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Hemagglutinin Residues of Recent Human A(H3N2) Influenza Viruses That Contribute to the Inability to Agglutinate Chicken Erythrocytes

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To identify the molecular determinants contributing to the inability of recent human influenza A(H3N2) viruses to agglutinate chicken erythrocytes, phenotypic revertants were selected upon passage in eggs or MDCK cells. The Leu194Ile or Val226Ile substitutions were detected in their hemagglutinin (HA) sequence concomitantly with the phenotypic reversion. Remarkably, as little as 3.5% of variants bearing a Val226Ile substitution was found to confer the ability to agglutinate chicken erythrocytes to the virus population. Hemadsorption assays following transient expression of mutated HA proteins showed that the successive Gln226 → Leu → Ile → Val changes observed on natural isolates resulted in a progressive loss of the ability of the HA to bind chicken erythrocytes. The Val226Ile change maintained the preference of the HA for SAα2,6Gal over SAα2,3Gal and enhanced binding of the HA to α2,6Gal receptors present on chicken erythrocytes. In contrast, simultaneous Ser193Arg and Leu194Ile substitutions that were found to confer the ability to agglutinate sheep erythrocytes increased the affinity of the HA for SAα2,3Gal. © 2001 Academic Press

Key Words: influenza; hemagglutinin; hemadsorption; receptor; sialic acid.

INTRODUCTION

The techniques of hemagglutination and hemadsorption, first described 60 years ago (Hirst, 1941), remain crucial in the detection of influenza viruses and in the antigenic characterization of their hemagglutinin (HA) and neuraminidase. Both techniques are based on the interaction between the viral HA and its cellular receptors, the sialic acids (SA), linked to cell surface glycoproteins and glycolipids. The most widespread form of SA is an N-acetylneuraminic acid (Neu5Ac) with an acetylated amino group at position 5. Substitution of one of the hydrogen atoms in the methyl moiety of the acetyl group by a hydroxyl group results in N-glycolylneuraminic acid (Neu5Gc), which is common in many animal species, especially in horses, guinea pigs, sheep, pigs, and cows (Suzuki *et al.,* 1985, 2000; Hanaoka *et al.,* 1989; Kotani and Takasaki, 1997), but not in humans, except in the case of particular cancers (for review, see Herrler *et al.,* 1995; Schauer *et al.,* 1995; Traving and Schauer, 1998). The efficiency of binding of influenza viruses is dependent on both the type of SA and the type of linkage that connects the SA residue with the oligosaccharide of the receptor molecule. It has been shown that human viruses preferentially bind to SA attached to galactose (Gal) by an α2,6 linkage, while avian and equine influenza strains bind preferentially to α2,3Gal-linked SA (Rogers and Paulson, 1983; Rogers *et al.,* 1983; Matrosovich *et al.,* 2000). In the case of the H2 and H3 subtype viruses, residues at position 226 and 228 in the HA have been involved in this specificity, since the HAs of human viruses contain Leu and Ser at 226 and 228, while the HAs of avian viruses contain Gln and Gly at the equivalent positions (Rogers *et al.,* 1983; Connor *et al.,* 1994; Matrosovich *et al.,* 1997, 2000; Vines *et al.,* 1998). Mutagenesis experiments have demonstrated that a Leu-to-Gln mutation at position 226 of a human virus HA can alter its specificity from SAα2,6Gal to SAα2,3Gal, and that an additional Ser-to-Gly mutation at position 228 enhances the specificity toward SAα2,3Gal (Naeve *et al.,* 1984; Vines *et al.,* 1998). However, some experimental data indicate that these positions are not the exclusive determinants of linkage specificity (Daniels *et al.,* 1987). For instance, the X-31 variant with the Leu at position 226, but a Ser193Arg change, showed altered binding properties as compared to the X-31 parent virus, as it acquired the ability to agglutinate erythrocytes containing exclusively SAα2,3Gal linkages. In the case of influenza viruses of the H1 subtype, human and avian isolates also recognize α2,6 and α2,3 linkages, respectively, but both of their HAs contain Gln at position 226 and Gly at position 228. Other residues in HAs of this subtype, including 145, 186, and 225, can also influence receptor specificity (Matrosovich *et al.,* 1997; Ito *et al.,* 1998). Taken together, these observations suggest that the SAα2,6Gal/SAα2,3Gal specificity of a given influenza vi-

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rus is most likely to be determined by a complex set of residues in its HA protein.

Receptor specificity of the HA of influenza A viruses has been correlated with their ability to agglutinate erythrocytes from different animal species (Ito *et al.,* 1997a). Indeed, the distribution of the different types of sialic acids differs among them. Erythrocytes from horse and cow display mainly the NeuGcα2,3Gal. In contrast, those from chicken and human display both NeuAcα2,6Gal and NeuAcα2,3Gal. Equine and avian viruses have been shown to agglutinate erythrocytes from all species analyzed, while human influenza viruses usually agglutinate human, guinea pig, and chicken erythrocytes, but not those from horses or cows (Ito *et al.,* 1997a). However, recent clinical isolates of human A(H1N1) and A(H3N2) influenza viruses appear to have lost the ability to agglutinate chicken erythrocytes (Morishita *et al.,* 1993; Nobusawa *et al.,* 2000). For H1N1 viruses, it has been described that amino acid substitutions in the presumed receptor-binding site of the HA were responsible for this phenotypic change (Morishita *et al.,* 1996). For H3N2 viruses, several changes among the receptor-binding residues (such as residues 135, 190, 194, and 226) have been observed, but only the Glu190Asp substitution has been correlated to the loss of the ability to agglutinate chicken erythrocytes (Cox and Bender, 1995; Lindstrom *et al.,* 1996, 1998; Fitch *et al.,* 1997; Mori *et al.,* 1999; Nobusawa *et al.,* 2000).

To identify viral molecular determinants contributing to the loss of ability of recent human A(H3N2) viruses to bind chicken erythrocytes, we tried to select phenotypic revertants of viruses isolated in Paris in 1997 upon passage in embryonated eggs or in Madin–Darby canine kidney (MDCK) cells. Indeed, both processes have been shown to possibly modify the agglutination properties of influenza virus isolates (Azzi *et al.,* 1993; Burnet and Bull, 1943).

Here, we show that upon passage in eggs, but also in MDCK cells, phenotypic variants of recent A(H3N2) viruses can be selected that have recovered the ability to agglutinate chicken erythrocytes. We compared the sequences of the HA, NA, M1, and M2 genes of several variants with those of the viral isolates from which they were derived. Amino acid substitutions were only detected in the HA1 portion of the HA gene, in particular at residues 226, 193, and 194. Using a hemadsorption assay on HA expressed from cloned cDNA, we demonstrated that a single substitution at residue 226 (Val → Ile) or 194 (Leu → Ile) was sufficient to restore the ability to agglutinate chicken erythrocytes. Our data further suggested that the successive Gln → Leu → Ile → Val changes observed at residue 226 since the emergence of H3N2 viruses in humans in 1968 could be responsible for a progressive loss of the ability of the HA to bind chicken erythrocytes. In addition, we showed that simultaneous Ser193Arg and Leu194Ile substitutions conferred the ability to agglutinate sheep erythrocytes. Finally, using desialylated and then resialylated chicken erythrocytes for hemagglutination and hemadsorption tests, we investigated whether the phenotypic changes associated with the Val226Ile as well as with the simultaneous Ser193Arg and Leu194Ile substitutions corresponded to a change in the receptor specificity of the HA.

RESULTS

Selection of phenotypic revertants of recent influenza A(H3N2) viruses that recovered the ability to agglutinate chicken erythrocytes

Recent A(H3N2) viruses have been shown to be unable to agglutinate chicken erythrocytes although they retain the ability to agglutinate guinea pig or human erythrocytes. To determine the molecular basis of the inability to bind chicken erythrocytes of these viruses, phenotypic revertants were selected from three representative viruses isolated in Paris in 1997 (P896, P906, and P908) upon passage on MDCK cells or on embryonated eggs. The ability of the P896, P906, and P908 viruses to agglutinate erythrocytes from different species was examined before and during passage history. Growth on MDCK cells or eggs did not alter the ability of P896 or P908 to agglutinate guinea pig or human erythrocytes nor their inability to agglutinate sheep and horse erythrocytes (Table 1). Interestingly, the P906 virus acquired the ability to agglutinate sheep erythrocytes upon two passages in eggs (C1E2, Table 1). Although none of the P896, P906, and P908 isolates (C1) were able to agglutinate chicken erythrocytes, this property was acquired upon 14 or 5 passages on MDCK cells, for P896 and P908, respectively, or following one or two passages in eggs, for P906 and P908, respectively (Table 1). Sequences of the HA, NA, M1, and M2 genes of the P896, P906, and P908 viruses were examined upon isolation on MDCK cells (C1) and following passage on MDCK cells (Cn) or one or two passages on eggs (C1E1, C1E2) (Tables 1 and 2). The amino acid sequences of the NA, M1, and M2 genes were conserved. In contrast, variations of the amino acid sequence of the HA1 portion of the HA protein were detected (Table 2).

The HA1 sequences of the initial isolates (C1) of P896 and P906 were identical and showed six differences with the corresponding sequence of P908 (including the Asp133Asn change which is predicted to create a potential glycosylation site) (Table 2). The Leu194Ile change was detected in the P906 HA sequence at the same time as the recovery of binding to chicken erythrocytes (C1E1, Table 1) and may have thus contributed to this phenotypic change. Similarly, the concomitant detection of the Val226Ile substitution and the ability to agglutinate chicken erythrocytes (at C14 and C1E2 for P896 and P908, respectively, Table 1) suggested a possible correlation. An Asp53Asn change was also observed at C14

TABLE 1

Hemagglutination of Erythrocytes from Different Animal Species and Amino Acid Changes in the HA Upon Passage of Recent A(H3N2) Viruses in Eggs or MDCK Cells

Viruses	Passage history[a]	Hemagglutination with erythrocytes from[b]:					Change in HA	
		Guinea pig	Human	Chicken	Sheep	Horse	aa[c] 194	aa[c] 226
P896	C1	128	128	<2	<2	<2	Leu	Val
	C14	128	64	**16**	<2	<2	Leu	**Ile**
P908	C1	128	128	<2	<2	<2	Leu	Val
	C5	128	128	**4**	NT	NT	Leu	Val[d]
	C7	64	128	**32**	NT	NT	Leu	Val[e]
	C8	64	128	**32**	NT	NT	Leu	**Ile**
	C10	128	64	**16**	<2	<2	Leu	**Ile**
	C14	64	64	**16**	NT	NT	Leu	**Ile**
	C1E1	16	32	<2	NT	NT	NT	NT
	C1E2	128	128	**128**	NT	NT	Leu	**Ile**
P906	C1	256	256	<2	<2	<2	Leu	Val
	C14	128	64	<2	<2	<2	NT	NT
	C1E1	256	2048	**512**	<2	<2	**Ile**	Val
	C1E2	256	1024	**256**	32	<2	**Ile**[f]	Val

Note. NT, not tested. Changes associated with the ability to agglutinate chicken erythrocytes are indicated in bold characters and those associated with the ability to agglutinate sheep erythrocytes are underlined.

[a] CxEy where x refers to the number of passages in MDCK cells and y to the number of passages in eggs.

[b] Titers are expressed as the reciprocal of the highest virus dilution producing hemagglutination.

[c] Amino acid.

[d] Ile in 3.5% of individual cDNA clones.

[e] Ile in 33.3% of individual cDNA clones.

[f] In association with the Ser193 Arg substitution.

for the P896 virus; however, it seemed less likely to be implicated in the ability to bind chicken erythrocytes since residue 53 is far from the receptor binding site (Wiley *et al.,* 1981). Interestingly, during passages of P908 on MDCK cells, agglutination of chicken erythrocytes was detected at C5, while the Val226Ile change was detected only at C8. Molecular cloning of PCR products derived from the P908-HA1 cDNA at C5 and C7 was performed. Sequence analysis of individual clones revealed the presence of the Val226Ile substitution in a

TABLE 2

Amino Acid Changes in the HA1 Portion of the HA Protein for Recent A(H3N2) Viruses Upon Passage in MDCK Cells or Eggs

Virus	Passage history*	Amino acid residue at position:											
		53	57	91	92	122	133	186	193	194	226	246	275
P896	C1	D	Q	S	T	K	D	S	S	L	V	N	D
	C2	D	Q	S	T	K	D	I	S	L	V	N	D
	C14	N	Q	S	T	K	D	S	S	L	I	N	D
P906	C1	D	Q	S	T	K	D	S	S	L	V	N	D
	C5	D	Q	S	T	K	D	S	S	L	V	S	D
	C10	D	Q	S	T	K	D	S	S	L	V	S	D
	C1E1	D	Q	S	T	K	D	S	S	I	V	N	D
	C1E2	D	Q	S	T	K	D	S	R	I	V	N	D
P908	C1	D	R	T	K	N	N	S	S	L	V	N	G
	C8	D	R	T	K	N	N	S	S	L	I	N	G
	C10	D	R	T	K	N	N	S	S	L	I	N	G
	C14	D	R	T	K	N	N	S	S	L	I	N	G

Note. No differences in the HA2 portion of the HA protein were detected.

* CxEy where x refers to the number of passages in MDCK cells and y to the number of passages in eggs.

small proportion (1 of 29 cDNA clones analyzed, 3.5%) of the viral population at C5, and in a larger proportion (7 of 21 cDNA clones analyzed, 33.3%) at C7 (Table 1). These observations indicated that the virus population was mixed with regard to the nature of residue 226 as early as at C5 and suggested that a small proportion (as low as 3.5%) of variants bearing an Ile at position 226 could be sufficient to confer to the virus population the ability to agglutinate chicken erythrocytes.

The ability of P906 to agglutinate sheep erythrocytes seemed to be correlated with the Ser193Arg substitution detected at C1E2 (Tables 1 and 2). However, the Leu194Ile substitution detected after a single egg passage at C1E1 and still present at C1E2 (Tables 1 and 2) could also have played a role in the phenotypic change. The occurrence of both an Ile at position 194 and an Arg at position 193 have already been reported to result from egg adaptation (Hardy *et al.,* 1995; Robertson, 1993).

A few additional sequence changes were detected that were not correlated with a modification of the agglutination properties of the virus. For P896, a Ser186Ile substitution was detected in the HA1 upon two passages on MDCK cells (C2, Table 2). This substitution, which has been described before as related to egg adaptation and to an increased affinity for SAα2,3Gal (Gambaryan *et al.,* 1999), reverted at C14. For P906, an Asn246Ser substitution predicted to suppress a glycosylation site in HA1, was detected from passage C5 up to C10 (Table 2). In contrast, the Asn246 residue remained conserved upon two passages of P906 in eggs (Table 2), although its modification as a result of egg adaptation of human H3 viruses has been reported by others (Ito *et al.,* 1997b; Katz *et al.,* 1990).

Correlation between HA1 amino acid substitutions and hemadsorption activity on chicken and sheep erythrocytes

We used a hemadsorption assay to confirm that the ability of the P908- or P906-derived HA to bind chicken erythrocytes and the Val226Ile or Leu194Ile substitution detected in the HA1 were indeed correlated. The HA genes were cloned from P908 and P906 isolates (C1, Val226–Leu194), from P908 at C10 (Ile226–Leu194), and from P906 at C1E1 (Val226–Ile194). The HA-encoding sequences were cloned into plasmid pCI under the control of the CMV early promoter. The HA levels at the surface of transiently transfected COS-1 cells were similar for the wild-type (wt) and variant proteins, as determined by flow cytometry analysis (data not shown). Hemadsorption assays were performed as described under Materials and Methods using COS-1 cells transfected with the wt or variant HAs, and guinea pig or chicken erythrocytes. As shown in Fig. 1A, the wt HA of P908 or P906 (Val226–Leu194) presented hemadsorption activity with guinea pig but not with chicken erythrocytes. In contrast, the P908-derived HA with Ile226 or the P906-derived HA with Ile194 showed hemadsorption activity with chicken erythrocytes, while retaining the same hemadsorption activity with guinea pig erythrocytes as the wt proteins.

Similar experiments were performed to elucidate the molecular basis of the acquisition of the ability to agglutinate sheep erythrocytes by some P906-derived variants. The HA proteins derived from P906 at C1 (Ser193–Leu194), C1E1 (Ser193–Ile194), and C1E2 (Arg193–Ile194), as well as a P906-HA mutant (Arg193–Leu194) generated by site-directed mutagenesis, were transiently expressed in COS-1 cells. The hemadsorption assays clearly showed that the Leu194Ile or Ser193Arg substitutions alone were not able to confer to the P906-HA the ability to bind to sheep erythrocytes, whereas the P906-HA-derived protein with both substitutions showed hemadsorption activity on sheep erythrocytes (Fig. 1B).

Analysis of glycosidic linkages between SA and galactose of glycoconjugates on the surface of animal erythrocytes

To investigate whether the phenotypic changes associated with the Val226Ile and the Ser193Arg + Leu194Ile substitutions corresponded to a change in the receptor specificity of the HA, we first examined the nature of the SA linkages present at the surface of erythrocytes from different species by flow cytometry analysis. Such data were available for human, chicken, and horse erythrocytes (Ito *et al.,* 1997a), but to our knowledge, not for guinea pig and sheep erythrocytes. Here, the amounts of SAα2,3Gal and SAα2,6Gal present at the surface of guinea pig and sheep erythrocytes were compared with those at the surface of human and chicken erythrocytes, using linkage-specific lectins and flow cytometry analysis as described under Materials and Methods. The data shown in Fig. 2A revealed that guinea pig, chicken, and human erythrocytes displayed both types of linkages, while sheep erythrocytes presented only SAα2,3Gal moieties. Noticeably, the proportion of SAα2,3Gal relative to SAα2,6Gal appeared higher on chicken erythrocytes than on guinea pig or human erythrocytes and could therefore be determinant with respect to agglutination.

To study the receptor specificity of P896- and P908-derived viruses containing a Val or an Ile at residue 226 of the HA, and of P906-derived viruses bearing a Ser193/Leu194 or Arg193/Ile194 sequence in the HA protein, desialylated chicken erythrocytes were reconstituted with either α2,6- or α2,3Gal-linked SA and used for virus hemagglutination. The levels of SA on resialylated cells were compared to those on native chicken erythrocytes by flow cytometry analysis (Fig. 2B). The α2,6-resialylated cells displayed a higher level of SAα2,6Gal than native erythrocytes, and they were agglutinated by the reference strains A/Sydney/5/97 and A/PR/8/34 as effi-

A pCI P908/Val226 P908/Ile226 P906/Leu194 P906/Ile194
Guinea pig
Chicken
B
Sheep
pCI P906/Ser193+Leu194 P906/Ser193+Ile194 P906/Arg193+Leu194 P906/Arg193+Ile194

FIG. 1. (A) Effect of substitutions at residues 194 or 226 of the HA from P906 and P908 viruses on the efficiency of hemadsorption of chicken and guinea pig erythrocytes. (B) Effect of substitutions at residues 193 and/or 194 of the HA of P906 on the efficiency of hemadsorption of sheep erythrocytes. COS-1 cells were transfected with pCI or pCI-HA plasmids that expressed the wt or mutant HA proteins, as indicated. The hemadsorption assay was performed as described under Materials and Methods using chicken and guinea pig (A) or sheep (B) erythrocytes, as indicated.

ciently as native erythrocytes (Table 3). The variants P896-C14 (Ile226) and P908-C10 (Ile226) that had recovered the ability to agglutinate native chicken erythrocytes agglutinated SAα2,6Gal-resialylated erythrocytes as well (Table 3). Remarkably, the parent viruses P896 and P908 (C2 passage, Val226), that were unable to agglutinate

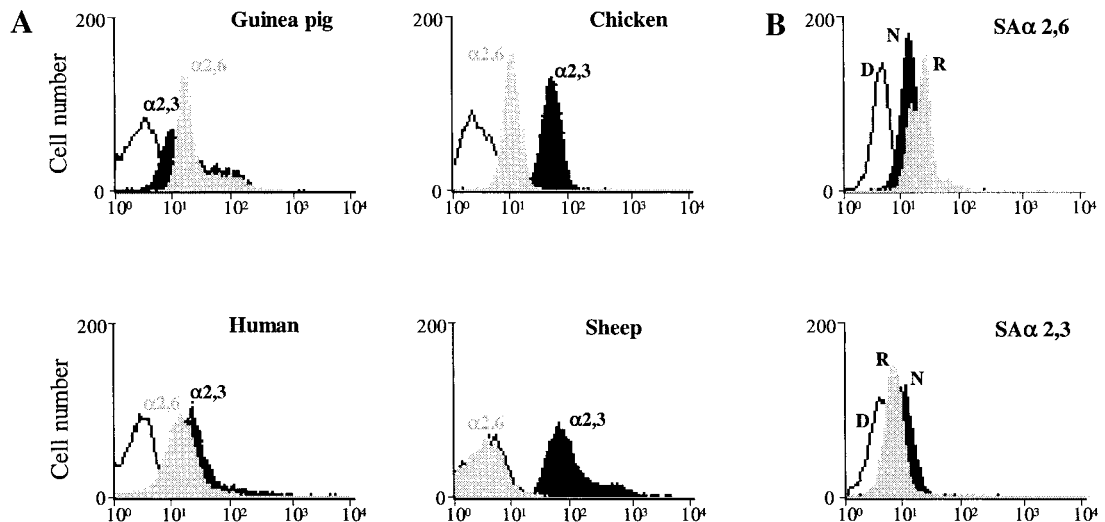

FIG. 2. (A) Comparison of the relative amounts of SAα2,3Gal and SAα2,6Gal on the surface of guinea pig, chicken, human, and sheep erythrocytes. The cells were incubated with DIG-labeled *Sambucus nigra* agglutinin (specific of SAα2,6Gal, gray histograms); DIG-labeled *Maackia amurensis* agglutinin (specific of SAα2,3Gal, black histograms); or without lectins (negative control, open histograms). Lectins attached to the erythrocytes were detected with FITC-labeled anti-DIG antibodies and flow cytometry analysis was performed, as described under Materials and Methods. Total amount of SA for chicken and human erythrocytes is 325 and 617 nmol/ml, respectively, according to Nobusawa *et al.* (2000); it is 600 and 530 nmol/ml for human and guinea pig erythrocytes, respectively, according to Shukla and Schauer (1982). (B) Relative amounts of SAα2,6- or α2,3-Gal on native (N, black histograms), desialylated (D, open histograms), or resialylated (R, gray histograms) chicken erythrocytes. Desialylation and resialylation with α2,6-sialyltransferase (SAα2,6) or α2,3-sialyltransferase (SAα2,3) were performed as described under Materials and Methods.

TABLE 3

Receptor Specificities of A(H3N2) Viruses P896, P906, and P908

Virus	Passage history[a]	Agglutination of chicken erythrocytes:			
		Native	Desialylated	Resialylated NeuAcα2,6Gal	Resialylated NeuAcα2,3Gal
P896	C2 (Val226)[b]	<2	<2	64	<2
	C14 (Ile 226)	16	<2	64	<2
P908	C2 (Val226)	<2	<2	64	<2
	C10 (Ile226)	32	<2	128	<2
P906	C2 (Ser193/Leu194)	<2	<2	32	<2
	C1E2 (Arg193/Ile194)	256	<2	256	128
A/Sydney/5/97[c]	Unknown[e] (Ile226/Ser193/Ile194)	128	<2	128	64
A/PR/8/34[d]	Unknown	1024	<2	2048	1024

[a] Cx Ey where x refers to the number of passages in MDCK cells and y to the number of passages in eggs.
[b] Amino acid residues present at the indicated positions from the HA1 protein sequence (H3 numbering).
[c] A(H3N2) reference strain.
[d] A(H1N1) reference strain.
[e] This virus has undergone multiple passages on eggs and was produced on MDCK cells for this analysis.

native chicken erythrocytes which display both SAα2,6Gal and SAα2,3Gal, appeared to agglutinate resialylated erythrocytes containing only the SAα2,6Gal linkage with an efficiency similar to their respective C14 and C10 variants. These results taken together suggest that the α2,6 sialyloligosaccharides at the surface of native erythrocytes have some characteristics that distinguish them from those at the surface of resialylated erythrocytes. The α2,3-resialylated cells displayed less SAα2,3Gal moieties than native chicken erythrocytes (Fig. 2B), but however they were efficiently agglutinated by the A/PR/8/34 virus and, surprisingly, by the A/Sydney/5/97 H3N2 reference strain which displayed an Ile226 (Table 3). Unlike α2,6 resialylated cells, they were not agglutinated by any of the parent or variant P896 and P908 viruses, whether harboring a Val or an Ile at position 226 of the HA (Table 3). Remarkably, the P906-C1E2 variant that had acquired the ability to agglutinate sheep erythrocytes (Table 1), but not the P906-C2 parental virus, agglutinated SAα2,3Gal-resialylated erythrocytes (Table 3). This observation, together with the fact that sheep erythrocytes only display SAα2,3Gal (Fig. 2A), suggested that the simultaneous Ser193Arg and Leu194Ile changes conferred to the HA the ability to bind to SAα2,3Gal.

Effect of various amino acid substitutions at position 226 of the HA protein on hemadsorption activity on native and α2,6- or α2,3-resialylated chicken erythrocytes

Since the emergence of human influenza A(H3N2) viruses in 1968, four major amino acid residues were described at position 226 of the HA molecule: Leu (1968/1991), Gln (1992/1994), Ile (1994/1997), and more recently Val (1996–2000) (Bush *et al.,* 1999; Cox and Bender, 1995; Fitch *et al.,* 1997; Lindstrom *et al.,* 1996, 1998; Mori *et al.,* 1999). Influenza A(H3N2) viruses that were unable to agglutinate chicken erythrocytes were first detected at the beginning of the 1990s. To evaluate the influence of each amino acid found at position 226 on the efficiency of binding to chicken erythrocytes, the P908-HA sequence was modified by site-directed mutagenesis, so as to substitute the Val residue at position 226 by a Gln, Leu, Ile, Asp (an acidic amino acid), His (a basic amino acid), or Ala residue.

The expression levels of the wt and mutated HA proteins at the cell surface were first examined by flow cytometry analysis and were found to be similar (data not shown). The wt and mutant HAs were compared using a hemadsorption assay as described earlier. Bound erythrocytes were disrupted and the concentration of hemoglobin in the lysate was evaluated by measuring the absorbance at 540 nm. As shown in Fig. 3A, for the Ile, Leu, and Gln 226 mutants, hemadsorption of chicken erythrocytes was clearly increased as compared to the wt (Val226) HA, while hemadsorption of guinea pig erythrocytes was less variable between mutated and wt HAs. For the His226 mutant, a twofold increase of hemadsorption of chicken erythrocytes was observed, whereas hemadsorption of guinea pig erythrocytes was similar to that of wt HA. The HA with an Ala226 residue showed a lower hemadsorption activity than the wt on guinea pig erythrocytes, while the HA with an Asp226 residue showed no hemadsorption activity. We next tested the wt (Val226), Ile, Leu, and Gln mutant HAs for their ability to bind to either native, α2,6-, or α2,3-resialylated chicken erythrocytes in the hemadsorption assay. As shown in Fig. 3B, the HA of the A/PR/8/34 (H1N1) reference strain used as a control exhibited high hemadsorption activity on native as well as on α2,6- or α2,3-resialylated erythrocytes. Hemadsorption activity on α2,6-resialylated cells was variable and correlated with hemadsorption of

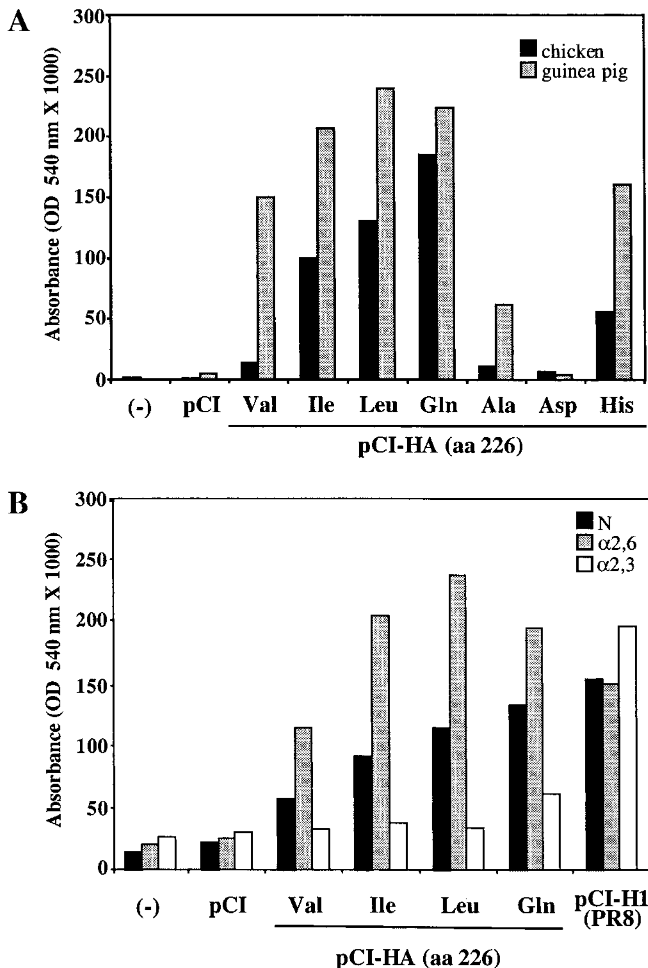

FIG. 3. Hemadsorption activity of wt (Val226) and mutant HA proteins. The hemadsorption assay was performed as described under Materials and Methods on COS-1 cells transfected with pCI or pCI-HA plasmids that expressed wt or mutant HA proteins derived from the P908 virus, or with pCI-H1 that expressed the HA from the A/PR/8/34 reference strain, as indicated. The hemoglobin released by lysis of bound erythrocytes was measured by absorbance at 540 nm. (−) Control nontransfected COS-1 cells. (A) Hemadsorption activity was measured on chicken (black histograms) and guinea pig (gray histograms) erythrocytes. (B) Hemadsorption activity was measured on native (N) chicken erythrocytes (black histograms), or on chicken erythrocytes desialylated and resialylated with α2,6- (gray histograms) or α2,3-sialyltransferase (white histograms). Experiments were repeated three times, and representative data are shown.

native erythrocytes, both being significantly higher for the mutant HAs than for the HA with a Val226 residue (Fig. 3B). In contrast, none of the wt or mutant HAs showed significant hemadsorption activity on α2,3-resialylated erythrocytes (Fig. 3B), in accordance with the results of hemagglutination experiments (Table 3).

DISCUSSION

Whereas several studies have shown that isolation of human influenza viruses in eggs selects variants with amino acid substitutions in the HA molecule, MDCK cells have not been found to readily select receptor-binding variants (Govorkova *et al.,* 1999; Gubareva *et al.,* 1994; Hardy *et al.,* 1995; Katz *et al.,* 1987; Meyer *et al.,* 1993; Robertson, 1993; Robertson *et al.,* 1987). Ito *et al.* (1997b) suggested that the fact that allantoic cells display only the SAα2,3Gal type of linkage could be responsible for selection of variants with SAα2,3Gal specificity in the allantois of chicken eggs. Nevertheless, we demonstrated here that growth on both MDCK cells and eggs allowed the selection of variants with amino acid substitutions in the receptor binding site of the HA. Since MDCK cells display both SAα2,3Gal and SAα2,6Gal receptors (Govorkova *et al.,* 1999; Ito *et al.,* 1997b), the nature of the selective pressure exerted by growth on these cells is unclear.

In this report, we demonstrated that the nature of the residues at position 226 or 194 in the HA protein may contribute to the inability of recent influenza H3N2 isolates to agglutinate chicken erythrocytes. Variations at residue 194 have already been observed, mostly in an experimental context, and are likely to result from host cell adaptation (Govorkova *et al.,* 1999; Hardy *et al.,* 1995; Lindstrom *et al.,* 1996). We focused on variations at residue 226 because they have been observed among circulating strains of influenza viruses. Residue 226 has been shown to be the most critical residue within the HA receptor binding pocket with respect to host specificity of influenza viruses of the H3 subtype (Martin *et al.,* 1998; Naeve *et al.,* 1984). Indeed, the introduction of viruses with an avian-derived H3 in the human population in 1968 was rapidly followed by a Gln226Leu change in the HA (Bean *et al.,* 1992), which has been shown to correspond to a switch from SAα2,3Gal to SAα2,6Gal receptor specificity (Connor *et al.,* 1994; Matrosovich *et al.,* 1997; Rogers *et al.,* 1983; Vines *et al.,* 1998). Surprisingly, residue 226 later reverted to the ancestral Gln. Indeed, most circulating strains isolated between 1992 and 1994 contained a Gln at position 226, but cocirculating strains with an Ile at the same position were also described (Bush *et al.,* 1999; Fitch *et al.,* 1997). Interestingly, phylogenetic analysis of the HA1 portion of the H3 reveals that the Leu226Gln revertant viruses were clustered in an uncompetitive side branch and soon stopped evolving, while the strains containing Ile226 evolved further and were incorporated into the successful trunk lineage (Bush *et al.,* 1999; Fitch *et al.,* 1991, 1997). Viruses with an Ile226 residue evolved more recently to Val226 (Bush *et al.,* 1999; Fitch *et al.,* 1997; Lindstrom *et al.,* 1996). Using a hemadsorption assay, we demonstrated that the successive changes Gln → Leu → Ile → Val at residue 226, which were observed since the human H3N2 viruses emerged, were responsible for the progressive loss of the HA ability to bind chicken erythrocytes.

A Glu190Asp substitution in the HA of human H3N2 influenza viruses isolated recently in Japan was also shown by Nobusawa *et al.* (2000) to be implicated in the

inability of recent H3N2 viruses to agglutinate chicken erythrocytes. However, it is important to note that although all the HA proteins included in our study showed an Asp190, some of these were nonetheless able to bind chicken erythrocytes. This observation suggests that the phenotypic change induced by a given substitution in the HA may depend on other characteristics of the amino acid sequence of the protein. Also, it cannot be excluded that the phenotypic change observed at the level of the HA is not the only factor that determines the modification of agglutination properties of the virus. For H1N1 viruses, a possible role in the binding to erythrocytes has been suggested for proteins other than the HA, such as the NA or M1 (Morishita *et al.,* 1993, 1996; Tong *et al.,* 1998). However, in the case of our H3N2 variants, we found no changes in the NA or M segments that may have contributed to the acquisition of the ability to agglutinate chicken erythrocytes.

Residues 190 and 226 are both part of the receptor binding site of the hemagglutinin (Weis *et al.,* 1988; Wilson *et al.,* 1981). Residue 226 has been shown to be determinant for the relative ability of the HA to interact with SAα2,6Gal or SAα2,3Gal receptors. However, it is not clear how variations of this residue could influence the ability of H3N2 viruses to agglutinate chicken erythrocytes, since these display both SAα2,6Gal and SAα2,3Gal. Our experiments using linkage-specific lectins and flow cytometry analysis demonstrated that chicken erythrocytes displayed a lower proportion of SAα2,6Gal relative to SAα2,3Gal than guinea pig and human erythrocytes. Accordingly, analysis of the amount of SA released from erythrocytes using a specific α2,3 sialidase showed that 56% of the total SA were α2,3 linked for chicken erythrocytes, compared to 28% for human erythrocytes (Nobusawa *et al.,* 2000). Given the fact that the total amount of sialic acid, as measured by several teams using different methods, has been repeatedly found to be about twofold lower at the surface of chicken erythrocytes (Eylar *et al.,* 1962; Sarris and Palade, 1979; Shukla and Schauer, 1982; Rogers *et al.,* 1986), it can be estimated that the density of SAα2,6Gal is about threefold lower on chicken erythrocytes as compared to human or guinea pig erythrocytes, while the density of SAα2,3Gal is similar on both cell types. This extrapolation to relative SA densities is allowed, as the three types of cells are in the same size range.

A hypothesis is that the low density of SAα2,6Gal receptors on chicken erythrocytes is the limiting factor for their agglutination by recent H3N2 viruses. In support of the above hypothesis, we found that resialylated chicken erythrocytes displayed more SAα2,6Gal linkages than native chicken erythrocytes and that these resialylated erythrocytes were agglutinated by recent human H3N2 viruses. Another explanation would rely on the possibility that the SA receptors present on native chicken erythrocytes are linked to different types of saccharides than to the Galβ(1,4)GlcNAc unit, which is recognized by the rat liver 2,6-sialyltransferase used in our experiments. Indeed, the SAα2,6 that we detected on the chicken erythrocytes by flow cytometry analysis using the SNA lectin may be linked either to D-galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc), given the specificity of this lectin. It could thus be hypothesized that the nature of residue 226 could affect the ability of the HA to discriminate between the different types of underlying saccharide rings of the SA receptor molecule, as has been previously suggested for residue 190 by Nobusawa *et al.* (2000). Experiments to address this possibility are currently in progress.

The phenotype of recent H3N2 isolates could also be related to an inability of their HA to bind SAα2,3Gal moieties. However, neither the wt viruses with Val226 nor the phenotypic revertants with Ile226 agglutinated sheep erythrocytes, which display mostly SAα2,3Gal as documented here by flow cytometry analysis, and by others using biochemical methods (Kotani and Takasaki, 1997; Krotkiewski, 1988). Consistently, viruses with Ile226 did not agglutinate erythrocytes resialylated with SAα2,3Gal, whereas they did agglutinate those resialylated with SAα2,6Gal. Furthermore, mutant HAs with Ile or Leu226 showed significantly higher hemadsorption activity than the HA with Val226 on α2,6- but not on α2,3-resialylated erythrocytes. These findings suggested that the Val226Ile substitution that conferred the ability to bind chicken erythrocytes to the HA did not change the specificity of the HA toward SAα2,3Gal, but rather enhanced binding affinity of the HA for SAα2,6Gal. Surprisingly, the A/Sydney/5/97 (H3N2) reference strain was found to agglutinate α2,3- as well as α2,6-resialylated erythrocytes despite the presence of an Ile at position 226 and a Ser at position 193 of the HA. This observation suggests that other determinants of the amino acid sequence of the HA that contribute to its ability to bind SAα2,3Gal may have been acquired upon multiple passage of this virus in eggs. Remarkably, the mutant P908-HA with Gln226 did not agglutinate α2,3-resialylated erythrocytes, whereas the presence of a Gln at residue 226 of H3 proteins derived from avian viruses has been shown to be determinant for binding to SAα2,3Gal (Rogers *et al.,* 1983; Vines *et al.,* 1998). This observation further emphasizes the fact that other determinants than residue 226 are involved in the ability to bind SAα2,3Gal. These may have evolved along with residue 226 upon circulation of the 1968 reassortant-derived H3N2 viruses in the human population.

Unlike the Val226Ile substitution, the concomitant Ser193Arg and Leu194Ile substitutions detected in the HA of the P906 variants at C1E2 conferred the ability to agglutinate sheep erythrocytes, and they are therefore of particular interest. Here, we demonstrated that this ability was correlated with the capacity to recognize the SAα2,3Gal linkage mostly present in sheep erythrocytes.

It has been reported by Daniels *et al.* (1987) that a variant of the X-31 virus with a Ser193Arg change acquired the ability to agglutinate erythrocytes containing only SAα2,3Gal, while retaining its ability to agglutinate those containing only SAα2,6Gal. Since residue 193 is relatively far from the region of the receptor binding site (Eisen *et al.,* 1997), it was speculated that this residue might interact with more underlying saccharide rings of the SA receptor molecules (Matrosovich *et al.,* 2000). The nature of residue 193 could be particularly determinant for the binding of the HA to SA of the Neu5Gc type, which are also present on sheep erythrocytes (Kotani and Takasaki, 1997; Skehel and Wiley, 2000). The role of residue 193 in the recognition of sheep erythrocytes and hence, in the specificity of the HA toward NeuAcα2,3Gal or NeuGcα2,3Gal receptors, is currently being examined.

Interestingly, during passages of the P908 virus on MDCK cells, we observed agglutination of chicken erythrocytes as early as at C5, while the Val226Ile change was detected only at C8. By sequencing individual clones of the HA gene, we found that at C5, a small proportion (3.5%) of variants bearing an Ile at position 226 was sufficient to confer the ability to agglutinate chicken erythrocytes to the virus population. This may be the reason no HA change associated with the recovery of chicken erythrocytes agglutination following passage on MDCK cells could be detected in previous studies that relied on the sequencing of PCR products (Azzi *et al.,* 1993; Morishita *et al.,* 1993).

MATERIALS AND METHODS

Viruses and cells

Human influenza A(H3N2) viruses A/Paris/896/97 (P896), A/Paris/906/97 (P906), and A/Paris/908/97 (P908) used in this study were isolated on Madin–Darby canine kidney cells directly from clinical specimens (nasal swabs) and passaged either 14 times in these cells or twice in 11-day-old embryonated chicken eggs. The collected infectious culture fluids were stored at −80°C. These viruses were antigenically and genetically related to the A/Nanchang/933/95 (H3N2) reference strain, as were all influenza A(H3N2) viruses isolated in France during the 1996/1997 season. MDCK cells were cultivated in Eagle's minimal essential medium (MEM) containing 8 mM tricine, 40 μg/ml gentamicin, and 5% fetal calf serum (FCS). COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and supplemented with 10% FCS.

Hemagglutination test

The hemagglutination test was performed at room temperature with a 0.5% suspension of guinea pig, chicken, human, horse, or sheep erythrocytes in PBS (Charles River), as described previously (Dowdle *et al.,* 1979).

Sequencing of the NA and M genes

Viral RNA was extracted from allantoic fluids or cell culture supernatants using the Trizol reagent (Life Technologies). cDNAs were synthesized using the 5′-AGCAAAAGCAGG-3′ oligonucleotide and the AMV-reverse transcriptase (Promega). The neuraminidase and matrix sequences were amplified by PCR using, respectively, the oligonucleotide pairs 5′-AGCAAAAGCAGGAGTGAAGATG-3′ and 5′-CAAGGAGTTTTTTTCTAAAATTGCG-3′ or 5′-AGCAAAAGCAGGTAGATATTGAAA-3′ and 5′-AGTAGAAACAAGGTAGTTTTTTACT-3′ and the AmpliTaq enzyme (Perkin–Elmer). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using N2 and M-specific primers, a Big Dye terminator sequencing kit (Perkin–Elmer), and an automated sequencer (ABI Prism 377, Perkin–Elmer).

Sequencing and cloning of the HA genes

Viral RNA was extracted as indicated above. For sequencing purposes, the entire HA coding sequences were amplified by PCR using oligonucleotide pairs 5′-AAGCAGGGGATAATTCTATTAACC-3′ and 5′-AGAAACAAGGGTGTTTTTAATTACT-3′. The nucleotide and protein sequences are available from GenBank under Accession Nos. AF363502, AF363503, and AF363504. For cloning purposes, the HA coding sequences were amplified by PCR using oligonucleotides 5′-GCAGACGCGTCATGAAGACTATCATTGCTTTGAG-3′ and 5′-GTGCGTCGACTCAAATGCAAATGTTGCACCTAA-3′, which, respectively, included a *Mlu*I and a *Sal*I restriction enzyme site (underlined). The PCR products were cloned between the *Mlu*I and *Sal*I sites of the pCI expression vector (Promega) downstream of the CMV immediate-early enhancer/promoter. Positive clones were sequenced as indicated above.

Construction of mutants

cDNAs encoding a mutant P908-HA with a Val226 → Leu, Gln, Ile, Ala, Asp, or His substitution or a mutant P906-HA with a Ser193 → Arg substitution were generated following an overlap extension PCR protocol (Pogulis *et al.,* 1996) using the pCI-HA plasmid as a template. Proofreading *Pwo* polymerase (Roche) was used for PCR amplification. The sequence of the primers used to generate overlapping PCR products and the subsequent fusion PCR product can be obtained from the authors upon request. The fusion PCR products containing the desired mutations were subcloned into pCI-P908/HA or -P906/HA plasmids, using the *Nsi*I and *Xba*I or *Mlu*I and *Xba*I restriction sites, respectively. Positive clones were sequenced as indicated above.

Hemadsorption assay on HA expressing cells

The procedures used for the hemadsorption assay were as described previously (Morishita *et al.,* 1996; Vines *et al.,* 1998). Briefly, 1.5×10^6 COS-1 cells in 35-mm dishes were transfected with 2 μg purified pCI-HA plasmid mixed with 6 μl Fugene reagent (Roche) according to the manufacturer's recommendations. After 16 h incubation at 37°C, the medium was replaced with 2 ml DMEM supplemented with 10% FCS, and the cells were incubated for 24 h at 37°C. After removal of the medium, cells were washed twice with PBS containing 10% FCS, treated with *Vibrio cholerae* sialidase (5.5 mU/ml, Roche) for 1 h at 37°C, washed twice again with PBS–10% FCS, and incubated for 1 h at 4°C with chilled 1% chicken or guinea pig erythrocyte suspensions (Charles River) in PBS. The cells were washed five times with PBS and fixed with methanol. After being air dried, the cells were stained with a 1:20 dilution of Giemsa solution (Reactifs RAL) and examined using a Leica DMRB microscope and the CollSNAP software. Alternatively, adsorbed erythrocytes were disrupted with 1 ml lysis buffer (CAT ELISA Kit, Roche) and the concentration of hemoglobin in the lysate was evaluated by measuring the absorbance at 540 nm.

Flow cytometry analysis

At 40 h posttransfection, COS-1 cells transfected with pCI-HA plasmids were washed twice with PBS–0.02% NaN_3 and then incubated with 1 ml PBS–2 mM EDTA for 20 min at 37°C. The cells were maintained in suspension, spun down, washed twice with PBS–0.02% NaN_3, and incubated for 30 min at 4°C with an anti-A/Sydney/5/97 (H3N2) virus rabbit serum, diluted 1:1000 in PBS–0.02% NaN_3–1% BSA. The cells were then washed once with PBS–0.02% NaN_3 and incubated for 30 min at 4°C with fluorescein-labeled goat anti-rabbit immunoglobulin antibodies (Southern Biotechnology Associates Inc.), diluted 1:200 in PBS–0.02% NaN_3–1% BSA. Following two washes and fixation in PBS containing 2% paraformaldehyde, the cells were analyzed for fluorescence intensity on a FACScalibur fluorospectrometer (Becton–Dickinson).

For analysis of the relative amounts of SAα2,3Gal and SAα2,6Gal moieties on the surface of guinea pig, chicken, human, and sheep erythrocytes, flow cytometry analysis was performed using linkage-specific lectins (Glycan Differentiation Kit, Roche), as described previously (Govorkova *et al.,* 1999; Ito *et al.,* 1997a). In brief, 5×10^6 erythrocytes were incubated at 4°C for 1 h with 0.1 μg/ml digoxigenin (DIG)-labeled *Sambucus nigra* agglutinin 1 (SNA 1), which specifically binds to Neu5Ac/Neu5Gc-SAα2,6Gal/GalNAc residues (Shibuya *et al.,* 1987, 1988), or with 0.5 μg/ml DIG-labeled *Maackia amurensis* agglutinin (MAA), specific for Neu5Ac/Neu5Gc-SAα2,3Gal residues (Wang and Cummings, 1988; Knibbs *et al.,* 1991). Control cells were incubated without lectins. After two washes with cold PBS the cells were incubated for 30 min at 4°C with fluorescein-conjugated anti-DIG antibodies (Roche) diluted 1:200 in PBS, washed twice with PBS, and then analyzed for fluorescence intensity on a FACScalibur fluorospectrometer (Becton–Dickinson).

Hemagglutination receptor specificity assay

Chicken erythrocytes were enzymatically modified to contain either the NeuAcα2,6Gal or the NeuAcα2,3Gal linkages as previously described (Kosa *et al.,* 1993). Briefly, 10^9 cells were desialylated by incubation at 37°C during 30 min with 1 ml TMB containing 200 mU neuraminidase from *V. cholerae* (Roche). Resialylation was performed on 60 μl packed erythrocytes resuspended in 40 μl CSB containing 1.5 mM CMP-AcNeu (Roche) and incubated at 37°C for 4 h with 6 mU α2,6- or α2,3-sialyltransferase (Roche) to generate NeuAcα2,6Galβ1,4GlcNAc or NeuAcα2,3-Galβ1,4GlcNAc/-Galβ1,3GlcNAc moieties, respectively. The efficiency of desialylation and resialylation was controlled by flow cytometry analysis using a lectin specific for the SAα2,6Gal or SAα2,3Gal linkage as described above. Hemagglutination and hemadsorption assays were performed at 4°C using native, desialylated, and resialylated erythrocytes.

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