Studies on the molecular basis for loss of the ability of recent influenza A (H1N1) virus strains to agglutinate chicken erythrocytes

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Recent strains of influenza A but not B viruses have lost the ability to agglutinate chicken red blood cells (CRBC). The H1N1 viruses isolated in Japan during the 1991/92 season could be divided into two groups. Group 1 viruses (A/Aichi/4/92 and A/Aichi/7/92) agglutinated goose red blood cells (GRBC) and CRBC, while group 2 viruses (A/Aichi/24/92 and A/Aichi/26/92) did not agglutinate CRBC. There were no amino acid differences between them in the haemagglutinin (HA) polypeptide. Reassortment experiments between a group 1 virus (A/Aichi/4/92) or a group 2 virus (A/Aichi/24/92) and the A/WSN/33 influenza A (H1N1) virus strain suggested that the HA gene products of the viruses of both groups had lost the capacity to agglutinate CRBC. The HA proteins expressed on Cos cells by transfecting the cDNAs of the virus HA gene of A/Aichi/4/92 and A/Aichi/24/92 agglutinated GRBC but not CRBC.

These experiments indicated that the HA proteins of H1N1 viruses of both groups isolated in 1992 had lost the ability to agglutinate CRBC even though the group 1 virions showed haemagglutinating capacity with CRBC. By using the cDNAs of the HA gene of seven natural isolates obtained from 1977 to 1992, it was found that the expressed HA proteins of influenza A (H1N1) viruses isolated since 1988 had lost the ability to agglutinate CRBC. Experiments with chimeric and point-mutated HA cDNAs of A/Aichi/24/92 showed that an amino acid change at residue 225, which occurred after 1986, and a cluster of amino acid changes at residues 193, 196 and 197, which occurred before 1986, were responsible for loss of the ability to agglutinate CRBC. Egg-adapted virus derived from A/Aichi/24/92 had one amino acid change at residue 225 compared to the parental virus.

Introduction

Since the observation (Hirst, 1941) of haemagglutination by influenza A viruses, haemagglutination has proven to be an extremely valuable technique for virus identification, quantification and purification. Influenza A virus agglutinates red blood cells of several avian and animal species because it binds sialic acid on the surface of these cells. From the time that haemagglutination was first observed, chicken, human or guinea-pig erythrocytes have been commonly used. Weis et al. (1988) identified the structure of the sialic acid-binding region of the haemagglutinin (HA) protein by X-ray analysis. Nobusawa et al. (1988) observed that site-directed mutation of the receptor binding site of the HA protein affected the ability of HA protein to bind to red blood cells.

Influenza viruses grown in MDCK cells are antigenically more like the original clinical samples than those grown in embryonated chicken eggs (Robertson et al., 1985; Katz et al., 1990). Furthermore, the efficiency of isolation of influenza viruses with MDCK cells is generally higher than with embryonated chicken eggs. Therefore, in Japan, influenza viruses are usually cultured and isolated with MDCK cells. Recently, there have been many reports from the regional public health institutes that hardly any of the influenza A (H1N1) and A (H3N2) viruses isolated from MDCK cells agglutinated chicken red blood cells (CRBC). We found that influenza A (H1N1) viruses isolated in the 1991/92 influenza season in Japan could be divided into two different groups according to the characteristics of haemagglutination with...
CRBC. Group 1 viruses agglutinated goose, human and guinea-pig erythrocytes and CRBC, while group 2 viruses agglutinated the first three but not CRBC. Azzi et al. (1993) also reported the same phenomenon in recently isolated H1N1 viruses. The candidate viral protein for host-specific haemagglutination was considered to be the HA glycoprotein. However, comparison of the amino acid sequences of the HA polypeptide deduced from the base sequences of the HA gene of different groups of H1N1 viruses isolated in 1992 showed no amino acid differences (Morishita et al., 1993). Therefore, it was suspected that some modification of the HA protein of group 1 or group 2 viruses had occurred as a result of the action of product(s) of other gene(s), resulting in the loss or gain of the ability to agglutinate CRBC. In order to answer the questions (1) whether or not the HA proteins of group 1 and 2 viruses retain the ability to agglutinate CRBC, and (2) which gene product affects the phenotype of HA, we carried out reassortment and gene expression experiments. In this report, we present evidence that the HA proteins of group 1 and 2 viruses isolated in 1992 have lost the ability to agglutinate CRBC, and that the amino acid change(s) responsible had already occurred in 1988.

Methods

- **Viruses.** The viruses in this study were isolated from school children in Aichi prefecture in Japan by using MDCK cells and were passaged in MDCK cells. A/Nagano/1396/88 and A/Nagano/1605/88 were kindly supplied by K. Nakamura at the Nagano Research Institute for Public Health and Pollution.

- **Haemagglutination test.** The haemagglutination test was done at 4°C with 0.5% CRBC or goose red blood cells (GRBC).

- **Reassortment experiments.** MDCK cells were mixed-infected with 2 p.f.u. per cell of A/Aichi/4/92 (group 1) or A/Aichi/24/92 (group 2) and A/WSN/33 viruses. Progeny viruses were plaque-purified in MDCK cells without trypsin.

- **Identification of the gene constellation in reassortant viruses.** The HA gene was identified by the haemagglutination-inhibition test using anti-A/Yamagata/32/89 (H1N1) ferret and anti-A/WSN/33 rabbit sera. Other genes were identified by partial primers for PCR were TGTAAGGGGCAATTCTCC (1971-1990) (PB1), CAGGAAACAGCTATGCTAGTACTAAACAA (3’ end of influenza A virus; the underlined sequence is the sequence complementary to M13RV).

- **Site-directed mutagenesis.** The HA1 region of the HA cDNA (EcoRI-Smal fragment) was inserted into pUC19. Primer U/Earl is CGCCCTTTCTGGCCA-TCTATCATACA (underlining marks the recognition site of Earl and italic letters indicate the coding sequence of the HA gene from amino acid 192 to 196; the amino acid at residue 192 was changed to that of A/Aichi/24/92 type). Primer A/Earl is CGCCCTTTCTGGCCCTTTT-GGTCCCTCT (underlining marks the recognition site of Earl and italic letters indicate the coding sequence of the HA gene from amino acid 192 to 186). PCR products obtained with U/Earl (antisense) and M4 (sense) from A/USSR/77 HA cDNA, and with A/Earl (sense) and RV (antisense) from A/Aichi/24/92 HA cDNA were cleaved with XhoI and Earl, and HindIII and Earl, respectively. The fragments obtained were ligated and inserted into the EcoRI/HindIII site in pUC19. The EcoRI-Smal fragment of chimeric HA cDNA was ligated to the Smal-Xbal fragment of HA DNA of A/USSR/77 and inserted into pME18S expression vector.

- **Haemadsorption assay of HA cDNA.** Each cDNA (200 ng) in 20 μl of Eagle’s minimum essential medium (MEM) without serum (MEMO) was incubated with 20 μl of diluted ripofectamine (36 μl of 6/83, A/England/333/80 and A/Brazil/11/78 viruses were amplified by RT-PCR. The sense and antisense primers were 5’-specific AGCAAAACGGGAAA (1-17) and 3’-specific TTAATCCGCCAC-ATAGCC (1775-1757), respectively. The second PCR was done with a 5’-specific primer containing an EcoRI site and a 3’-specific primer containing a XbaI site. cDNA was digested with EcoRI and Smal or Smal and XbaI (the H1 HA gene contains one Smal site in the HA1 region and two EcoRI sites in the HA2 region). These fragments were ligated and inserted into EcoRI and XbaI sites in pME18S expression vector (kindly supplied by K. Maruyama at Tokyo Medical and Dental University). Three clones were isolated independently and a sample of each was used for the expression experiments.
ripenectamine/400 μl of MEM0) for 15 min at room temperature. Cos
cells (0.5 × 10⁵ cells per 18 mm coverslip) which had been prepared 18 h
earlier were washed with MEM0 and then 0.16 ml of MEM0 was added.
The DNA and ripenectamine mixture (40 μl) was added to the medium
and the cells were incubated for 6 h. The medium was changed to MEM
containing 10% fetal calf serum (MEM10) and further incubated for 42 to
46 h at 37 °C. The medium was changed to MEM0 4 h before assay. The
MEM0 was removed and then 0.5% CRBC or GRBC was added to the
culture, which was then incubated for 15 min at room temperature.
Unadsorbed RBC were washed out with MEM0 and cells were stained
with crystal violet for 10 s.

Results

Host-specific haemagglutination

Influenza A (H1N1) viruses isolated before the 1991/92
influenza season and A (H3N2) viruses isolated before the
1992/93 influenza season in Aichi prefecture agglutinated
CRBC. During the 1991/92 season, for H1N1 viruses, and the
1992/93 season, for H3N2 viruses, isolates could be divided
into two groups according to their host-specific haemag-
glutination (group 1 viruses agglutinated GRBC and CRBC,
group 2 viruses did not agglutinate CRBC). A retrospective
study showed that group 2 H1N1 viruses had been isolated in
the 1987/88 season in Nagano prefecture (A/Nagano/
1605/88) (data not shown). In the 1994/95 season, small
epidemics of H1N1 viruses occurred in Japan, but these viruses
did not agglutinate CRBC. In our previous study (Morishita et
al., 1993), we observed no differences in the amino acid
sequence of HA polypeptides of H1N1 viruses for group 1 and
2 viruses isolated in 1992. We suspected that some modi-
fication of the HA protein of group 1 or group 2 viruses was
caused by the product(s) of other gene(s), resulting in loss or
gain of the ability to agglutinate CRBC.

The gene responsible for host-specific
haemagglutination

To find the H1N1 virus gene responsible for host-specific
haemagglutination, reassortment experiments were done.
MDCK cells were mixed-infected with A/Aichi/24/92 (group
2) and A/WSN/33 viruses. Sixty plaques were randomly
picked and tested for their ability to agglutinate GRBC and
CRBC. Fifty-three and seven clones belonged to the group 1
and 2 phenotypes, respectively. The latter clones were
examined for their gene constellations (Table 1a). All
reassortant clones with the group 2 phenotype had the HA
gene derived from A/Aichi/24/92. The other 53 clones had
the HA gene of A/WSN/33. These results indicated that the
HA protein of A/Aichi/24/92 is responsible for loss of the
ability to agglutinate CRBC.

Table 1. Genotype and HA titre of reassortant virus clones

The gene constellations were identified as described in Methods. ND, Not determined.

(a) Reassortants between A/Aichi/24/92 and WSN/33

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(b) Reassortants between A/Aichi/4/92 and WSN/33

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Reassortment between A/Aichi/4/92 (group 1) and A/WSN/33 viruses

Because the sequences of the HA gene of A/Aichi/24/92 (group 2) and A/Aichi/4/92 (group 1) were the same (Morishita et al., 1993), the HA protein of group 1 might not have the ability to agglutinate CRBC. From reassortment experiments between A/Aichi/4/92 and A/WSN/33 viruses, reassortant viruses with the group 2 phenotype (PW10 and PW70) were isolated, which had the HA gene from A/Aichi/4/92 and the M gene of A/WSN/33 (Table I b). On the other hand, the reassortant viruses (PW13 and PW15) which had the HA and M genes of A/Aichi/4/92 agglutinated CRBC. Thirty strains which had the HA gene derived from A/WSN/33 agglutinated CRBC. These results suggested that the HA protein of A/Aichi/4/92 had lost the ability to agglutinate CRBC and that the M gene product of A/Aichi/4/92 might affect the HA phenotype of the virus so as to restore the ability to agglutinate CRBC.

Expression of the HA genes of group 1 and 2 viruses

To confirm the above results and to study the HA phenotype without the effects of other virus products, we expressed the cDNAs of the HA genes of the viruses of both groups on Cos cells and also that of the A/USSR/90/77 (H1N1) virus (USSR-HA cDNA) as a control. Both GRBC and CRBC adsorbed to Cos cells transfected with USSR-HA cDNA (Fig. 1 a, b). Adsorption of CRBC was greater than that of GRBC. On the other hand, GRBC but not CRBC adsorbed to
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**Fig. 2. Haemadsorption of chimeric HA cDNAs.** The preparation of point mutant HA proteins and chimeric HA proteins with A/Aichi/24/92 and A/USSR/77 HA cDNAs is described in Methods. White bars indicate the HA region from A/Aichi/24/92 and black bars indicate that from A/USSR/77. Vertical lines in A/Aichi/24/92 indicate amino acid differences from A/USSR/77. The exact amino acid differences are shown in Fig. 3. The point mutants at residues 225, 193, 196 and 197 are indicated by circles in each HA gene. The haemadsorption test was performed as described in Methods.

Cos cells transfected with A/Aichi/24/92 HA cDNA (Fig. 1c, d). The expressed HA cDNA of A/Aichi/4/92 was also unable to adsorb CRBC (see Fig. 3). These experiments clearly showed that the expressed HA proteins (HA\textsuperscript{Ex}) of both virus groups agglutinated GRBC but had lost the ability to agglutinate CRBC.

The portion of the HA gene responsible for host-specific haemagglutination

To find the portion of the HA\textsuperscript{Ex} protein responsible for loss of the ability to agglutinate CRBC, we prepared several chimeric cDNAs. A/Aichi/24/92 (group 2) and USSR-HA cDNAs were cleaved with the restriction endonucleases NspV, BbsI and SmaI, and the fragments were ligated (Fig. 2). These enzymes cleaved HA cDNAs at amino acid residues 123, 208 and 239, respectively. The results for #3 chimeric HA showed that the portion of A/Aichi/24/92 between amino acid residues 123 to 239 was responsible for loss of the phenotype; however, #4 and #5 chimeric HAs, which contain amino acid residues 123 to 208 and 208 to 239 of A/Aichi/24/92, respectively, agglutinated CRBC, which suggested that more than one amino acid was responsible for loss of the ability to agglutinate CRBC.
Agglutination of CRBC by expressed HA proteins of natural isolates

To investigate the differences in agglutination phenotype of the HA \( \text{Ex} \) protein of natural influenza A (H1N1) virus isolates, we prepared HA cDNAs of A/Brazil/11/78, A/England/333/80, A/Dunedin/6/83 and A/Yamagata/120/86, and expressed them on Cos cells. The differences in amino acid sequences between these proteins are shown in Fig. 3. The HA \( \text{Ex} \) proteins of these HA cDNAs adsorbed to both GRBC and CRBC (Fig. 3). Two types of virus strains were isolated in Nagano prefecture in the 1987/88 influenza season. A/Nagano/1396/88 (group 1) and A/Nagano/1605/88 (group 2) had three amino acid differences in the HA polypeptide. The HA cDNAs of A/Nagano/1396/88 and A/Nagano/1605/88 expressed on Cos cells did not agglutinate CRBC (Fig. 3). So the difference in amino acid sequences between the HA proteins of two viruses isolated in the same year did not affect the ability to agglutinate CRBC.

These results indicated that the HA gene product of the H1N1 virus had already lost the ability to agglutinate CRBC in 1988.

Amino acid residues on the HA molecule responsible for loss of the ability to agglutinate CRBC

From the experiments on expression of chimeric HA cDNAs, it was clear that the portion of HA responsible for loss of the ability to agglutinate CRBC resided in residues 123 to 239 in the HA1 region (#3). Comparison of amino acid sequences in the HA1 region among natural isolates suggested that residues 160, 225 or 210 were the candidate amino acids for loss of the ability to agglutinate CRBC. We constructed point mutant HA cDNAs and carried out a haemadsorption assay (Fig. 2). A point mutation at residue 210 from asparagine (N) to serine (S) in chimeric protein #4 maintained the haemadsorption ability. However, a point mutation at residue 225 from glycine (G) to aspartic acid (D) in protein #4...
drastically reduced the haemadsorption ability (data not shown). A point mutation at residue 225 in A/Aichi/24/92 from D to G (protein #7) resulted in the ability to adsorb CRBC, while a point mutation at residue 210 in A/Aichi/24/92 from S to N did not. These results indicated that amino acid residue 225 was one of those responsible for the loss of agglutinating ability. A point mutation at residue 225 in A/USSR/77 from G to D (#6) did not affect haemadsorption ability. This result was compatible with those obtained from chimeric HA experiments which showed that a change in a residue at 123 to 208 and another in a residue at 208 to 239 are both necessary for the ability to agglutinate CRBC to be lost (#3, #4 and #5). Next we tried to identify another of the residues responsible within the stretch 123 to 208. The amino acid change at residue 160 which occurred in another of the residues responsible within the stretch 123 to 208. The amino acid change in a residue at 160 which occurred in 1988 showed that this amino acid was not the one responsible (data not shown).

Because many amino acid changes occurred in this region before 1986, we prepared a particular chimeric protein, #8, in order to determine the candidate residues. This chimeric HA protein, expressed on Cos cells, did not adsorb to CRBC, indicating that another of the amino acids responsible was either 193, 196 and 197. Point mutants with any one of the amino acid changes in Aichi/24/92 were unable to agglutinate CRBC (#9, #10 and #11), while the triple mutant with changes at residues 193, 196 and 197 did agglutinate CRBC.

**HA sequence of an egg-adapted virus derived from Aichi/24/92 virus (group 2)**

A/Aichi/24/92 had lost the ability to agglutinate CRBC. We obtained egg-adapted virus A-24-3E from A/Aichi/24/92 virus after three passages in embryonated chicken eggs. The HA titre of A-24-3E was 1024 with CRBC. The amino acid sequence of A-24-3E deduced from the nucleotide sequence of the HA gene indicated only one amino acid change, from D to G at residue 225, from the parental A/Aichi/24/92. This amino acid change restored the ability to haemagglutinate CRBC (Fig. 2).

**Discussion**

In this study we have shown that the HA protein of influenza A (H1N1) virus lost the ability to agglutinate CRBC after 1988 as a result of four amino acid changes compared with the A/USSR/90/77 virus. One of the amino acids responsible is residue 225, which is in the receptor binding site on the left edge of the receptor binding pocket. Some viruses isolated before 1988 have this change from G to D (for instance, A/Dunedin/6/83). However, a single amino acid change at residue 225 is not enough to cause loss of ability to agglutinate CRBC (Fig. 2). Experiments with chimeric HA proteins produced from A/Aichi/24/92 and A/USSR/77 indicated that another amino acid(s) located between residues 123 to 208 is necessary. Many amino acid changes occurred in this region before 1986 (Fig. 3), so we prepared a chimeric protein, #8, in order to determine the candidate residues. This protein was constructed using the restriction enzyme EarI; this enzyme has different cutting and recognition sites, so with its use any chimeric protein can be constructed. Chimeric protein #8 showed that the amino acid residues responsible for host-specific haemagglutination were located within residues 193 to 208, and that residues 193, 196 and 197 were the candidates (Fig. 3). Mutation of each amino acid showed that a change in any one residue was not enough to change the phenotype of the HA protein. These amino acid residues are not included in the receptor binding site but are located on the rim near amino acid residues which are included in the receptor binding site (residues 190, 194 and 195) (Nobusawa & Nakajima, 1988).

From these data it was suggested that minor modification at the rim of the receptor binding site on the HA protein occurred before 1986 as a result of changes at residues 193, 196 and 197, but this modification did not affect binding of HA protein to CRBC in the absence of an additional amino acid change at residue 225. Mainstream changes observed in our study (Fig. 3) were also seen in virus strains isolated in other countries (except the change at residue 275 after 1990) (Rocha et al., 1993; Pyhäälä et al., 1995). Substitution at residue 225 had been described as being associated with adaptation to grow in eggs (Robertson et al., 1991; Rocha et al., 1993). However, substitution at residue 225 was observed in virus strains isolated after 1986.

The amino acid changes on the HA molecule reduce or increase the extent of haemagglutination. Yewdell et al. (1986) isolated adsorptive mutants of an H3N2 virus in the presence of a mixture of monoclonal antibodies against haemagglutinin. These mutants possessed increased avidity for binding CRBC and had amino acid changes from the parental virus at residues 185, 231 or 244. In our work, the HA protein had decreased ability to bind CRBC. However, it is not clear why avidity for human and guine erythrocytes was unchanged.

Binding of influenza A viruses to erythrocytes involves two distinct specificities for sialic acid a2,6-Gal and a2,3-Gal linkages (Rogers et al., 1983; Suzuki et al., 1986). However, the receptor-binding specificity does not affect the ability to agglutinate CRBC (Nobusawa et al., 1991).

As described in Results, recent influenza A (H3N2) viruses also show similar host-specific haemagglutination. We did not find specific amino acid differences in the HA polypeptide between group 1 and group 2 H3N2 viruses isolated in the same year (data not shown). We suspect that the mechanism for host-specific haemagglutination by H3N2 viruses is similar to that of H1N1 viruses. It is interesting that two subtype viruses suddenly lost the ability to agglutinate CRBC. We cannot as yet explain the evolutionary or epidemiological significance of this phenomenon. Further study of the mechanism may help to answer these questions.
The results of reassortment (Table 1b) and expression (Fig. 3) experiments, indicated that the HA protein of group 1 viruses had lost the ability to agglutinate CRBC, but that group 1 virions retained the ability to agglutinate CRBC. Therefore, some modification to the HA protein of group 1 virus might be brought about by some other viral protein(s), restoring its ability to agglutinate CRBC. Reassortment experiments between A/Aichi/4/92 and A/WSN/33 viruses suggested that the M gene might affect the phenotype (Table 1b). The HA gene of reassortants PW10 and PW13 was expressed on Cos cells and it was confirmed that these HA proteins lost the ability to agglutinate CRBC. The phenotype of these reassortants was maintained for at least three passages, indicating that it was genetically determined. We suspected that the M gene might be responsible for the acquisition of haemagglutination of CRBC in A/Aichi/4/92 but not in A/Aichi/24/92. However, we did not find any amino acid differences between A/Aichi/24/92 and Aichi/4/92 in the coding and noncoding regions of the M1 and M2 proteins. One possible explanation is that the M gene product of A/WSN/33 does not fit the HA protein of A/Aichi/4/92, resulting in the group 2 phenotype. Detailed analysis of the modification of the HA protein and determination of the virus protein(s) responsible remains to be carried out.

References


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