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Amino-acid change on the antigenic region B1 of H3 haemagglutinin may be a trigger for the emergence of drift strain of influenza A virus

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SUMMARY

Sera from 27 children and eight older persons, which had been collected in 1998 and 1999 and showed haemagglutination–inhibition (HI) activity against influenza A/Sydney/5/97 (H3N2) strain, were characterized with a binding assay using chimeric haemagglutinin (HA) proteins between A/Aichi/2/68 (A/AI/68) and A/Sydney/5/97 (A/SD/97) strains. Sera from the young children had a tendency to recognize only the antigenic site B1 of the HA1 region. On the other hand, sera of the older individuals were fully reactive to all antigenic sites of HA1 except antigenic site D. Recent epidemic strains, A/Panama/2007/99 (A/PM/99)-like viruses have differences in amino acids in antigenic sites A, C, and B2 but not B1. However, human antisera obtained even from young children had HI activity to Panama-like viruses. The limited epidemic of A/PM/99-like viruses may have been due to the existence of antibody against B1, which had been produced in response to infection by the A/SD/97-like viruses.

INTRODUCTION

Influenza is an acute infectious respiratory disease caused by influenza viruses. As yet, influenza cannot be controlled by vaccines and epidemics of influenza occur every winter. These epidemics are thought to be caused by the influenza viruses that have escaped from host defence mechanisms of acquired immunity by altering the antigenicity of the haemagglutinin (HA) protein. From analyses of natural and laboratory-selected antigenic variants, 4–5 antigenic sites on the HA molecule have been identified with H3N2 [1, 2] and H1N1 [3, 4] viruses. Mutants which escape neutralization by a single monoclonal antibody (mAb) can be obtained at a frequency of 10^{-4} to 10^{-6} [5–7] and usually cannot be isolated *in vitro* with a mixture of mAbs [8]. Post-infection human sera contain polyclonal antibodies with varying specificity towards the HA protein. Antigenic drift strains have been thought to result from the accumulation of a series of amino-acid changes in antigenically important regions of the HA molecule. Wilson and Cox [9] observed that new drift variants of epidemiological importance generally have four or more amino-acid substitutions located in two or more of the antigenic sites. However, the manner in which amino-acid changes accumulate during passages in human populations remains undetermined. In order to obtain a better understanding of the molecular mechanisms responsible for antigenic drift of influenza viruses in human populations, characterization of human antisera is required. Studies to detect antigenic sites or antigenic variations on the HA protein have

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been carried out frequently using non-human sera [8, 10–12]. However, studies employing human sera have been limited [13, 14].

We previously presented a new method for analysing the human antibodies against influenza H3 subtype viruses using chimeric HA proteins [14]. Convalescent human sera obtained from patients infected with A/Kamata/14/91 (A/KM/91)(H3N2)-like viruses did not bind to the expressed HA protein of the A/AI/68 strain on the COS cells but did bind to that of the A/KM/91 strain. Binding assays with these convalescent sera and chimeric HA proteins between A/AI/68 and A/KM/91 strains showed that each person had a different response to antigenic regions of the HA protein. This result was similar to that obtained with a competition binding assay using mouse mAbs in a study conducted by Wang et al. [13]. We showed that three convalescent (A-1, -2, -3) sera derived from young patients (<5 years) bound only to antigenic site B1. Haaheim [15] reported that the reactivity of sera of young children (0–5 years) to a monoclonal (185/1) variant of A/Texas/1/77(H3N2) strain was reduced, whereas adult sera (⩾15 years) were fully reactive. These results suggested that the emergence of a drift strain might be due to limited antigen recognition by young children. However, since our previous study was conducted on a rather small scale, additional research on a larger group of young children was needed to strengthen our hypothesis. In the present experiments, we increased the number of human sera that had been collected during 1998–1999 for an epidemiological programme prior to immunization with influenza vaccines. Although we did not do a background study on each serum, these specimens may be more or less typical of the general population and we used them to study whether influenza virus infection in young children promotes antigenic drift.

METHODS

Serum

Seventy-four human serum samples, which were obtained in 1998 and 1999 and showed haemagglutination–inhibition (HI) activity to the A/SD/97 virus, were used in these experiments. Ten of the 1998 and six of the 1999 sera, which were negative to A/SD/97, were also used as negative controls. For analysis of reactivity of sera in epitope recognition, we used 35 specimens, which showed an HI titre of more than 40 against A/SD/97.

Construction of chimeric HA cDNAs

Vaccine viruses (A/Sydney/5/97 and A/Panama/2007/99) were obtained from the National Institute of Infectious Diseases, Japan. HA cDNAs of A/AI/68, A/SD/97, and A/PM/99 viruses were cloned and inserted into pME18S expression vector using *Eco*RI and *Xba*I sites [16]. In order to create suitable chimeric HA proteins, *Ear*I sites were inserted into the HA cDNAs of A/AI/68 and A/SD/97 viruses as described previously [14]. Primer sets for creating cleavage sites at amino-acid residues 200, 240, 170, 150 were

(5′ > GCGCTCTTCACAGTCTCTAC(+);
5′ > GCGCTCTTCACTGTGACTCTCCC(−)),

(5′ > GCGCTCTTCGGGGTTACTTC(+);
5′ > GCGCTCTTCACCCCGAGGAGCAATT(−)),

(5′ > GAACTCTTCTGTACATTTGGG(+);
5′ > GAACTCTTCGTACAATTTGTCA(−)),

(5′ > GAACTCTTCATTGAACTGGTTG(+);
5′ > GAACTCTTCTCAATATACTGAA(−)),

respectively. The *Ear*I recognition sites are underlined. For cleavage at amino-acid residue 110, the *Kpn*I site was used. Chimeric HA cDNAs were inserted into the pME18S expression vector. Furthermore, a chimeric HA protein between A/PM/99 and A/AI/68 viruses was constructed at residue 150 using the same primers described above.

Immunofluorescent staining of the HA protein from expressed cDNA

Immunofluorescent staining of the HA protein from expressed cDNA was performed as described previously [16]. For quantitative fluorescein isothiocyanate (FITC) assays, two experiments were carried out in duplicate. The difference in values was not more than twofold and the smaller value was used.

Virus

Inactivated A/SD/97 and A/PM/99 viruses for the HI test described in the text were purchased from Denka Seiken (Tokyo, Japan).

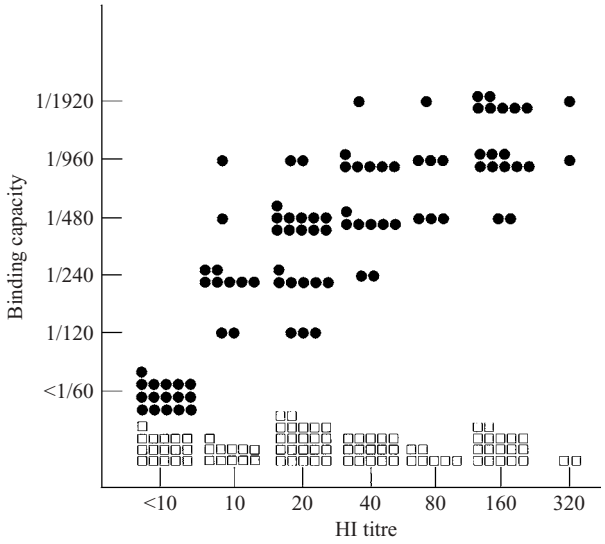

Fig. 1. Relationship between HI titres and binding capacity of human sera against chimeric proteins. Chimeric HA (SD/AI and AI/SD) proteins were expressed in COS cells and stained with diluted human sera and FITC-labelled anti-human goat serum. The vertical axis indicates the binding capacity of the chimeric protein. The horizontal axis shows the HI titre of each serum. The binding capacity was estimated using the highest dilution point. Closed circles and open squares show the binding capacity of each serum to SD/AI and AI/SD, respectively.

RESULTS

HI activity and binding assays

We constructed chimeric HA cDNAs between HA cDNAs of A/AI/68 and A/SD/97 at the *Nde*I site corresponding to amino-acid residue 304. The 68/SD HA cDNA consisted of the HA1 region from A/Aichi/68 HA cDNA and the HA2 region from A/SD/97 HA cDNA, and SD/68 HA cDNA consisted of the HA1 region from A/SD/97 HA cDNA and the HA2 region from A/AI/68 HA cDNA. Both chimeric proteins were expressed in COS cells and showed haemadsorption and fusion activity of the same degree as that of the parental HA proteins (results not shown).

The binding of human sera to these HA proteins was assayed. The capacity to bind to SD/68 and 68/SD HA proteins was measured by FITC staining using limiting dilutions of antisera. The HI titre and binding capacity of each serum against the HA protein of SD/68 was correlated (Fig. 1). However, none of the sera was reactive to 68/SD HA protein. Therefore, it was concluded that the human sera used in this experiment did not contain antibodies to the HA2 region of HA protein.

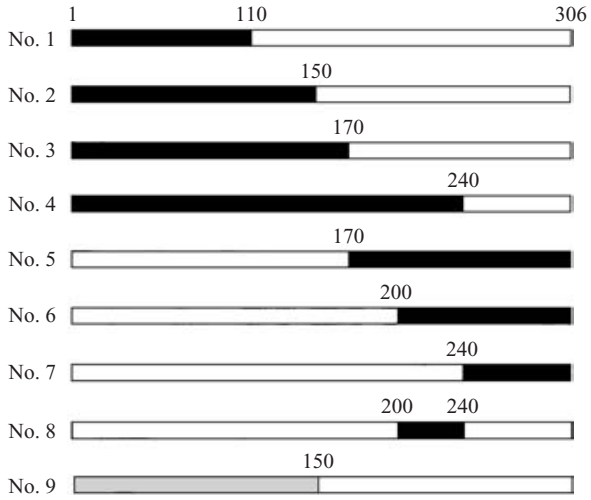

Fig. 2. Structure of the chimeric proteins used in this study. ■, Sydney/05/97; □, Aichi/2/68; ▨, Panama/2007/99.

Further analysis of reactivity of the human sera with the HA1 region of chimeric HA protein

Figure 2 shows the constructed chimeric proteins used in this experiment. All chimeric proteins were detected with FITC staining using mAb no. 35 [14], which could detect the expressed HA proteins of A/AI/68, A/SD/97 and all chimeric HA cDNAs. All 35 human sera used in this experiment had HI titres of 40 or greater to the A/SD/97 virus. Table 1 shows a summary of the binding experiments. We compared the sera of two groups ($\leqslant$11 years and $\geqslant$12 years) with respect to their ability to bind to each chimeric protein. Figure 3(*a*, *b*) shows the binding capacities of these groups. A chimeric protein incorporating the 150–170 amino-acid region of the A/SD/97 HA protein could not be obtained. Therefore, the binding of this region was estimated by comparing to the binding of nos. 2 and 3 chimeric proteins. The sera of children $<$11 years which bound to the 1–110 (no. 1) and 1–150 (no. 2) regions of the A/SD/97 HA protein constituted only 3·7% (1/27) and 22·2% (6/27) respectively, of the children of this group. However, sera which bound to the 1–170 (no. 3) and 1–240 (no. 4) regions of this protein were numerous, at 92·6% (25/27) and 96·1% (25/26) respectively. Only 1 out of 26 (3·8%) sera bound to regions 170–306 (no. 5), 200–306 (no. 6) and 240–306 (no. 7). From these results, it was suggested that the sera of children not more than 11 years old recognized mainly the 150–170 amino-acid region (antigenic B1) of the A/SD/97 HA protein. On the other hand, the sera of individuals who were 12 years or older (Fig. 3*b*) could

Table 1. *Binding capacity of human sera to chimeric HA proteins*

Sample	Age (years)	HI titre (A/Sydney/05/97)	Binding capacity to chimeric HA proteins							
			No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
mAb no. 35*	—	—	3200	3200	3200	3200	3200	3200	3200	3200
1	0	160	<60	<60	240	240	<60	<60	<60	<60
2	2	160	<60	<60	60	120	<60	<60	<60	<60
3	2	80	<60	<60	120	60	<60	<60	<60	<60
4	3	80	<60	<60	<60	120	<60	<60	<60	<60
5	3	160	<60	<60	480	120	<60	<60	<60	<60
6	4	80	<60	60	120	120	<60	<60	<60	<60
7	4	160	<60	<60	60	120	<60	<60	<60	<60
8	4	40	<60	<60	120	240	<60	<60	<60	<60
9	4	160	<60	120	480	960	<60	<60	<60	<60
10	4	160	<60	120	480	480	<60	<60	<60	<60
11	5	160	<60	240	240	480	<60	<60	<60	<60
12	6	320	<60	<60	60	120	<60	<60	<60	<60
13	6	160	—†	<60	960	960	—	—	—	—
14	6	160	—	<60	240	480	<60	<60	<60	—
15	7	40	—	<60	<60	—	<60	<60	<60	—
16	7	40	—	<60	120	<60	<60	<60	<60	—
17	7	80	<60	<60	240	120	<60	<60	<60	<60
18	7	160	<60	<60	240	960	<60	<60	<60	<60
19	8	160	<60	<60	240	480	<60	<60	<60	<60
20	8	80	<60	<60	480	480	<60	<60	<60	<60
21	9	40	—	240	480	480	<60	<60	<60	—
22	9	80	—	<60	120	240	<60	<60	<60	—
23	10	160	<60	<60	60	240	<60	<60	<60	<60
24	10	80	240	240	480	480	240	240	480	<60
25	10	160	<60	<60	480	1920	<60	<60	<60	<60
26	10	40	<60	<60	120	60	<60	<60	<60	<60
27	11	40	—	<60	240	120	<60	<60	<60	—
28	12	80	—	<60	1920	960	<60	<60	<60	—
29	14	160	—	240	960	480	240	240	480	—
30	16	320	—	<60	960	960	<60	<60	<60	<60
31	17	40	120	240	240	—	<60	<60	<60	<60
32	19	160	<60	960	960	240	<60	<60	<60	<60
33	30	40	480	240	480	240	480	960	960	<60
34	31	40	240	120	240	60	60	240	240	<60
35	39	40	240	120	60	60	<60	60	60	<60

Expressed chimeric HAs were stained with each serum and anti-human IgG as described in the Materials and Methods. The binding capacity was established using the highest dilution point.

* Monoclonal antibody.

† —, Not done.

bind to all chimeric proteins except region 200–240 (no. 8), corresponding to antigenic site D of the H3 HA protein.

Binding characterization of human sera directed against A/PM/99 viruses

Figure 4 shows the reported number of patients with influenza-like illness in Japan. After the large epidemic of A/SD/97-like viruses, the number of patients with influenza-like illness decreased rapidly, even after the appearance of A/PM/99-like viruses [17].

The HI test using ferret antiserum to A/SD/97 showed decreased activity against A/PM/99 in 1/16 (Table 2), 1/8 [18] and in 1/4 [19], suggesting that the A/PM/99 virus is an antigenic drift strain. Amino-acid changes in the antigenic determinant sites on

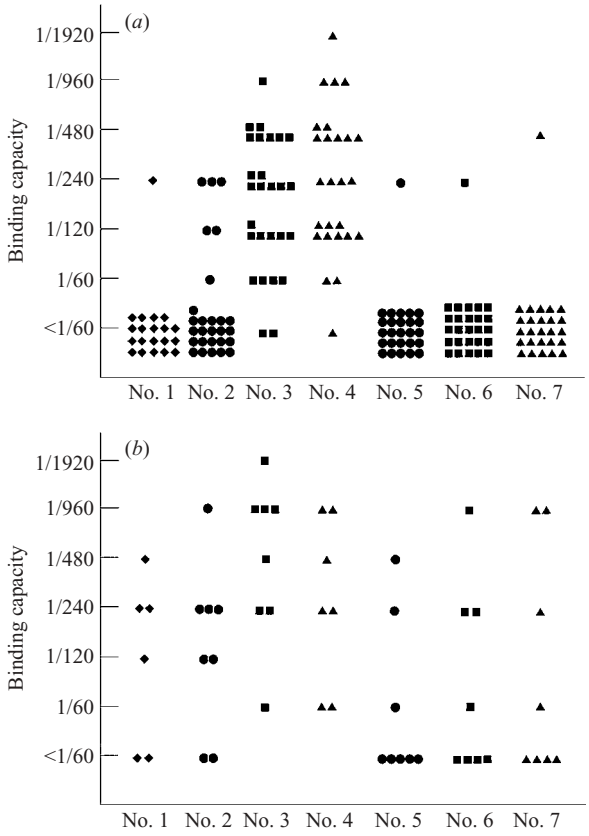

Fig. 3. The capacity of human sera to bind to chimeric protein. Chimeric HA proteins were expressed in COS cells and stained with diluted human sera and FITC-labelled anti-human goat serum. The binding capacity was estimated using highest dilution point. (*a*) Sera of patient $\leqslant$11 years. (*b*) Sera of patients aged $\geqslant$12 years.

the HA protein of the A/PM/99 virus compared to the A/SD/97 virus were observed at residues 57 (site C), 137 (site A), 142 (site A), 144 (site A), 192 (site B2), and 194 (site B2). We constructed a chimeric HA cDNA (no. 9) between A/PM/99 (1–150) and A/AI/68 HA cDNAs and the binding of each serum to chimeric HA protein no. 9 was compared to those between A/SD/97 and A/AI/68 (nos. 1 and 2) (Table 2).

Sample nos. 3, 7, 23 and 25 of young children had no binding ability to antigenic site A of the HA1 of A/SD/97 virus (no. 2). These sera also did not bind to antigenic site A of the HA1 of A/PM/99 virus (no. 9). Therefore the possibility was ruled out that these young children had been infected with A/PM/99-like viruses and had antibody against site A of the HA1 of A/PM/99 virus. Among sera obtained from children $<$11 years, five (sample nos. 6, 9, 10, 11 and 24) had the ability to bind to antigenic site A of A/SD/97 (no. 2). However, all sera lost the capacity to bind to antigenic site A of A/PM/99 HA (no. 9). Therefore, these young children had also been infected with A/SD/97-like viruses. As for the older individuals, sample no. 32 lost binding activity towards chimeric protein no. 9, but most sera did not. The latter sera had antibodies against the 1–110 region of HA1 of A/SD/97 HA (no. 1). Because amino acids in the antigenic site C/E of A/PM/99 are similar to those of A/SD/97, except the amino acid at residue 57, the binding of these samples to region 1–150 of A/PM/99 HA might result from binding to the 1–110 amino-acid region of HA of A/SD/97. However, we could not exclude the possibility that these individuals had acquired anti-Panama antibodies by infection with A/PM/99-like viruses. Another amino-acid difference between A/SD/97 and A/PM/99 in antigenic regions was at residues 192 and 194 in site B2. We did not analyse the effect of these amino-acid changes on the binding activity of human sera in this experiment, because sera from children not more than 11 years old did not have antibodies towards site B2. As yet, no amino-acid change in region B1 has been found in A/PM/99 or in the currently circulating viruses of the 2001/2002 season in Aichi Prefecture in Japan (results not shown). From HI assays of these sera to study reactivity to the A/PM/99 virus, all sera had HI titres that were similar to those seen with the A/SD/97 virus (Table 2) (except sample no. 32).

DISCUSSION

Our study material consisted of 74 human sera that had been collected from June to October of 1998 and 1999 and showed HI activity to the A/SD/97 virus. These sera did not recognize the HA protein of the A/AI/68 strain but did recognize that of the A/SD/97 strain. Binding assays to study reactivity of these sera to chimeric proteins between A/AI/68 and A/SD/97 strains revealed that these sera did not contain antibodies that bound to the HA2 region. Our results confirmed the observation of Cox and Brokstad [20], who indicated that after vaccination, antibodies were detected to subunit HA1 but hardly any to HA2, as determined by a radio-immunoblot assay. However controversial results were also reported by Styk et al. [21] and Qiu et al. [22] who reported that human convalescent sera showed a higher titre in binding to ^{125}I-labelled HA2 than to ^{125}I-labelled HA1. We could not explain this discrepancy. The use of mAbs against the HA2 region (mAb nos. 66 and 116) revealed that

Table 2. *Binding capacity of human sera to chimeric HA protein with A/Panama/2007/99*

Sample		HI value			Binding capacity to chimeric HA proteins		
		Age (yr)	(A/SD/97)	(A/PM/99)	No. 1	No. 2	No. 9
Ferret sera	A/SD/97	—	640	40–80	—	—	—
	A/PM/99	—	640	320	—	—	—
	3	2	80	80	<60	<60	<60
	5	3	160	80	<60	<60	60
	7	4	160	80	<60	<60	<60
	23	10	160	80	<60	<60	<60
	25	10	160	80	<60	<60	<60
	6	4	80	40	<60	60	<60
	9	4	160	80	<60	120	<60
	10	4	160	160	<60	120	<60
	11	5	160	80	<60	240	<60
	24	10	80	20	240	240	<60
	30	16	320	80	<60	<60	<60
	31	17	40	20	120	240	120
	32	19	160	10	<60	960	<60
	33	30	40	20	480	240	480
	34	31	40	20	240	120	240
	35	39	40	20	240	120	60

Ferret sera were obtained from the Institute of Infectious Diseases in Japan.

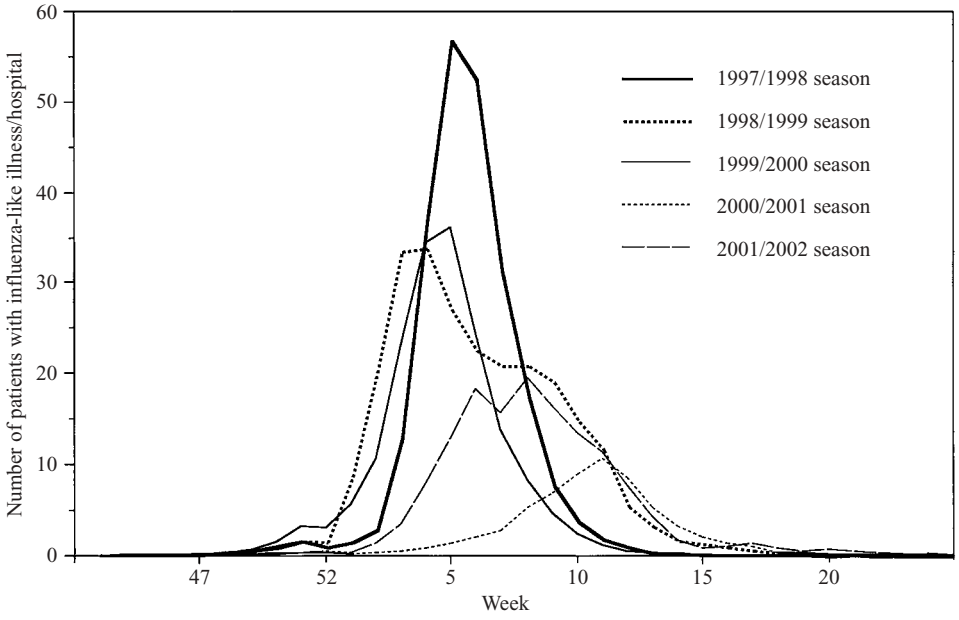

Fig. 4. Reported numbers of patients with influenza-like illness in Japan [17].

the antigenicity of this region was retained by the chimeric HA (68/SD) without any change (results not shown).

In our previous study [14], convalescent sera of young children also recognized antigenic site B1, but the observation was drawn from a small number of samples. In the present large-scale study, we were able to confirm that young children reacted more specifically to a limited region of the HA protein. In the previous report, we suggested that human sera did not bind to antigenic site D and obtained the same results in the present experiments. In a study of DNA

vaccination of mice, we observed that immunization of mice with DNA or virus particles induced serum antibodies that bound to antigenic site D [23]. Therefore, this antigenic site might be murine-specific.

As shown in Figure 4, after a relatively large epidemic of A/SD/97-like viruses in the 1997/1998 season, epidemics by H3 viruses have remained small up to the 2001/2002 season. In comparison with A/SD/97 strains and epidemic strains isolated in the most recent season, differences in amino acids were seen in antigenic sites A, C, and B2. Young children whose sera reacted with site A of the A/SD/97 virus failed to bind to site A of A/PM/99 (Table 2). Therefore, young children were mainly infected with A/SD/97-like viruses but not with A/PM/99-like viruses. Judging from HI values of human sera against A/PM/99, sera of these children were still fully reactive with A/PM/99. This might be explained by the fact that these sera had antibodies that bind to the unchanged site B1 of the HA protein of A/PM/99. Therefore, human antisera of either young or older persons had HI activity to A/PM/99-like viruses. The limited epidemic of A/PM/99-like viruses may have been due to the existence of antibody against B1, which had been produced in response to infection by the A/SD/97-like viruses.

We assumed that an amino-acid change in site B1 might act as a trigger for the emergence of a drift strain. Because young children had antibodies to a limited region of the HA protein, a mutant virus with a change on site B1 of the HA molecule might have more easily escaped in young children compared to mutants with changes on antigenic sites A, C, and/or B2. In spite of our assumption, A/PM/99-like viruses that have circulated had amino-acid changes on antigenic sites A, C, and B2 but not on antigenic site B1. One possibility for their appearance is that amino-acid changes in sites A, C, and B2 in A/PM/99-like viruses occurred not by escaping directly from antigenic pressure but rather had only a slight advantage for propagation among human populations compared to A/SD/97-like viruses. On the other hand, HI activity of sample no. 32 revealed that some people have antibody that predominantly binds to site A of the A/SD/97 virus, suggesting that a mutation at antigenic site A would allow the virus to escape from antigenic pressure. In our study, although this sample might be representative of only a minor population, we could not exclude the possibility that on a worldwide scale, individuals of this type may be numerous. Analysis of human sera showed that A/PM/99-like viruses do not constitute a drift strain but rather a quasi-species [24] among A/SD/97-like viruses, in other words, A/PM/99-like viruses did not undergo enough change to cause an epidemic. Different results in the HI test using ferret sera and human sera with A/SD/97 and A/PM/99 strains, may be explained first, by a difference in the response of the different species to antigenic determinant sites of the HA protein and secondly, each of the A/SD/97 and A/PM/99 strains contains strain-specific amino-acid substitutions that were not possessed by epidemic viruses. Among six amino-acid substitutions observed in antigenic sites between A/SD/97 and A/PM/99 strains, three amino-acid substitutions at residues 57 (site C), 137 (site A), and 192 (site B2) were mainstream amino-acid substitutions which became fixed in most of the subsequent isolated strains, but three substitutions (142 and 144 on site A, 194 on site B2) were strain-specific (K. Sato et al., unpublished results). The number of amino-acid substitutions of epidemic A/PM/99-like viruses was reduced and does not fit to the criteria of drift viruses observed by Wilson and Cox [9].

We could not determine here whether the infection of young children with influenza A virus promotes antigenic drift, because we could not explain why amino-acid changes occurred in antigenic sites A, C, and B2. However, we are convinced that large-scale molecular analysis of human sera collected from the general population is not only useful in understanding the mechanisms for antigenic drift but can also help in predicting the emergence of future drift strains.

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