analysis showed that B/Singapore/222/79 (lineage B), and B/Ibaraki/2/85 or B/Victoria/2/87 (lineage A) crossed antigenically, but these three strains and B/Yamagata/16/88 or B/Hong Kong/22/89 (lineage B) did not [5,7]. In 18 cases of reinfection analysed here, reinfection with the same and different lineages occurred in 7 and 11 cases, respectively. The existence of viruses which do not cross-react at all antigenically was presumed to increase the chance of reinfection with influenza B virus. The influenza B viruses isolated in 1984/85 and 1987/88 were close antigenically except B/Ibaraki/2/85. Of 234 persons who were infected in the 1984/85 influenza season, 10 (4.3%) were reinfected in the 1987/88 influenza season. This number is comparable with that (3%) of those who were infected in the 1987/88 influenza season and reinfected in the 1989/90 influenza season. This indicates that reinfection with influenza B virus occurred between antigenically close viruses. However, no cases of reinfection in the same influenza season were observed in this study.

The HA1 polypeptides of influenza B viruses were plotted on the evolutionary tree of influenza B virus. The three-dimensional structure of the HA molecule of the influenza B virus has not been determined. However, most of the important structural features of the type A influenza virus HAs are conserved in the influenza B virus HA [12]. Most of the amino-acid changes shown in Fig. 2 are located on the globular head of the HA molecule. The B/Ibaraki/2/85 virus, which was used as a vaccine strain in Japan, has seven strain-specific changes (121, 196, 273, 275, 286, 301, and 312), and antigenically four-fold or greater differences from most other viruses of lineage A. The primary infection viruses (1A–7A) and reinfection viruses (1B–7B) of lineage A were close antigenically. Between them, there were four mainstream amino-acid changes (73, 116, 136, and 163). Each of

these viruses has only a few strain-specific changes except 2A (five amino-acid changes) and 2B (four amino-acid changes), which were not shared between primary infection and reinfection viruses. Reinfection influenza B viruses did not have specific amino-acid differences from the epidemic viruses. Because the influenza B viruses isolated in the same influenza seasons were close antigenically, most of the strain-specific changes may not affect the antigenic structures even though the changes were located on the antigenic sites of the HA molecule [13].

A number of vaccine strains of influenza B virus have been used in Japan since 1962. However, influenza control through vaccination was unsuccessful [14], and the vaccination policy for students was changed from mass to individual in 1986. Recent studies involving antigenic and genetic analysis of epidemic viruses suggest that antigenic variation of influenza B virus is a major obstacle to effective control of influenza by vaccination [5]. In the present study, among seven children who had primary infections in 1985 and reinfections in 1988, four were vaccinated. The occurrence of reinfection with antigenically close influenza B viruses in children even after natural infection may partly explain the relative inefficiency of the vaccine [15]. The lowered antibody response may play a part in the reinfection with influenza B virus, because reinfection in the same influenza season was not observed in the present study.

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