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Analysis of the host-specific haemagglutination of influenza A(H1N1) viruses isolated in the 1995/6 season

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SUMMARY

Two phenotypes of human influenza A(H1N1) virus are currently circulating in Japan. One (group 1) agglutinates both chicken and goose red blood cells (CRBC and GRBC), the other (group 2) agglutinates GRBC but not CRBC. In the 1995/6 season, group 2 viruses accounted for 70% of the H1N1 viruses isolated in MDCK cells. The 1995/6 viruses were located on two branches of the genetic tree. One branch contained both group 1 and group 2 viruses and the other branch contained only group 2 viruses. Group 2 viruses had aspartic acid at residue 225 in the haemagglutinin (HA) protein, the key amino acid residue for group 2 phenotype. The HA protein of group 1 viruses had a change from aspartic acid to asparagine at residue 225 and the expressed HA protein of these viruses adsorbed CRBC. Serial passage of group 2 viruses in MDCK cells or embryonated chicken eggs caused these viruses to gain the ability to agglutinate CRBC. MDCK-adapted viruses had the same amino acid sequences of HA polypeptide as the original ones, but egg-adapted viruses had changed amino acid sequences. The expressed HA protein from one egg-adapted virus that originally belonged to group 2 adsorbed CRBC.

INTRODUCTION

Influenza viruses grown in embryonated eggs are often antigenically different from those grown in MDCK cells, and the viruses grown in MDCK cells reflect the original clinical samples more closely than those grown in eggs [1–5]. Furthermore, the efficiency of isolation of influenza A viruses in MDCK cells is generally higher than in eggs. Therefore, in Japan, influenza viruses are currently isolated in MDCK cells.

In the 1991/2 influenza season, 50% of H1N1 viruses isolated in MDCK cells agglutinated chicken red blood cells (CRBC) (group 1), whereas the other 50% were barely able to agglutinate CRBC (group 2). We determined that an amino acid change at residue 225 from glycine to aspartic acid in the haemagglutinin (HA) protein after 1986 was responsible for the loss of the ability to agglutinate CRBC [6]. H1N1 viruses isolated in MDCK cells after 1986 have an amino acid change at residue 225 as a mainstream change and group 1 viruses also had the same amino acid change at this residue [7]. Because the expressed HA gene product of group 1 viruses could not adsorb CRBC, some modification of the HA protein after translation was suggested [6, 8]. The phenotype of group 1 viruses was stably maintained in MDCK cells

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but that of group 2 viruses was unstable. After several passages of group 2 viruses in MDCK cells, the viruses gained the ability to agglutinate CRBC [8, 9]. In the epidemic of influenza in the 1995/6 season in Japan, H1N1 viruses were the major viruses. We found that group 2 viruses were still predominant and group 1 viruses had the change in amino acid at the key point.

MATERIALS AND METHODS

Viruses

The viruses in this study were isolated from schoolchildren in the Aichi and Tokyo areas in Japan in MDCK cells and passaged in these cells or allantoic cavities of 11-day-old chick embryos.

Haemagglutination (HA) test

The HA test was performed at 4 °C with 0·5% CRBC or goose red blood cells (GRBC), according to the procedure described by Dowdle and colleagues [10].

Cloning of HA genes

Complementary DNA (cDNA) of the HA gene from the viruses was amplified by the reverse transcription (RT)-PCR method as described previously [6]. Two sets of sense and antisense primer s(#1 and #2) were #1–1:5′ > AGCAAAAGCAGGGGAAA (1–17), #1–2:5′ > TTAATCCGCAGCATAGCCAG (1178-1159) and #2–1:5 > GAGGTCTGTTTGGAGCCATT (1060-1079), #2–2: 5′ > AGTAGAAACAAGGGTGTTTT (1778-1759), respectively, numbered according to the positive sequence of the HA gene of A/PR/8/34 [11]. The second PCR was performed with the #1-1 primer containing an *Eco*R I site and #1-2. Synthesized cDNA was inserted into the pT7Blue T- vector (Novagen) for sequencing. The inserted HA1 cDNA was cut out with *Eco*R I and *Sma* I and the fragments exchanged with the corresponding region of pME18S-USSR as described previously [6].

Site-specific mutagenesis

The HA1 region of the HA cDNA (*Eco*R I-Sma I fragment) derived from A/166/95 or A/25/96 virus was inserted into pUC19. Site-specific mutagenesis was done by the PCR mutagenic procedure as described previously [6]. The mutant primers were TTGATTTCTTATTTTGGG(aspartic acid to asparagine at residue 225 for A/166/95 HA1cDNA) and TTGACCTCTTACTTTGGG (aspartic acid to glycine at residue 225 for A/25/96 HA1cDNA).

Haemadsorption assay of cells transfected with HAcDNA

Haemadsorption assay was performed as described previously [6]. Briefly, each cDNA (200 ng) in Eagle's minimum essential medium (MEM) without serum (MEMO) was incubated with lipofectamine for 15 min at room temperature. COS cells ($0{\cdot}5 \times 10^5$ cells/18 mm coverglass) which had been prepared 18 h earlier were washed with MEMO, the DNA and lipofectamine mixture was added, and then the cells were incubated for 6 h. The medium was changed to MEM containing 10% foetal calf serum (MEM10) and the cells were further incubated for 42–46 h at 37 °C. The medium was changed to MEMO 4 h before haemadsorption assay.

RESULTS

Haemagglutination properties of the H1N1 viruses isolated in the 1995/6 influenza season

Of 168 H1N1 strains isolated in the 1995/6 season, 51 (30%) belonged to group 1 and 117 (70%) belonged to group 2 phenotype. Group 1 viruses were isolated in 1996 and group 2 viruses in 1995 and 1996. Group 1 viruses retained the haemagglutination property to both CRBC and GRBC, while group 2 viruses gained the ability to agglutinate CRBC after three passages in MDCK cells (Table 1). Antigenically, viruses of both groups were similar to the A/Yamagata/32/89 strain.

Amino acid sequences of the HA polypeptide of H1N1 isolates

Table 2 shows the changes in amino acids in the HA1 and HA2 regions of 16 H1N1 strains isolated in the 1995/6 season in Japan from those of the A/Aichi/24/92 (A/24/92) strain. The numbers of changed amino acid in the HA1 region of H1N1 virus isolated in 1995/6 were 4–10 and those in the HA2 region were 6–7. Difference in amino acid in the HA2 region was seen only at residue HA2 47. The

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Table 1. *Haemagglutinin titre of influenza A(H1N1) virus with chicken and goose red blood cells*

Virus group	Virus strains	Chicken red blood cells passage no.*					Goose red blood cells passage no.*				
		1	2	3	4	5	1	2	3	4	5
1	A/Aichi/30/96	256	512	256	128	128	256	512	256	256	256
	A/Aichi/31/96	256	256	512	128	128	256	512	256	256	256
2	A/Aichi/166/95	< 4	4	64	128	128	128	256	512	256	256
	A/Aichi/25/96	< 4	4	64	128	128	128	256	512	256	256
	A/Aichi/26/96	< 4	16	256	128	128	128	256	512	256	256

* Viruses were passaged in MDCK cells.

phylogenic tree of the HA1 region from 1986 is shown in Figure 1. The changed amino acids are shown on the tree. H1N1 isolates in the 1995/6 season were located on two branches. One branch (A) contains both group 1 and group 2 viruses. Four strains isolated in late 1995 had the group 2 phenotype. Seven strains of group 1 viruses isolated in 1996 had the amino acid change at residue 225 from aspartic acid to asparagine and were derived from group 2 viruses isolated in late 1995. On the other hand, five strains isolated in early 1996 which had the group 2 phenotype were located on the other branch (B).

Properties of the expressed HA protein of H1N1 viruses isolated in MDCK cells

In order to determine whether HA protein of group 1 or 2 viruses isolated in 1995/6 had the ability to adsorb CRBC, the HA gene of the virus belonging to each group was expressed on COS cells. The expressed HA protein from A/Aichi/166/95 (A/166/95) virus (group 2) on branch A and A/Aichi/25/96 (A/25/96) virus (group 2) on branch B adsorbed GRBC but not CRBC. The site-specific mutagenesis at residue 225 from aspartic acid to glycine in the HA protein of A/25/96 restored the ability to adsorb CRBC (data not shown). Therefore, aspartic acid at residue 225 still was the key point for adsorbing CRBC. On the other hand, the expressed HA protein from A/Aichi/30/96 (A/30/96) virus (group 1) on branch A adsorbed both GRBC and CRBC (Fig. 2).

Amino acid sequences of the HA polypeptide of the viruses in throat swabs

The amino acid sequences of the HA polypeptide of the viruses in throat swabs were deduced from the sequences of cDNA obtained by the RT-PCR method. As shown in Table 2, the amino acid sequences of the MDCK isolates and the viruses in throat swabs were the same. Thus, the viruses isolated in MDCK cells reflected genetically the viruses in humans as reported previously [2, 4, 5].

Phenotypes and amino acid sequences of the HA polypeptide of MDCK-adapted virus

After passages in MDCK cells, group 2 viruses changed to the group 1 phenotype (Table 1). Comparison of the amino acid sequences of the HA polypeptide of phenotypically changed viruses with those of the parental viruses showed no amino acid changes (Table 2).

Amino acid sequences of the HA polypeptide of egg-adapted virus

A group 1 virus (A/30/96) adapted to eggs easily and the virus was recovered (A/30/96E). On the other hand, group 2 viruses hardly grew in eggs and three passages were needed to obtain high HA titre viruses in eggs (A/166/95E and A/25/96E). Egg-adapted viruses restored the haemagglutinating ability of CRBC. Amino acid sequences of these viruses were compared with those of the parental viruses (Table 2). In our previous paper, we reported that egg-adapted A/24/92E had an amino acid change at residue 225 from aspartic acid to glycine and that this amino acid change caused the recovery of haemagglutinability of CRBC [6]. The HA protein of A/166/95E had amino acid changes at residues 189 and 207 from glycine to arginine and serine to tyrosine, respectively, while the HA protein of A/25/96E had an amino acid change at residue 156 from glutamic acid to lysine. The

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Table 2. *Amino acid changes in the HA1 polypeptide of influenza A(H1N1) strains isolated in 1995/6 season from that of A/Aichi/24/92 virus*

		Amino acid change																						
		HA1																HA2						
Virus	Group	56	104	133	137a	149	156	189	207	218	223	225	240	244	253	275	317	46	47	67	72	124	135	169
MDCK isolates																								
A/24/92	2	I	D	T	A	R	E	G	S	A	V	D	G	I	A	D	V	N	G	G	K	S	T	S
KT/381/95*	2	T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	G	A	T	N	N	N
KT/391/95	2	T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	G	A	T	N	N	N
A/166/95**	2	T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	R	A	T	N	N	N
A/181/95	2	T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	R	A	T	N	N	N
A/9/96	2	T	D	S	T	K	E	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
A/25/96	2	T	N	S	T	K	E	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
A/26/96	2	T	N	S	T	K	E	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
A/38/96	2	T	D	S	T	K	E	G	S	A	V	D	G	M	A	G	A	D	G	A	T	N	N	N
KT/100/96	2	T	D	T	T	K	E	G	S	A	V	D	G	I	A	G	V	D	G	A	T	N	N	N
KT/69/96	1	T	D	T	A	K	E	G	S	A	I	N	E	I	A	G	V	D	G	A	T	N	N	N
KT/90/96	1	T	D	T	A	K	E	G	S	A	I	N	E	I	A	G	V	D	G	A	T	N	N	N
KT/93/96	1	T	D	T	A	K	E	G	S	A	I	N	E	I	A	G	V	D	G	A	T	N	N	N
A/27/96	1	T	D	T	A	K	E	G	S	A	I	N	G	I	A	G	V	D	R	A	T	N	N	N
A/29/96	1	T	D	T	A	K	E	G	S	A	I	N	G	I	A	G	V	D	R	A	T	N	N	N
A/30/96	1	T	D	T	A	K	E	G	S	A	I	N	G	I	A	G	V	D	R	A	T	N	N	N
A/31/96	1	T	D	T	A	K	E	G	S	A	I	N	G	I	A	G	V	D	R	A	T	N	N	N
Swab																								
KT/381/95S		T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	G	A	T	N	N	N
A/166/95S		T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	R	A	T	N	N	N
MDCK adapt																								
A/166/95M	1	T	D	T	A	K	E	G	S	A	I	D	G	I	A	G	V	D	G	A	T	N	N	N
A/25/96M	1	T	N	S	T	K	E	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
A/26/96M	1	T	N	S	T	K	E	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
Egg adapt																								
A/24/92E	1	I	D	T	A	R	E	G	S	A	V	N	G	I	A	D	V	N	G	G	K	S	T	S
A/166/95E	1	T	D	T	A	K	E	R	Y	A	I	D	G	I	A	G	V	D	R	A	T	N	N	N
A/25/96E	1	T	N	S	T	K	K	G	S	T	V	D	G	M	V	G	A	D	G	A	T	N	N	N
A/30/96E	1	T	D	T	A	K	E	G	Y	A	I	N	G	I	A	G	V	D	R	A	T	N	N	N

The amino acid residues are numbered corresponding to the numbering of the H3 subtype according to the alignment of Winter and colleagues [11] and Cox and colleagues [13]. The amino acids which were absent in the H3 subtype are numbered using alphabetic suffixes. Only positions which drifted from the HA polypeptide of the A/Aichi/24/92 (A/24/92) strain are shown. Viruses isolated in Aichi prefecture are shown as A and those isolated in Tokyo as KT. Swab signifies throat swab sample. S, M, and E after the isolation year indicate swab sample, MDCK-adapted, and egg-adapted, respectively.

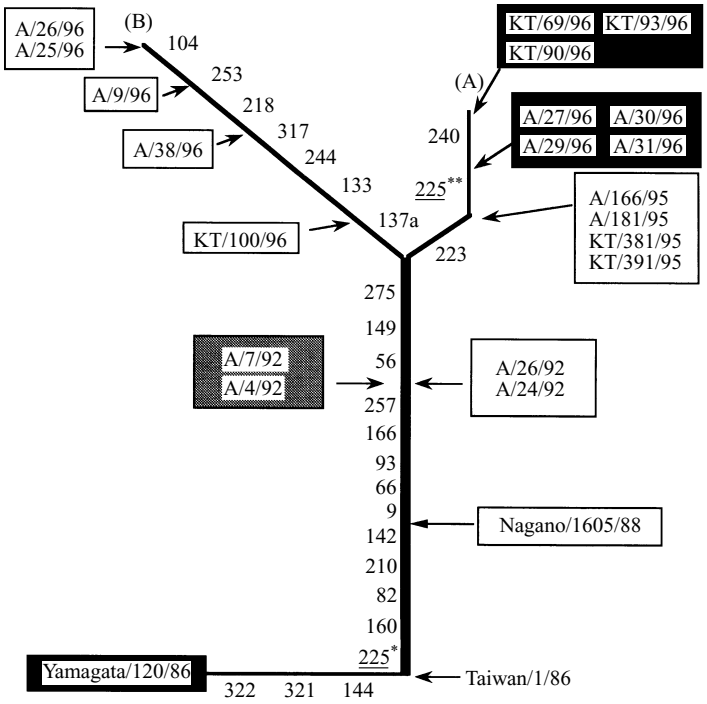

Fig. 1. The evolutionary tree for the HA1 polypeptides of the influenza A (H1N1) virus strain isolated between 1986 and 1996. Numbers refer to the mainstream amino acid changes that have become fixed in most of the subsequent strains (vertical lines), or to strain-specific amino acid changes on the side branches. Grey square indicates group 1 viruses isolated in 1991/2, black squares indicate group 1 viruses isolated in 1995/6 and white squares indicate group 2 viruses. KT, A/Kamata; A, A/Aichi. 225* and 225**, amino acid changes from glycine → aspartic acid and from aspartic acid → asparagine, respectively.

expressed HA protein from A/166/95E did not adsorb CRBC, and therefore, the recovery of haemagglutinability of the virus was not due to the changes in amino acids in the HA protein. On the other hand, the expressed HA protein of A/25/96E adsorbed CRBC in spite of having an amino acid change only at residue 156. A/30/96E, which easily adapted to eggs, had an amino acid change at residue 207 from serine to tyrosine.

DISCUSSION

After the appearance of influenza A(H1N1) viruses in 1977, the HA protein of H1N1 viruses gradually changed in structure [6, 12–16]. Two N-glycosylation sites (amino acid residues 63 and 129) were added after 1983 and one glycosylation site (amino acid residue 160) was lost after 1986. Amino acid changes at residues 193, 197, and 198 before 1986, and the following change at residue 225 from glycine to aspartic acid after 1986 resulted in the HA protein losing its ability to agglutinate CRBC [6]. This phenomenon (host-specific haemagglutination) became clear in the 1991/2 influenza season in Japan [8]. In that influenza season, 50% of H1N1 isolates were of a phenotype that did not agglutinate CRBC (group 2). However, this phenotype changed to group 1 phenotype that can agglutinate CRBC during serial passages in MDCK cells. Group 2 phenotype could be maintained only by plaque to plaque passages [8]. After the 1991/2 season, a small number of H1N1 viruses were isolated in the 1994/5 season in Japan but no H1N1 viruses were isolated in the Aichi and Tokyo areas until the 1995/6 season. In this season, the H1N1 epidemic occurred all over Japan and 70% of the H1N1 isolates in the Aichi and Tokyo areas were group 2. The amino acid at residue 225 of these viruses was still aspartic acid and the HA protein expressed in COS cells could not adsorb CRBC. The phenotype of these group 2 viruses was also unstable in MDCK cells.

It has been reported that the HA sequences of the viruses isolated in MDCK cells were similar to those directly sequenced from swab samples [2, 4, 5]. The amino acid sequences obtained from the swab samples were the same as those of MDCK-isolated viruses. The HA protein of all of seven randomly selected group 1 viruses isolated in early 1996 contained asparagine at residue 225. These viruses were derived from group 2 viruses located on branch A (Fig. 1).

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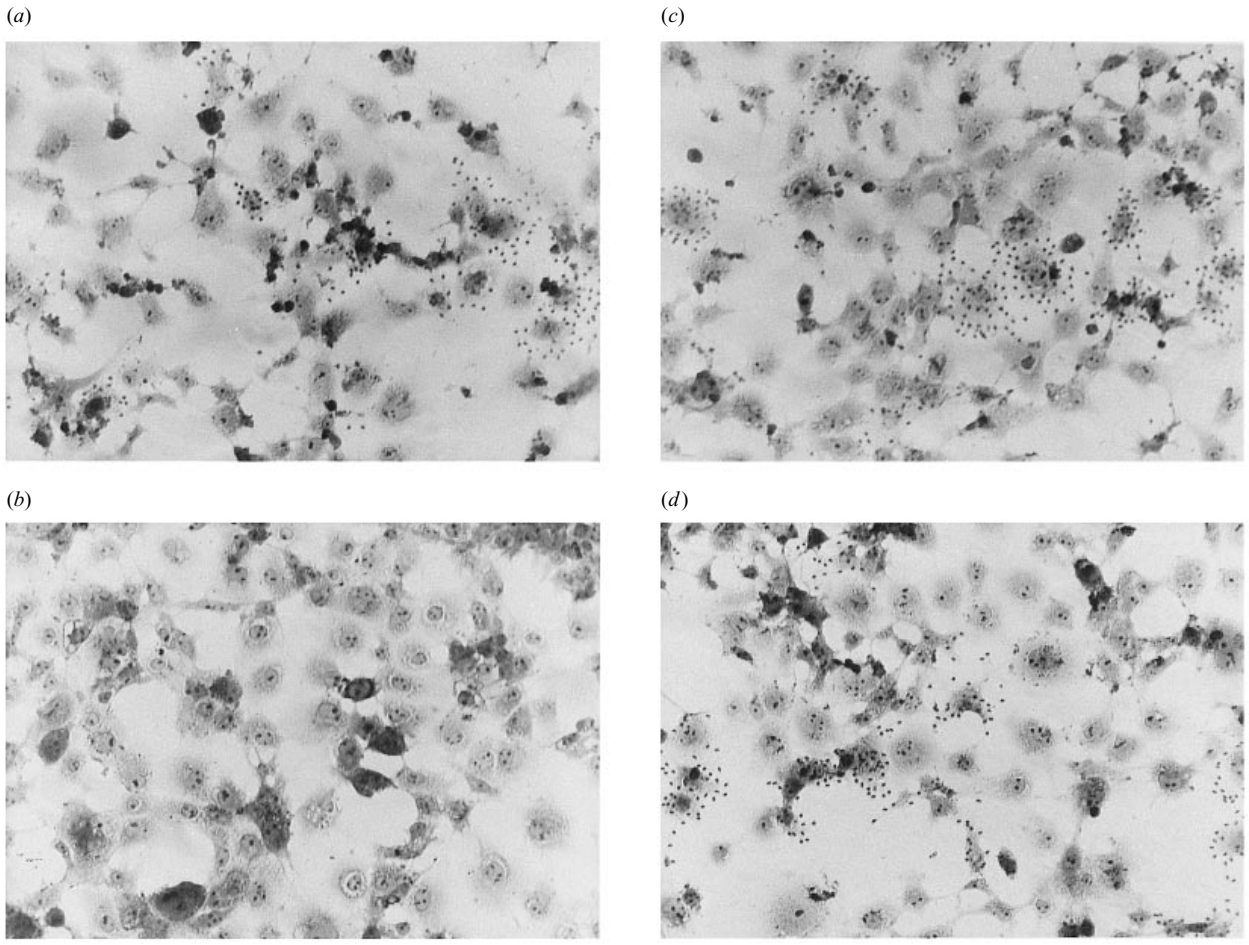

Fig. 2. Adsorption of GRBC and CRBC to COS cells transfected with A/Aichi/166/95 (group 2, 1st passage) and A/Aichi/30/96 (group 1, 1st passage) HAcDNAs. (*a*) A/Aichi/166/95 HAcDNA and GRBC; (*b*) A/Aichi/166/95 HAcDNA and CRBC; (*c*) A/Aichi/30/96 HAcDNA and GRBC; (*d*) A/Aichi/30/96 HAcDNA and CRBC. The haemadsorption test was performed as described in Materials and methods.

The expressed HA protein of A/30/96 (group 1) in COS cells could adsorb CRBC, unlike the group 1 viruses isolated in the 1991/2 season whose expressed HA protein could not adsorb CRBC. A point mutation at residue 225 from aspartic acid to asparagine in the HA protein of A/166/95 restored the ability to adsorb CRBC (data not shown).

It has been reported that typical substitutions in amino acids due to egg adaptation of H1N1 viruses are glutamic acid to lysine at amino acid residue 189, aspartic acid to asparagine at residue 190, and aspartic acid to glycine or asparagine at residue 225 [3, 4]. Group 1 viruses in the 1995/6 season had asparagine at residue 225 as often seen in egg-adapted viruses and this residue is in the receptor binding site on the left edge of the receptor binding pocket [17, 18]. A/25/96E has aspartic acid at residue 225 and lysine at residue 156 and the expressed HA protein of A/25/96E adsorbed CRBC. This shows that the amino acid change at residue 156 from glutamic acid to lysine can also cause host-specific haemagglutination. Residue 156 is located at the tip of the HA molecule and is immediately adjacent to the receptor-binding site. A/166/95E has aspartic acid at residue 225 and could agglutinate CRBC, but the expressed HA protein of A/166/95E could not adsorb CRBC. For A/166/95E, some other gene product(s) may help binding of its HA to CRBC as well as MDCK-adapted viruses. It is worth noting that the HA protein of each egg-adapted virus had changes in amino acids in the HA1 region but not in the HA2 region. This was clearly different from the phenotypically changed viruses adapted to MDCK cells, which had no amino acid changes in the HA protein (Table 2).

The mechanism of phenotypic change from group 2 to group 1 by passaging in MDCK cells is still not

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clear. Hausman and colleagues [19] showed the participation of N1 neuraminidase of influenza A/FPV/Rostock/34 in haemadsorbing activity. However, the N1 protein of A/Aichi/24/92M (MDCK-adapted virus derived from A/24/92; group 1 [8]) expressed in COS cells did not show any haem-adsorbing activity either with GRBC or CRBC (unpublished data). Therefore, neuraminidase of MDCK-adapted virus might not directly participate in the haemagglutination of CRBC.

As reported previously [6], recent human influenza A (H3N2) viruses were also divided into group 1 and group 2 phenotypes. For H3N2 viruses, all strains changed to the group 2 phenotype after the 1993/4 season. However, similar to H1N1 viruses, serial passages of H3N2 viruses in MDCK cells also changed the group 2 phenotype to group 1 without a mutation on the HA gene.

The reduced haemagglutinating ability of CRBC of recently isolated influenza viruses is similar to that of the classical O-D change [9, 20, 21]. The classical O (original) virus isolated in the amniotic cavity could not agglutinate CRBC. However, the O virus passaged in the allantoic cavity could agglutinate CRBC to become a D (derived) virus. This O-D change was not observed in H2N2, H3N2, and new H1N1 viruses until recently in Japan. Azzi and colleagues [9] found the same phenomenon in the 1983 H1N1 viruses in Italy.

It is interesting that two subtype viruses (H1N1 and H3N2) suddenly lost the ability to agglutinate CRBC.

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