Virology 258, 232–239 (1999)

Effects of Egg-Adaptation on the Receptor-Binding Properties of Human Influenza A and B Viruses

A. S. Gambaryan,* J. S. Robertson,† and M. N. Matrosovich*,‡,[1]

*M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, 142782 Moscow, Russia; †National Institute for Biological Standards and Control, Blanche Lane, Potters Bar, Herts EN6 3QG, United Kingdom; and ‡Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, Tennessee 38105

Received December 11, 1998; returned to author for revision March 2, 1999; accepted March 31, 1999

Propagation of human influenza viruses in embryonated chicken eggs (CE) results in the selection of variants with amino acid substitutions near the receptor-binding site of the hemagglutinin (HA) molecule. To evaluate the mechanisms by which these substitutions enable human virus growth in CE, we studied the binding of 10 human influenza A (H1N1, H3N2) and B strains, isolated and propagated solely in MDCK cells, and of their egg-adapted counterparts to preparations of cellular membranes, gangliosides, sialylglycoproteins, and sialyloligosaccharides. All egg-adapted variants differed from nonadapted strains by increased binding to the plasma membranes of chorio-allantoic (CAM) cells of CE and by the ability to bind to CAM gangliosides. In addition, there was no decrease in affinity for inhibitors within allantoic fluid. These findings indicate that growth of human influenza viruses in CE is restricted because of their inefficient binding to receptors on CAM cells and that gangliosides can play an important role in virus binding and/or penetration. The effects of the egg-adaptation substitutions on the receptor-binding properties of the viruses include (i) enhancement of virus binding to the terminal Sia(α2–3)Gal determinant (substitutions in HA positions 190, 225 of H1N1 strains and in position 186 of H3N2 strains); (ii) a decrease of steric interference with more distant parts of the Sia(α2–3Gal)-containing receptors (a loss of glycosylation sites in positions 163 of H1 HA and 187 of type B HA); and (iii) enhanced ionic interactions with the negatively charged molecules due to charged substitutions at the tip of the HA [187, 189, 190 (H1), and 145, 156 (H3)]. Concomitantly with enhanced binding to Sia(α2–3)Gal-terminated receptors, all egg-adapted variants decreased their affinity for equine macroglobulin, a glycoprotein bearing terminal 6′-sialyl(N-acetyllactosamine)-moieties.

INTRODUCTION

Influenza virus infection is initiated by virus attachment to terminal sialyloligosaccharide receptor determinants of cell surface glycoproteins and/or gangliosides. Two major types of terminal sialyl-galactose moieties are encountered in nature, Sia(α2–6)Gal and Sia(α2–3)Gal; human influenza viruses preferentially bind to the former (reviewed by Paulson, 1985; Suzuki, 1994). This binding specificity of human influenza viruses appears to be maintained due to a predominance of Sia(α2–6)Gal residues on the surface of ciliated cells of human respiratory epithelium and an abundance of Sia(α2–3)Gal moieties in human respiratory mucins (Baum and Paulson, 1990; Couceiro *et al.,* 1993).

Propagation of human influenza A and B viruses that are present in clinical material in the allantoic cavity of embryonated chicken eggs (CE) results in the selection of variants with amino acid substitutions around the receptor-binding site of the hemagglutinin (HA) (reviewed by Robertson, 1993). Several variants with different substitutions can be derived from a single human specimen and usually only one, although sometimes two, amino acid substitutions are found in each variant. Viruses that belong to different HA types and subtypes acquire distinct (type- and subtype specific) substitutions during egg adaptation. Unlike egg-adapted variants, human viruses isolated in Madin–Darby canine kidney (MDCK) cell cultures are usually homogeneous and identical to viruses replicating in humans, at least in their HA (Katz *et al.,* 1990; Robertson *et al.,* 1990, 1991; Katz and Webster, 1992).

Ito *et al.* (1997) provided a plausible explanation for the selection of influenza virus variants in CE and for the absence of such selection in MDCK cells. Using sialic acid-binding lectins for the detection of sialo-sugar determinants on the cell surface, they found that Sia(α2–3)Gal moieties but not Sia(α2–6)Gal moieties are exposed on the cells of the chicken embryo chorio-allantoic membrane (CAM), the site of influenza virus replication in CE. They also found that non-egg-adapted human H3N2 viruses bound poorly to the Sia(α2–3)Gal-containing gangliosides and displayed a high affinity for Sia(α2–6)Gal-containing gangliosides, whereas the egg-adapted variants often changed the receptor-binding specificity toward preferential recognition of the Sia(α2–3)Gal. In contrast to CAM cells, MDCK cells were found to express both Sia(α2–6)Gal and Sia(α2–3)Gal moieties. This feature could explain why human H3 strains that

[1] To whom reprint requests should be addressed. Fax: (901) 523-2622. E-mail: Mikhail.Matrosovich@stjude.org.

0042-6822/99 $30.00

were isolated and passaged solely in MDCK cells accumulate no substitutions in the HA and retained their receptor-binding characteristics. We have previously confirmed and extended these data by showing that all non-egg-adapted human influenza A (H1 and H3) and B viruses, which were isolated and grown solely in MDCK cells, share a common high binding affinity for 6′-sialyl(*N*-acetyllactosamine) [6′SLN; Neu5Ac(α2–6)Gal(β1–4)GlcNAc] and that all their egg-adapted counterparts display enhanced binding to Neu5Ac(α2–3)Gal-containing synthetic glycopolymers (Gambaryan *et al.,* 1997). These data suggested that the CE selects receptor-binding variants of human viruses because the affinity of authentic human viruses for the Sia(α2–3)Gal-containing receptors on the target cells of the CAM is too low. However, this suggestion was not proved experimentally because no systematic studies on the comparison of binding to CAM cells of nonadapted and of the egg-adapted human viruses were performed. Furthermore, Hardy *et al.* (1995) reported that, besides the CAM cells, inhibitors present in the allantoic fluid could be responsible for the selection of at least some of the egg-adapted variants.

In this paper we have investigated whether egg adaptation always leads to enhancement of virus binding to receptors on their target cells of the CAM and/or whether egg-adapted variants arise by their ability to escape binding to inhibitors present in the allantoic fluid. We have also studied the molecular effects of distinct substitutions in the HAs of egg-adapted human viruses on their receptor-binding characteristics. To investigate these points, we compared non-egg-adapted MDCK-grown human influenza A (H1N1, H3N2) and B strains with their egg-adapted counterparts for their binding to preparations of CAM cell plasma membranes, gangliosides derived from the CAM and from MDCK cells and to soluble sialylglycoproteins and sialyloligosaccharides containing Sia(α2–3)Gal and Sia(α2–6)Gal residues.

RESULTS AND DISCUSSION

Binding to CAM membranes and gangliosides *vs* egg inhibitors

As the first part of this study, we investigated how the adaptation of human influenza viruses to growth in allantoic cavity of CE alters their binding to cellular receptors and to intercellular inhibitors present in this host system. We compared nonadapted viruses (they will be referred to as M viruses) and their egg-adapted counterparts (E viruses) for their binding to plasma membranes of CAM cells, to total CAM gangliosides, and to ovomucin, the sialylglycoprotein inhibitor from hen's eggs. Because differences in the glycosylation of virus proteins in different hosts cells can significantly effect their receptor-binding properties (Schulze, 1997; Gambaryan *et al.,* 1998 and references therein), the egg-adapted viruses were passaged once in MDCK cells before their comparison with nonadapted MDCK-grown parents.

All human influenza A and B non-egg-adapted viruses bound poorly to the plasma membranes of CAM cells, did not bind at all to the CAM cell gangliosides (Table 1), and had low affinity for ovomucin and normal allantoic fluid (Ovo and AF, see data in the Table 2). In contrast to M viruses, all their egg-adapted variants bound much better to CAM cells and bound to CAM gangliosides. Because the binding of egg-adapted variants to Ovo and AF either increased or did not change, we concluded that none of the egg-adapted variants analyzed in this study directly escaped binding to inhibitors present in the allantoic cavity of CE. These findings show that the selective pressures on human influenza viruses in CE primarily favor the selection of variants with enhanced binding to receptors on CAM cells.

In parallel to MDCK-grown E viruses, egg-grown preparations of the same viruses were tested (Table 1). In accord with our previous observation (Gambaryan *et al.,* 1998), the egg-grown viruses bound better both to CAM cell membranes and to CAM gangliosides than did the same virus strains grown in MDCK cells (Table 1). This phenomenon indicates most likely that some of the carbohydrates attached at the top of the HA directly participate in the virus interactions with the receptors of CAM cells.

Molecular species on the CAM cells that serve as productive receptors for influenza viruses have not been defined. In the CAM plasma membranes, both glycoproteins and gangliosides could be responsible for virus attachment (reviewed by Paulson, 1985; Suzuki, 1994). Remarkably, there appeared to be an absolute correlation between the inability of the non-egg-adapted viruses to grow in CE and their inability to bind to CAM gangliosides. Moreover non-egg-adapted viruses bound to gangliosides isolated from susceptible MDCK cells (Table 1). This correlation between the ability of the virus to grow in particular cells and its binding to gangliosides from these cells allows us to hypothesize that interaction with gangliosides represents an important step in the virus attachment, endocytosis, or penetration into the cell.

Molecular mechanisms of increased binding to CAM receptors

CAM cells predominantly express Sia(α2–3)Gal-terminated sialylglycoconjugates (Ito *et al.,* 1987). To specify possible molecular mechanisms, by which egg-adapted viruses increase their binding to such receptors, we determined the affinity of the viruses for a number of soluble receptor analogues (Table 2). We also analyzed the location of egg-adaptation substitutions in the three-dimensional model of influenza virus H3 HA complexes with sialyloligosaccharides (Eisen *et al.,* 1997; Fig. 1) to assist interpretation of the binding data.

The nonadapted viruses revealed distinct type and

TABLE 1

Relative Percent Binding of Influenza Viruses to Preparations of Plasma Membranes of CAM Cells and Total Gangliosides Isolated from CAM and MDCK Cells[a]

Virus[b]	Substitution in the HA1[c] of egg-adapted variants	CAM cell membranes		Gangliosides isolated: CAM cells		Gangliosides isolated: MDCK cells
		M host[d]	E host[d]	M host[d]	E host[d]	M host[d]
H1N1						
A/Chr/157/83M		10		<3		30
A/Chr/157/83E-32	163N → K*	100	100	5	15	
A/Chr/157/83E-5b	187N → K	100	100	20	30	
A/Chr/157/83E-5a	189E → K	70	100	60	100	
A/Chr/157/83E-49a	190D → N	100	100	60	90	
A/Chr/157/83E-26	225D → N	80	80	20	40	
A/Chr/157/83E-21b	225D → G	100	100	20	40	
A/NIB/23/89M[e]		10		<3		25
A/NIB/4/88E[e]	225D → G	90		40		
A/NIB/49/88E[e]	190D → N	80	100	70	100	
H3N2						
A/NIB/19/89M		5		<3		25
A/NIB/19/89E	186S → I	40	100	20	100	
A/NIB/47/89M		5		<3		50
A/NIB/47/89E	156E → K	80	100	60	100	
A/NIB/44/90M		5		<3		50
A/NIB/44/90E	186S → I	50	100	20	100	
A/NIB/3/90M		5		<3		20
A/NIB/3/90E	145N → K	30	100	15	100	
B						
B/NIB/15/88M[e]		5		<3		20
B/NIB/25/88E[e]	189T → P/I*	100	100	100	80	
B/NIB/48/90M		20		<3		40
B/NIB/48/90E-p1	189T → A*	100	100	100	70	

[a] Binding to the preparations of plasma membranes and of gangliosides from chorio-allantoic membrane and MDCK cells was determined in a solid phase overlay assay as described under Materials and Methods. The relative binding represents the percentage of number of binding sites for the virus on a particular substrate with respect to the number of binding sites for the same virus on the non-coated plastic in the absence of blocking agent (positive control).

[b] "M" after the strain name indicates that the virus was isolated and propagated solely in MDCK cells. "E" designates variants that were adapted to growth in embryonated chicken eggs.

[c] Amino acid substitutions in the HA1 portion of the HA of egg-adapted variants with respect to the non-egg-adapted counterpart. H3 numbering system in accord with the alignment of Matrosovich *et al.* (1993) is used for H1 influenza A and for type B HA. Substitutions marked by an asterisk result in a loss of glycosylation site.

[d] Host system in which the virus was grown for the binding studies. M host, MDCK cells; E host, embryonated eggs.

[e] The M variants of B/NIB/15/88 and B/NIB/25/88 and M variants of A/NIB/23/89, A/NIB/4/88, and A/NIB/49/88 had identical amino acid sequences of the HA1 part of the HA.

subtype-specific patterns of binding to the monovalent receptor analogues. H1N1 strains bound to sialyloligosaccharides LSTc and 6′SLN significantly stronger than to free *N*-acetylneuraminic acid (Neu5Ac), 3′SL, and 6′SL. This pattern suggested that the asialo portions of the former two analogues interacted with the HA, whereas the asialic parts of 3′SL and 6′SL did not. In contrast to H1 viruses, H3N2 strains bound to Neu5Ac, 6′SL, and 6′SLN with the same affinity and displayed decreased binding to 3′SL, indicating some interference between the asialic part of 3′SL and the HA. A characteristic distinction of type B viruses was their low affinity for free Neu5Ac as compared with the affinity for sialosides. Despite these distinctions, all non-egg-adapted viruses exhibited similar high affinity for 6′SLN (K_{ass} about 5–10 mM^{-1}) in accord with the known preference of human influenza viruses for 6′SLN-containing receptors (Matrosovich *et al.,* 1993, 1997; Gambaryan *et al.,* 1997).

Compared to nonadapted parents, the egg-adapted

TABLE 2

Binding of Influenza Viruses to Soluble Receptor Analogues[a]

Virus[b]	Substitution in HA1	Association constants of virus-analogue complexes, (mM^{-1} Neu5Ac)								
		αNeu5Ac	3′SL	6′SL	6′SLN	LSTc	EM	Ovo	AF[c]	DS[d]
H1N1										
A/Chr/157/83M		1.7	1.7	1.7	10	30	100,000	25	20	2
A/Chr/157/83E-32	163N → K*	1	2	0.5	2.5	7	10,000	50	40	10
A/Chr/157/83E-5b	187N → K	2.5	2.5	2.5	10	20	25,000	100	70	100
A/Chr/157/83E-5a	189E → K	3	3	5	30	30	15,000	1,000	500	500
A/Chr/157/83E-49a	190D → N	5	5	5	5	10	10,000	3,300	2,000	100
A/Chr/157/83E-26	225D → N	2.5	1.2	0.4	10	30	10,000	50	40	5
A/Chr/157/83E-21b	225D → G	7	10	3	10	25	3,000	100	80	10
A/NIB/23/89M[e]		1.7	2.5	1.7	12	25	100,000	70	40	
A/NIB/4/88E[e]	225D → G	5	5	1.2	5		10,000	1,000	300	
A/NIB/49/88E[e]	190D → N	5	5	5	5	7	20,000		1,000	
H3N2										
A/NIB/3/90M		10	2.5	10	10		1,000,000	5	4	<1
A/NIB/3/90E	145N → K	10	2	4			100,000	10	7	10
A/NIB/19/89M		6	3	10	10		1,000,000	5	4	5
A/NIB/19/89E	186S → I	8	10	12			300,000	5	4	5
A/NIB/44/90M		6	2	6	6		500,000	5	4	<1
A/NIB/44/90E	186S → I	10	10	10	10		200,000	5	4	<1
A/NIB/47/89M		6	2	6	6		500,000	5	4	<1
A/NIB/47/89E	156E → K	6	0.7	0.5	0.5		60,000	100	40	20
B										
B/NIB/15/88M[e]		2	6	10	10		50,000	10	10	<1
B/NIB/25/88E[e]	189T → I/P*	0.5	6	4			25,000	12	10	<1
B/NIB/48/90M		0.5	4	5	5		20,000	2	3	
B/NIB/48/90E-p1	189T → A*	0.2	5	2			10,000	6	5	
B/NIB/48/90E-p8	187N → S*	0.2	5	2			10,000	6	5	

[a] The binding was assayed and the association constants (K_{ass}) of virus-receptor analogue complexes were calculated as described under Materials and Methods. A higher value of K_{ass} corresponds to a higher binding affinity. EM, equine macroglobulin; Ovo, hen egg ovomucin; DS, dextran sulfate.

[b] All viruses were grown in MDCK cells to exclude effects of host-specific glycosylation on the receptor-binding properties.

[c] Noninfected allantoic fluid.

[d] The association constants for the virus complexes with dextran are expresses in $(\mu g/ml)^{-1}$.

[e] The M variants of B/NIB/15/88 and B/NIB/25/88 and M variants of A/NIB/23/89, A/NIB/4/88, and A/NIB/49/88 had identical amino acid sequences of the HA1 part of the HA.

strains changed the patterns of their affinities for free sialic acid and trisaccharides 3′SL, 6′SL, and 6′SLN (Table 2). These data indicated that all egg-adaptation substitutions affected the fine architecture of the HA receptor-binding site (RBS) and altered the interactions of the HA with the terminal sialic acid moiety and/or one to two penultimate sugar rings of the sialosides. The H1N1 and H3N2 egg-adapted variants with substitutions 190D → N, 225D → G (H1), and 186S → I (H3) bound 3′SL with at least twofold higher affinity than did non-adapted parents (Table 2). These effects appeared significant enough to suggest that enhanced binding of these variants to CAM cells was due to their stronger interaction with the terminal Sia(α2–3)Gal moieties of the receptors. The side chain of the amino acid in position 190 interacts with the 9-hydroxyl group of sialic acid (Weis *et al.,* 1988), whereas the side chain of residue 186 contacts residue 190 (Fig. 1). The main chain carbonyl oxygen of residue 225 was predicted to be hydrogen bonded to the 6-hydroxyl group of the galactose ring of 3′SL (Sauter *et al.,* 1992). Hence it is not unexpected that substitutions in these three positions of the HA changed the RBS and increased the virus affinity for Sia(α2–3)Gal.

Egg-adapted viruses with substitutions in the HA, 225D → N (H1N1), 145N → K, and 156E → K (H3N2) had a lower affinity for 3′SL than their nonadapted counterparts. Because all egg-adapted viruses bound stronger to CAM cell receptors (Table 1) and to 3′SL-containing sialylglycopolymers (Gambaryan *et al.,* 1997), we assumed that the negative effects of these substitutions on the interactions with the terminal receptor determinant must be counteracted and surpassed by improved HA

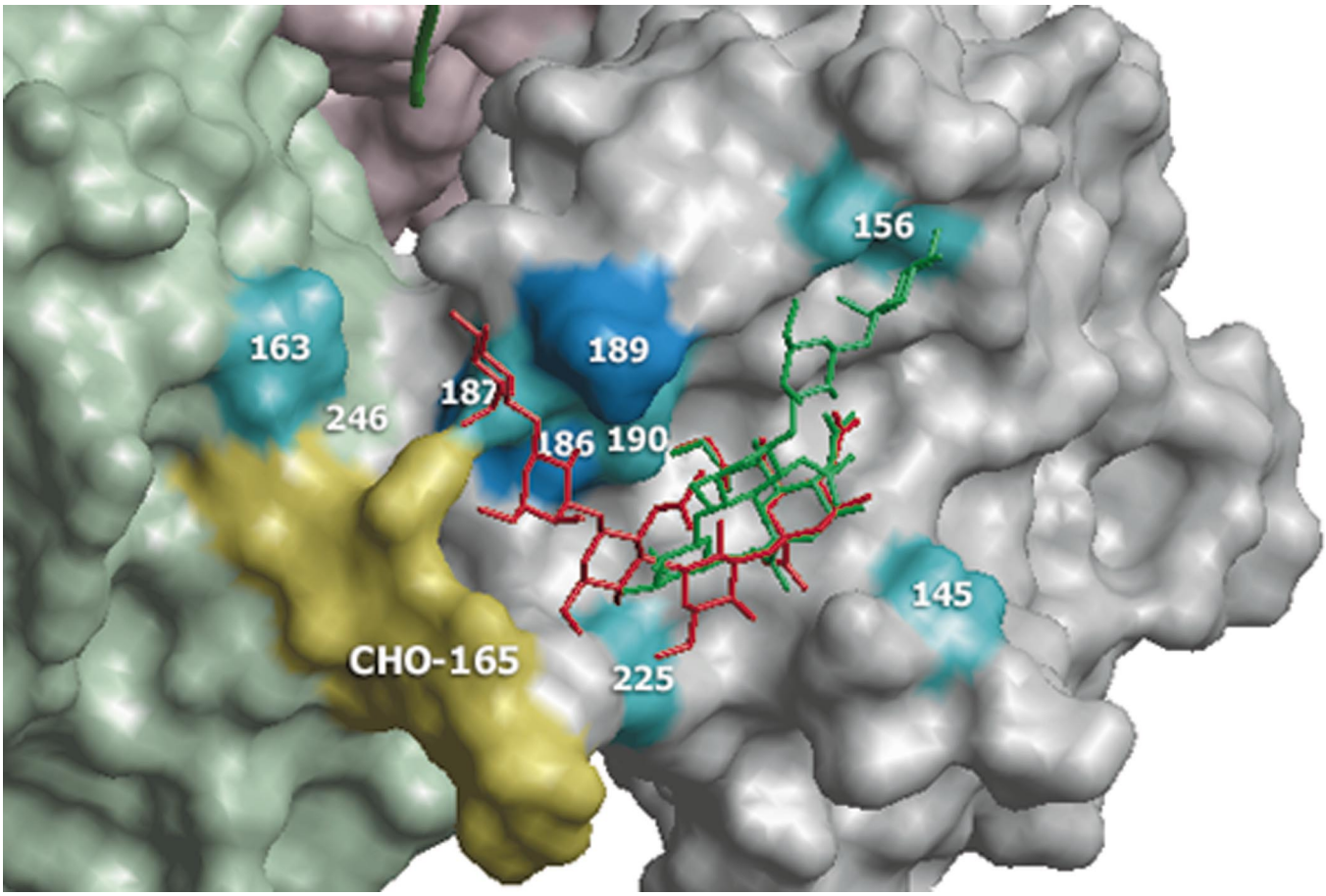

FIG. 1. Position of amino acid substitutions in the HAs of egg-adapted human influenza A (H1N1 and H3N2) and B viruses shown on the model of the X31 HA complex with sialyloligosaccharides (Eisen *et al.,* 1997). The HA monomers are painted in gray, light green, and light red. Substitutions are shown in cyan and in blue and numbered (H3 numbering according to the alignment of Matrosovich *et al.,* 1993). Carbohydrate attached at Asn_{165} of X31 HA is shown in yellow. Stick models of sialyloligosaccharides LSTa [Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc] (red) and LSTc [Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc] (green) from the corresponding individual complexes are superimposed on the same HA model. The figure was generated using WebLab ViewerPro 3.10 (Molecular Simulations, Inc., San Diego, CA).

interactions with the distant parts of the Sia(α2–3)Gal-containing receptors. For example, these substitutions could change the orientation of Sia(α2–3Gal) moiety in the RBS in a manner that would move the penultimate part of the receptor away from the carbohydrates in positions 163 (H1) and 246 (H3) (see below).

Effects of carbohydrates on binding

All egg-adapted type B viruses and one of the E variants of A/Chr/157/83 (H1N1) carried substitutions in a glycosylation sequon that led to a loss of the carbohydrates at HA positions 187 and 163, respectively. The affinity of these variants for 3′SL was not altered significantly compared with that of nonadapted viruses (Table 2), suggesting that a loss of carbohydrate did not change the virus interaction with terminal Sia(α2–3)Gal moieties of CAM cellular receptors. The structural model of the H3 HA complexed with natural pentasaccharide LSTa (Eisen *et al.,* 1997) predicts that the asialic parts of Sia(α2–3Gal)-containing sialyloligosaccharides would protrude toward the "left" side of the HA receptor-binding pocket (Fig. 1). It seems likely, therefore, that carbohydrates at position 187 of the type B HA (CHO_{187}) and at position 163 of H1N1 HA (CHO_{163}) sterically interfere with the accommodation of more distant parts of the receptors and that a deletion of these carbohydrates in the egg-adapted mutants resolves the interference. The presence of carbohydrates in this region of the HA appears to be a common feature of human influenza A and B viruses because H3N2 human strains also typically contain a carbohydrate attached to asparagine in the position 246 of the HA (see Fig. 1). Although the H3N2 egg-adapted mutants tested in this study preserved this carbohydrate, the loss of CHO_{246} as a result of egg adaptation of human H3 viruses has been reported by others (Wang *et al.,* 1989; Robertson, 1993; Ito *et al.,* 1997). Taken together, these data suggest that non-egg-adapted human influenza viruses bind weakly to Sia(α2–3)Gal-containing macromolecules because of sterical interference of their asialic portions with the left side of the RBS and that the presence of carbohydrates in this region of the HA can contribute to this interference.

The HAs of human influenza A and B viruses are believed to originate from the influenza viruses of wild

aquatic birds that crossed the species barrier (Webster *et al.,* 1992). It has been noticed that avian viruses contain the minimal number of carbohydrates in the globular head of the HA, whereas the number of carbohydrates in the HA head of human viruses increases during their evolution in humans (Inkster *et al.,* 1993; Matrosovich *et al.,* 1997). Our data suggest that additional carbohydrates in the region of the "left" side of the RBS can help the virus to escape neutralization by human respiratory mucins, which are known to contain predominantly Sia(α2–3)Gal determinants (Couceiro *et al.,* 1993 and references therein).

Effects of charged substitutions

Among the egg-adaptation substitutions in the HA of human influenza viruses, a striking predominance of charged substitutions over neutral ones was noticed (Robertson, 1993). To evaluate the possibility of such substitutions increasing nonspecific ionic interactions of the egg-adapted viruses with the negatively charged cells, we determined binding of the viruses to a macromolecular polyanion, dextran sulfate (DS, see Table 2), that served to mimic the negative charge of a cell surface but did not contain sialic acid receptors. The M viruses bound DS weakly, if at all (K_{ass}, $<$1–5 ml/μg). All "charged" substitutions in the HA of egg-adapted viruses increased their binding to DS, those in positions 187, 189, and 190 of H1 HA and in positions 145 and 156 of H3 HA having the highest effect. These data formally demonstrated for the first time that charged substitutions at the HA head can enhance nonspecific ionic interactions of the viruses with negatively charged macromolecules. This finding could explain why charged substitutions are so common in the egg-adapted variants of human viruses.

Decreased affinity of egg-adapted viruses for 6′-sialyl(*N*-acetyllactosamine)

We found previously that all non-egg-adapted human influenza A and B viruses share a high binding affinity for a synthetic sialylglycopolymer that carried moieties of 6′-sialyl(*N*-acetyllactosamine) attached to a hydrophilic macromolecular carrier (Gambaryan *et al.,* 1997). In this study, we assumed that equine macroglobulin (EM), a glycoprotein that contains 6′SLN-terminated biantennary carbohydrates (Hanaoka *et al.,* 1989), could be a more natural receptor-mimic for human influenza viruses. All egg-adapted variants bound to EM more weakly than their non-egg-adapted precursors (Table 2). Because neither CAM cells nor inhibitors present in the egg allantoic fluid appear to express 6′SLN residues, no negative selective pressure on the virus binding to 6′SLN could be expected. We concluded, therefore, that a decreased binding to EM was a consequence of the enhanced binding of the viruses to Sia(α2–3)Gal-specific receptors. This phenomenon could be explained by the opposite orientation of the asialo moieties of 2–3- and 2–6-linked sialosides with respect to the RBS (Fig. 1) and by the inability of the HA to accommodate both types of analogues equally well. It can be envisaged that changes in the position of the sialic acid that would resolve unfavorable contacts between the asialo moieties of 2–3-linked receptors and the "left" side of the RBS (see model of LSTa in Fig. 1) at the same time would create unfavorable contacts of the asialo moieties of 2–6-linked receptors and the "right" side of the RBS (Fig. 1, LSTc). The sialic acid moieties of LSTa and LSTc in their complexes with X31 HA (Eisen *et al.,* 1997) do not completely overlap (see Fig. 1). Although the difference is within the experimental error of X-ray analysis, this notion is consistent with our speculations about the energetically favorable accommodation of asialo moieties of 2–3- and 2–6-linked sialyloligossacharides requiring different orientations of the terminal sialic acid in the RBS.

Among the egg-adaptation substitutions in the HA, those in positions 145, 156, 190, and 225 of influenza A viruses had the highest negative effect on the binding to EM. The substitutions 190 and 225 (H1 strains) were particularly interesting. The amino acid substitutions in these positions are believed to be primarily responsible for the alterations of the receptor-binding specificity of H1 subtype avian HAs during their interspecies transmission to humans and pigs (Rogers and D'Souza, 1989; Matrosovich *et al.,* 1997; Matrosovich *et al.,* manuscript in preparation). Our present data confirm that amino acids in positions 190 and 225 are crucial for the H1 virus HA recognition of Sia(α2–6)Gal-containing receptors. We previously found that human H1 virus strains, unlike H3 influenza A and influenza B viruses, discriminated between 6′SLN and 6′SL (Matrosovich *et al.,* 1993; Gambaryan *et al.,* 1995, 1997). Interestingly, the substitution 190D $\rightarrow$ N completely abrogates this property of H1 viruses. Thus whereas most H1N1 human strains bound 6′SLN significantly better than they did 6′SL (Table 2), two variants with the mutation 190D $\rightarrow$ N bound both analogues with the same affinity. The molecular mechanisms of these effects are not clear.

As demonstrated in this study, egg adaptation of human viruses not only increases their affinity for Sia(α2–3)Gal-containing receptors but concomitantly impairs their ability to bind to Sia(α2–6)Gal-terminated receptors. These changes in the receptor-binding phenotype would likely decrease the fitness of egg-adapted viruses in humans because of their poorer attachment to target cells containing predominantly Sia(α2–6)Gal-moieties and of their higher sensitivity to neutralization by human respiratory mucins, which contain Sia(α2–3)Gal. This notion agrees with the report of Oxford *et al.* (1990), who demonstrated that an egg-adapted variant of type B virus was attenuated in virulence for volunteers.

MATERIALS AND METHODS

Viruses

Human influenza viruses isolated from clinical specimens and passaged solely in MDCK cells and their egg-adapted variants were obtained from the influenza virus repository of NIBSC (Potters Bar, UK). Isolation, egg-adaptation, and HA amino acid sequences of these viruses were described in previous publications (Robertson *et al.,* 1987, 1990, 1991; Meyer *et al.,* 1993; Robertson, 1993). For the studies on binding to soluble receptor analogues, both nonadapted and egg-adapted viruses were propagated in MDCK cells to permit valid comparison of their receptor-binding phenotypes. To evaluate effects of host-specific glycosylation on virus binding to cellular membranes and to gangliosides, egg-adapted viruses were grown both in MDCK cells and in CE, and these preparations were compared for their binding to solid phase adsorbed receptors (Table 1). In the other assays, only MDCK-grown viruses were used (Table 2).

Freshly collected infectious allantoic or culture fluid was clarified by low-speed centrifugation. The viruses were then pelleted by high-speed centrifugation, purified by centrifugation through 20% sucrose, resuspended in 60% glycerol–PBS solution containing 0.02% sodium azide to the final virus concentration of ~1 mg/ml, and stored at −20°C.

Cells membranes, gangliosides, sialyloligosaccharides, and sialylglycoproteins

Isolation of plasma membranes from CAM cells and total gangliosides from these cells, and the preparation of α2-macroglobulin from horse serum (EM) and hen egg ovomucin (Ovo) were as previously described (Gambaryan *et al.,* 1998). The sialic acid (Sia) content of sialylglycoproteins, their conjugates with horseradish peroxidase (HRP), and total chicken embryo allantoic fluid were assayed using an acidic ninhidrin method (Yao *et al.,* 1989).

Total gangliosides from MDCK cells were extracted and purified as described for the preparation of human placenta gangliosides (Taki *et al.,* 1988).

Free *N*-acetylneuraminic acid (Neu5Ac), 3′sialyllactose [3′SL, Neu5Ac(α2–3)Gal(β1–4)Glc], 6′sialyllactose [6′SL, Neu5Ac(α2–6)Gal(β1–4)Glc], bovine fetuin, and dextran sulfate (500 kDa) were purchased from Serva, Switzerland. 6′-sialyl(*N*-acetyllactosamine) [6′SLN, Neu5Ac(α2–6)Gal(β1–4)GlcNAc], and LSTc [Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc] were kindly provided by Dr. V.E.Piskarev, Nesmeyanov Institute of Organoelement Compounds, Moscow, Russia.

Solid-phase overlay binding assay

The binding of the viruses to plasma membranes of CAM cells and to gangliosides was assayed using the microplate absorption method described before (Gambaryan *et al.,* 1998). In brief, wells of 96-well polyvinyl chloride microplates were coated with CAM membranes or gangliosides and blocked by incubation with 0.02% solution of BSA in PBS for 2 h at 4°C. Serial twofold dilutions of viruses in 0.1% BSA–PBS solution were incubated in the wells for 4 h at 4°C. The viruses bound in the wells were detected by overlaying them with HRP-labeled fetuin. The amount of bound conjugate, which reflects the amount of the virus present in the wells, was quantified using *o*-phenylenediamine as a substrate. The absorbency at 490 nm was measured, and the data were converted to Scatchard plots (A_{490} *vs* A_{490}/C), where the concentration of the viruses was expressed in hemagglutination units. As a positive control for maximal virus binding, nonspecific physical virus absorption to the plastic was assayed. For this, the wells were not coated, the blocking step was omitted, and no BSA was present in the incubation mixture. All other conditions were the same as described above. The number of binding sites for the virus on a particular substrate was determined from the interception of the plots with the *x* axes. The number of binding sites on noncoated plastic (positive control) was taken as 100%, and the relative number of binding sites for the binding to plasma membranes and gangliosides was calculated with respect to this control. Replicate experiments were performed on different days and the results were averaged.

Competitive assay of virus binding to soluble receptor analogues

The affinity of the influenza viruses for soluble receptor analogs and for sialylgycoproteins present in noninfected allantoic fluid was assessed by using the solid-phase fetuin binding inhibition assay as previously described (Gambaryan and Matrosovich, 1992; Matrosovich *et al.,* 1993). This assay is based on the competition for binding sites on the viral particle between nonlabeled sialic acid-containing compounds and enzyme-labeled sialylglycoprotein fetuin. In brief, purified viruses diluted with PBS to a hemagglutination titer of 1:20–1:100 were adsorbed to the wells of fetuin-coated polystrene EIA microplates at 4°C overnight. After unbound virus was washed off with 0.01% Tween 80 in PBS (PBS-T), 0.05 ml of solution containing a fixed amount of HRP-labeled fetuin, and a variable amount of nonlabeled inhibitor was added to the plate, which was then incubated for 1 h at 2–4°C. The solutions were prepared in PBS supplemented with 0.02% BSA, 0.02% Tween 80, and 10 μM of the sialidase inhibitor Neu5Ac2en. After this incubation, the plates were washed with PBS-T, and the amount of labeled fetuin bound was determined by using the standard *o*-phenylenediamine chromogenic substrate.

The association constants of the virus complexes with receptor analogs were calculated for each concentration of the compound used in the competitive reaction, and the results were averaged. For these calculations, the

concentration of the sialic acid residues in the solution was used.

ACKNOWLEDGMENTS

We thank Dr. V.E. Piskarev for the generous gift of 6′SLN and LSTc and Dr. M.B. Eisen for crystallographic structures of influenza virus HA complexes with sialyloligosaccharides. This work was supported by grants from the Russian Foundation of Fundamental Investigations (95/97–04-11079), and the Russian state scientific and technical program "National Priorities in Medicine and Health Care." During the preparation of the manuscript, M.N. Matrosovich was supported by a Karnofsky fellowship from St. Jude Children's Research Hospital.

REFERENCES

Aytay, S. and Schulze, I. T. (1991). Single amino acid substitutions in the hemagglutinin can alter the host range and receptor binding properties of H1 strains of influenza A virus. *J. Virol.* **65,** 3022–3028.

Baum, L. G., and Paulson, J. C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. *Acta Histochem.* **XL,** (suppl.), 35–38.

Couceiro, J. N., Paulson, J. C., and Baum, L. G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res.* **29,** 155–165.

Eisen, M. B., Sabesan, S., Skehel, J. J., and Wiley, D. C. (1997). Binding of the influenza A virus to cell-surface receptors: Structures of five hemagglutinin-sialyloligosaccharide complexes determined by X-ray crystallography. *Virology* **232,** 19–31.

Gambaryan, A. S., and Matrosovich, M. N. (1992). A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. *J. Virol. Methods* **39,** 111–123.

Gambaryan, A. S., Marinina, V. P., Tuzikov, A. B., Bovin, N. V., Rudneva, I. A., Sinitsyn, B. V., Shilov, A. A., and Matrosovich, M. N. (1998). Effects of host-dependent glycosylation of hemagglutinin on receptor-binding properties of H1N1 human influenza A virus grown in MDCK cells and in embryonated eggs. *Virology* **247,** 170–177.

Gambaryan, A. S., Tuzikov, A. B., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., Robertson, J. S., Bovin, N. V., and Matrosovich, M. N. (1997). Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: Non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(*N*-acetyllactosamine). *Virology* **232,** 345–350.

Hanaoka, K., Pritchett, T. J., Takasaki, S., Kochibe, N., Sabesan, S., Paulson, J. C., and Kobata, A. (1989). 4-O-acetyl-N-acetylneuraminic acid in the N-linked carbohydrate structures of equine and guinea pig alpha 2-macroglobulins, potent inhibitors of influenza virus infection. *J. Biol. Chem.* **264,** 9842–9849.

Hardy, C. T., Young, S. A., Webster, R. G., Naeve, C. W., and Owens, R. J. (1995). Egg fluids and cells of the chorioallantoic membrane of embryonated chicken eggs can select different variants of influenza A (H3N2) viruses. *Virology* **211,** 302–306.

Inkster, M. D., Hinshaw, V. S., and Schulze, I. T. (1993). The hemagglutinins of duck and human H1 influenza viruses differ in sequence conservation and in glycosylation. *J. Virol.* **67,** 7436–7443.

Ito, T., Suzuki, Y., Takada, A., Kawamoto, A., Otsuki, K., Masuda, H., Yamada, M., Suzuki, T., Kida, H., and Kawaoka, Y. (1997). Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. *J. Virol.* **71,** 3357–3362.

Katz, J. M., Wang, M., and Webster, R. G. (1990). Direct sequencing of the HA gene of influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. *J. Virol.* **64,** 1808–1811.

Katz, J. M., and Webster, R. G. (1992). Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. *J. Gen. Virol.* **73,** 1159–1165.

Matrosovich, M. N., Gambaryan, A. S., Teneberg, S., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., Robertson, J. S., and Karlsson, K. A. (1997). Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology* **233,** 224–234.

Matrosovich, M. N., Gambaryan, A. S., Tuzikov, A. B., Byramova, N. E., Mochalova, L. V., Golbraikh, A. A., Shenderovich, M. D., Finne, J., and Bovin, N. V. (1993). Probing of the receptor-binding sites of the H1 and H3 influenza A and influenza B virus hemagglutinins by synthetic and natural sialosides. *Virology* **196,** 111–121.

Meyer, W. J., Wood, J. M., Major, D., Robertson, J. S., Webster, R. G., and Katz, J. M. (1993). Influence of host cell-mediated variation on the international surveillance of influenza A (H3N2) viruses. *Virology* **196,** 130–137.

Oxford, J. S., Schild, G. C., Corcoran, T., Newman, R., Major, D., Robertson, J., Bootman, J., Higgins, P., al-Nakib, W., and Tyrrell, D. A. (1990). A host-cell-selected variant of influenza B virus with a single nucleotide substitution in HA affecting a potential glycosylation site was attenuated in virulence for volunteers. *Arch. Virol.* **110,** 37–46.

Paulson, J. C. (1985). Interactions of animal viruses with cell surface receptors. *In*: "The Receptors" (M. Conn, Ed.), Vol. 2, pp. 131–219. Academic Press, Orlando, FL.

Robertson, J. S. (1993). Clinical influenza virus and the embryonated hen's eggs. *Rev. Med. Virol.* **3,** 97–106.

Robertson, J. S., Bootman, J. S., Newman, R., Oxford, J. S., Daniels, R. S., Webster, R. G., and Schild, G. C. (1987). Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. *Virology* **160,** 31–37.

Robertson, J. S., Bootman, J. S., Nicolson, C., Major, D., Robertson, E. W., and Wood, J. M. (1990). The hemagglutinin of influenza B virus present in clinical material is a single species identical to that of mammalian cell-grown virus. *Virology* **179,** 35–40.

Robertson, J. S., Nicolson, C., Bootman, J. S., Major, D., Robertson, E. W., and Wood, J. M. (1991). Sequence analysis of the haemagglutinin (HA) of influenza A (H1N1) viruses present in clinical material and comparison with the HA of laboratory-derived virus. *J. Gen. Virol.* **72,** 2671–2677.

Rogers, G. N., and D'Souza, B. L. (1989). Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* **173,** 317–322.

Sauter, N. K., Hanson, J. E., Glick, G. D., Brown, J. H., Crowther, R. L., Park, S. J., Skehel, J. J., and Wiley, D. C. (1992). Binding of influenza virus hemagglutinin to analogs of its cell-surface receptor, sialic acid: Analysis by proton nuclear magnetic resonance spectroscopy and X-ray crystallography. *Biochemistry* **31,** 9609–9621.

Schulze, I. T. (1997). Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. *J. Infect. Dis.* **176,** (suppl. 1), S24–S28.

Suzuki, Y. (1994). Gangliosides as influenza virus receptors. Variation of influenza viruses and their recognition of the receptor sialo-sugar chains. *Prog. Lipid Res.* **33,** 429–457.

Taki, T., Matsuo, K., Yamamoto, K., Matsubara, T., Hayashi, A., Abe, T., and Matsumoto, M. (1988). Human placenta gangliosides. *Lipids* **23,** 192–198.

Wang, M. L., Katz, J. M., and Webster, R. G. (1989). Extensive heterogeneity in the hemagglutinin of egg-grown influenza viruses from different patients. *Virology* **171,** 275–279.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56,** 152–179.

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988). Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333,** 426–431.

Yao, K., Ubuka, T., Masuoka, N., Kinuta, M., and Ikeda, T. (1989). Direct determination of bound sialic acids in sialoglycoproteins by acidic ninhydrin reaction. *Anal. Biochem.* **179,** 332–335.