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Host-mediated Selection of Influenza Virus Receptor Variants

SIALIC ACID-α2,6Gal-SPECIFIC CLONES OF A/DUCK/UKRAINE/1/63 REVERT TO SIALIC ACID-α2,3Gal-SPECIFIC WILD TYPE *IN OVO**

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Human and animal influenza A isolates of the H3 serotype preferentially bind SAα2,6Gal or SAα2,3Gal linkages (where SA represents sialic acid), respectively, on cell-surface sialyloligosaccharides. Previously, we have demonstrated selection of SAα2,3Gal-specific receptor variants of several human viruses which differed from the parent viruses by a single amino acid at residue 226 of the hemagglutinin which is located in the receptor binding pocket (Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. (1983) *Nature* 304, 76–78). In this report, the selection in the reverse direction was accomplished starting with a SAα2,3Gal-specific avian virus, A/duck/Ukraine/1/63 (H3N7), yielding SAα2,6Gal-specific variants that exhibit the receptor binding properties characteristic of the human isolates. Selection was again mediated at residue 226 of the hemagglutinin, in this case changing from Gln in the parent virus to Leu in the variants.

Although the SAα2,6Gal-specific avian virus variants were stable to passage in MDCK cells, they exhibited dramatic reversion to the SAα2,3Gal-specific phenotype of the parent virus during a single passage in chicken embryos. This was in contrast to the SAα2,6Gal-specific human virus isolates which were stable to passage in both hosts. The reversion of the avian virus variants in eggs provides compelling evidence for host-mediated selection of influenza virus receptor variants.

Influenza viruses exhibit considerable diversity in their ability to recognize specific sialyloligosaccharide structures as cell-surface receptor determinants (1–4), and it has become increasingly evident from laboratory models (5–8) that such specificity can provide the basis for changes in receptor binding that have been reported to occur during host adaptation (9–12). Indirect evidence that receptor binding properties may be important in the ecology of influenza viruses comes from the correlation between receptor specificity and species of origin observed for virus isolates bearing the H3 hemagglutinin (4). The human isolates of this serotype exhibit strong preferential binding to oligosaccharides terminating in SAα2,6Gal[1] sequences, while avian and equine H3 isolates preferentially bind SAα2,3Gal sequences. Furthermore, binding of cell-surface receptors by the human H3 isolates is very sensitive to inhibition by a glycoprotein present in horse serum, α_2-macroglobulin, whereas avian and equine isolates are not inhibited (7, 8). Thus, by growing human H3 isolates in the presence of horse serum, it has been possible to select variants with receptor properties that closely resemble those of avian and equine H3 isolates, *i.e.* that recognize oligosaccharides terminated by the SAα2,3Gal linkage and are insensitive to inhibition by equine α_2-macroglobulin. Sequence analysis of the genes for the hemagglutinins of these viruses revealed that they differed by a single nucleotide resulting in an amino acid change at residue 226 from leucine in the parental (SAα2,6Gal-specific) phenotype to glutamine in the variant with the avian type (SAα2,3Gal-specific) receptor specificity (7). In the three-dimensional structure of the H3 hemagglutinin reported by Wilson *et al.* (13), it was determined that amino acid 226 is located in the receptor binding pocket in the distal globular region of the molecule. How the difference of a single amino acid at this site can mediate dramatic changes in receptor binding specificity is still not clear.

This report describes a novel approach to the selection of receptor variants from an avian influenza virus, A/duck/Ukraine/1/63 (H3N7), distinguished from the parental, SAα2,3Gal-specific, virus by their ability to bind SAα2,6Gal sequences of cell-surface oligosaccharides. As reported for receptor variants of the human H3 isolates (7), the hemagglutinins of these avian variants differ from the parent by a single amino acid, at residue 226, located in the receptor binding pocket. Unlike the human H3 isolates, however, the SAα2,6Gal-specific variants of A/duck/Ukraine/1/63 rapidly revert to the parental, SAα2,3Gal-specific, phenotype when grown in the egg allantois but not when grown in mammalian cell culture. While the mechanism for this receptor shift remains unclear, these results appear to present a very clear

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[1] The abbreviations SAα2,6Gal and SAα2,3Gal refer to the terminal sialic acid (SA) linkage commonly found on glycoprotein oligosaccharides linked to asparagine and to threonine or serine, respectively (25, 26). Unless otherwise specified, SA refers to *N*-acetylneuraminic acid (NeuAc). Other abbreviations used include: NeuGc, N-glycolylneuraminic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine. pfu, plaque-forming units; HA, hemagglutination; HAI, hemagglutination inhibition assays.

example of host-mediated selection based on receptor specificity.

MATERIALS AND METHODS

Cells—Madin-Darby canine kidney (MDCK) cells (ATCC CCL34) were passaged and maintained in Eagle's minimum essential medium supplemented with 5% fetal calf serum and 100 units/ml of penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B.

Virus Growth—A/duck/Ukraine/1/63 (H3N7) was generously provided by Dr. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN. Cloned variants of A/Memphis/102/72 (H3N2) were produced from a seed stock, also obtained from Dr. Webster, by passage in the absence (M1/5) or presence (M1/HS8) of horse serum as previously described (8).

As standard procedure for growth in MDCK cell culture, monolayers were inoculated with virus, multiplicity of infection around 0.001, and incubated at 34 °C for 36–48 h in Eagle's minimum essential medium containing 2.5% bovine serum albumin and 20 μg/ml trypsin. At harvest, the medium was removed and centrifuged at 750 × *g* for 10 min to remove cell debris. Virus preparations were stored at −70 °C and assayed in parallel for hemagglutination activity and infectivity on MDCK cell monolayers in the presence of 20 μg/ml trypsin (14).

For growth of virus *in ovo*, 0.1 ml (10^4 pfu/ml) of MDCK cell lysates, diluted in phosphate-buffered saline containing antibiotics (15), was injected into the allantoic cavity of 10- to 11-day-old chicken embryos and incubated at 37 °C for 48 h. At harvest, eggs were cooled for 1 h at −20 °C; the allantoic fluid was collected and clarified at 7500 × *g* for 20 min. Virus was collected by centrifugation (5000 × *g* for 18 h), resuspended in phosphate-buffered saline, and stored at −25 °C.

Preparation of Derivatized Erythrocytes—The procedures for the enzymatic modification of human erythrocyte oligosaccharides have been previously described (4, 16). Briefly, native human erythrocytes were treated with *Vibrio cholera* sialidase (GIBCO) to remove sialic acid and abolish viral adsorption and hemagglutination. The sialic acid determinants were restored in a single, defined sequence by treatment of sialidase-treated (asialo) erythrocytes with CMP-[^{14}C] salic acid (New England Nuclear) and one of two highly purified mammalian sialyltransferases. The sialyltransferases employed in this study include the Galβ1,GalNAc α2,3-sialyltransferase,[2] purified from porcine submaxillary glands (17), and the Galβ1,4GlcNAc α2,6-sialyltransferase, purified from rat liver (18). The terminal sialyloligosaccharide sequence elaborated and the amount of sialic acid incorporated for each of these enzymes are, respectively, SAα2,3Galβ1,3GalNAc, 100–110 nmol/ml of packed erythrocytes, and SAα2,6Galβ1,4GlcNAc, 35–40 nmol/ml of packed erythrocytes.

Hemagglutination (HA) and Hemagglutination Inhibition (HAI) Assays—HA and HAI titers were determined in a Cooke microtiter system using a 1.5% suspension of native, asialo, or resialylated erythrocytes as previously described (8). The titration end points for both HA and HAI assays were determined after 60 min at room temperature.

Viral Adsorption to Derivatized Erythrocytes—For selection of receptor variants, adsorption of A/duck/Ukraine/1/63 to SAα2,6Gal-derivatized erythrocytes was performed essentially as previously described (4), except that virus was present in 15- to 30-fold higher concentrations. Virus (0.4 ml of MDCK cell lysates with HA titers of 64–128) was mixed with derivatized erythrocytes (5% (v/v) final concentration) and incubated for 5 min at room temperature. Erythrocytes and bound virus were pelleted by centrifugation at 3000 × *g* for 1 min, and the pellets were washed four times in fresh buffer. Adsorbed virus was eluted from erythrocytes with *Clostridium perfringens* sialidase (Sigma) as described under "Results."

RESULTS

Selection of Receptor Variants of A/Duck/Ukraine/1/63—While horse serum glycoprotein inhibitors have proven useful in the isolation of SAα2,3Gal-specific variants from human (SAα2,6Gal-specific) viruses, no glycoprotein yet examined has proven to be a selective inhibitor of hemagglutination of viruses specific for SAα2,3Gal, including human glycophorin which is presumably the predominant receptor on erythrocytes. Consequently, selection of SAα2,6Gal variants from SAα2,3Gal-specific avian isolates required a different approach. The procedure used, depicted in Fig. 1, involves selective adsorption and elution of the SAα2,3Gal-specific A/duck/Ukraine/1/63 with erythrocytes enzymatically modified to contain SAα2,6Gal sequences. First, virus was adsorbed to cells under conditions of large viral excess as previously described (7). The bound virus was eluted from cells by addition of *C. perfringens* sialidase (250 milliunits/ml) and incubation at 37 °C for 3 h. The virus released from erythrocytes was then grown in MDCK cell culture to amplify variants. After four such cycles, the eluted virus was plaqued on MDCK cells without intermediate amplification. Primary clones were initially screened for sensitivity to inhibition of growth in MDCK cells in the presence of horse serum and, finally, for receptor specificity by adsorption to derivatized erythrocytes. Of 64 primary clones, 5 were found to be specific for SAα2,6Gal linkages.

The receptor binding characteristics of five wild type clones and five cloned variants from A/duck/Ukraine/1/63 were compared to the parent virus, and the results are shown in Table I. Each of the virus preparations was examined for its ability to agglutinate native, sialidase-treated (asialo) and enzymatically resialylated erythrocytes containing SAα2,3Gal or SAα2,6Gal sequences and for sensitivity to equine α_2-macroglobulin, the major glycoprotein inhibitor of horse serum. The parent strain of A/duck/Ukraine/1/63-agglutin-

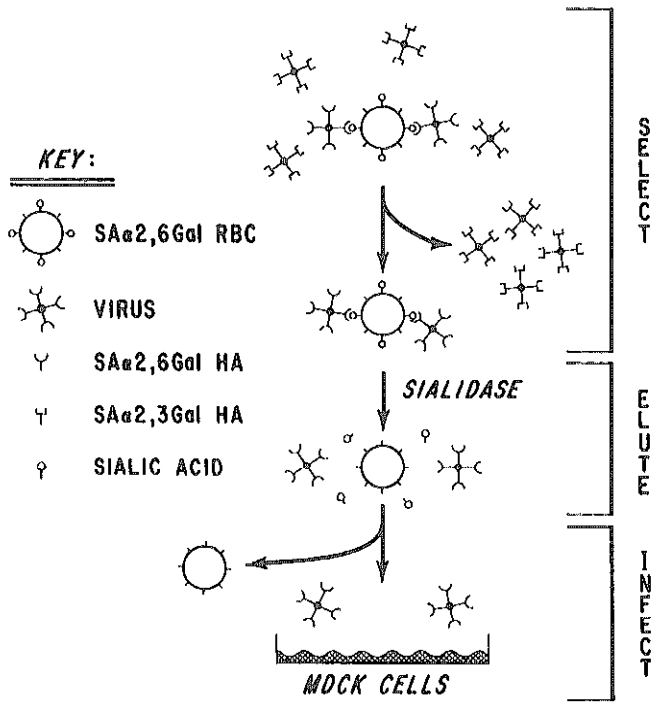

FIG. 1. **Selection of receptor variants by adsorption to derivatized erythrocytes.** The schematic depicts a simplified erythrocyte (*RBC*) enzymatically modified to contain sialic acid (*SA*) in the SAα2,6Gal linkage. Receptor variants which bind the SAα2,6Gal linkage adsorb to the erythrocytes, allowing separation from the predominant parent virus with the SAα2,3Gal-specific receptor phenotype. Subsequent elution of bound variants with bacterial sialidase followed by growth in MDCK cell culture yields a virus population enriched in the SAα2,6Gal-specific hemagglutinin. Details of the procedure are given in the text.

[2] Sialyltransferases are distinguished by prefixes indicating their preferred acceptor sequence and the isomeric linkage formed in the product. Thus, Galβ1,3GalNAc α2,3-sialyltransferase catalyzes the general reaction CMP-SA + Galβ1,3GalNAc→SAα2,3Galβ1,3GalNAc +CMP.

TABLE I

Characterization of receptor variants derived from A/duck/Ukraine/1/63 (H3N7)

Virus[a]	Agglutination of erythrocytes[b]				Hemagglutination inhibition[c] (equine α_2-M)	Amino acid at residue 226[d]
	Native	Asialo	SAα2,3Gal	SAα2,6Gal		
	HA titer				*HAI titer*	
A/duck/Ukraine/1/63 (parent)	64	0	64	0	160	Gln
Receptor variants						
UK6	32	0	64	0	80	
UK19	64	0	128	0	80	Gln
UK25	64	0	128	0	80	Gln
UK48	64	0	128	0	80	
UK62	64	0	128	0	80	
UK3	128	0	0	128	5120	
UK16	64	0	0	64	2560	
UK43	32	0	0	32	5120	Leu
UK49	64	0	0	128	2560	Leu
UK50	32	0	0	32	5120	

[a] Receptor variants of A/duck/Ukraine/1/63 were obtained as described in the text, plaque-purified three times, and grown in MDCK cell culture. Hemagglutination titrations were performed with MDCK cell lysates after removal of cell debris by low speed centrifugation (700 × *g* for 10 min).

[b] Human erythrocytes were either unmodified (Native), treated with *V. cholera* sialidase (asialo), or sialidase-treated cells resialylated with CMP-sialic acid and purified sialyltransferases as described under "Materials and Methods." The sialyloligosaccharide structures examined are SAα2,3Galβ1,3GalNAc (SAα2,3Gal) and SAα2,6Galβ1,4GlcNAc (SAα2,6Gal) commonly found as terminal sequences on *O*- and *N*-linked oligosaccharides, respectively. HA titers were determined as described and are expressed as the reciprocal of the greatest dilution of virus that produced agglutination (0 = <2).

[c] Hemagglutination inhibition by purified, heat-inactivated (30 min at 56 °C) equine α_2-macroglobulin (α_2-M) was performed as described. The initial concentration of inhibitor (3 mg/ml) was roughly the same concentration found in unfractionated serum (22). Results are expressed as the reciprocal of the highest dilution of α_2-macroglobulin causing inhibition of native erythrocyte agglutination by 4 hemagglutinating units of virus.

[d] Deduced from the nucleotide sequences of the HA_1 region of the hemagglutinin genes. Nucleotide sequences were determined using the dideoxynucleotide chain termination method with a primer extension system containing virus RNA, reverse transcriptase, and 5′-^{32}P-labeled synthetic oligonucleotide primers (7). The only amino acid sequence changes detected were at residue 226 as a result of changes in the triplet nucleotides 754–756 from CAG (glutamine, Gln) to CTG (leucine, Leu).

ated erythrocytes derivatized to contain the SAα2,3Gal linkage did not agglutinate SAα2,6Gal-derivatized cells and was not sensitive to hemagglutination inhibition by equine α_2-macroglobulin. The five wild type clones exhibited receptor properties identical to those of the parent strain. In contrast, five variants agglutinated cells modified to contain SAα2,6Gal linkages did not agglutinate SAα2,3Gal-derivatized cells and were sensitive to hemagglutination inhibition by equine α_2-macroglobulin. The receptor binding properties of these variants of A/duck/Ukraine/1/63, therefore, are very similar to those previously associated with the corresponding receptor type isolated for the human H3 isolates (4, 7, 8).

The complete amino acid sequences of the HA_1 region of the hemagglutinins expressed by the parent strain and representative SAα2,3Gal- and SAα2,6Gal-specific clones were deduced from the nucleotide sequences of their RNA genes as previously described (7). The only amino acid sequence changes detected were at residue 226. As summarized in Table I, amino acid 226 was glutamine in the parent strain and in the cloned wild type viruses while the cloned variants specific for SAα2,6Gal determinants had leucine at position 226. This residue is located in the distal portion of the molecule in the receptor pocket (13), and the presence of leucine or glutamine at amino acid 226 was previously observed to account for the different binding properties of the SAα2,6Gal-specific human H3 isolates and their SAα2,3Gal-specific receptor variants, respectively (7).

Changes in Receptor Specificity Associated with Growth in Eggs—During growth of the avian receptor variants in embryonated eggs for the purpose of sequence determination, the SAα2,6Gal-specific variants underwent a shift in receptor binding properties, reverting to the wild type SAα2,3Gal-specific, inhibitor-insensitive phenotype. To further examine this phenomenon, variants which had been plaque-purified (four successive plaque to plaque passes) and passaged once in MDCK cultures were seeded at various concentrations, ranging from 10^7 to 10 plaque forming units, in embryonated eggs and MDCK monolayers and grown for various lengths of time. Table II shows hemagglutination titrations, using derivatized red blood cells, of representative SAα2,3Gal-specific and SAα2,6Gal-specific cloned viruses after growth in the egg allantois (20 h at 37 °C) or MDCK cell culture (harvested at peak yield). The virus preparations were concentrated approximately 100-fold, by differential centrifugation, prior to assay to eliminate potential inhibitors of agglutination that may be present in allantoic fluid. Progeny from both of the SAα2,3Gal-specific isolates (UK19 and UK25) preferentially agglutinated erythrocytes modified to contain SAα2,3Gal determinants whether grown in MDCK cells or the embryonated egg. The weak agglutination of SAα2,6Gal-containing erythrocytes observed in both MDCK and egg grown virus is typical of concentrated preparations of A/duck/Ukraine/1/63 (4). The SAα2,6Gal-specific variants (UK43 and UK49), on the other hand, retained their specificity for SAα2,6Gal sequences when grown in MDCK cells, but exhibited an apparently dose-dependent gradient of reversion from the original SAα2,6Gal-specific phenotype to a wild type SAα2,3Gal-specific virus when grown in eggs. This receptor shift is especially evident at the higher infectious doses where one of the variants, UK43, agglutinated only SAα2,3Gal-derivatized cells and showed no agglutination of erythrocytes containing SAα2,6Gal sequences.

TABLE II

Receptor specificities of Ukraine variants after passage in the egg allantois and MDCK cell culture

Host	Infectious dose[a]	UK25		UK19		UK49		UK43	
		α2,3	α2,6	α2,3	α2,6	α2,3	α2,6	α2,3	α2,6
	pfu	*HA titer*[b]							
MDCK[c]	10^7	4,096	128	4,096	128	0	4,096	0	4,096
	10^6	8,192	128			0	4,096		
	10^5	8,192	128			0	8,192		
	10^4	8,192	128			0	16,384		
	10^3	8,192	256			16	7,182		
	10^2	8,192	128			0	16,384		
	10	8,192	256			0	16,384		
EGG[d]	10^7	8,192	256	512	0	256	128	256	0
	10^6	4,096	64	5,120	0	1,024	512	320	0
	10^5	4,096	64	2,048	0	2,048	2,048	1,280	640
	10^4	8,192	128	320	0	256	512	1,280	320
	10^3	16,384	512	320	0	64	128	2	2
	10^2	4,096	64	5,120	0	0	16	0	0
	10	1,024	16	128	0	0	2	0	0

[a] Virus seed stocks were prepared by growth of plaque-purified virus (4 successive plaque to plaque passes) in MDCK cell cultures. Infectious titers (plaque-forming units/ml) of the cell lysates were determined as described under "Material and Methods." These preparations had log pfu/HA ratios of 6.3 (UK19), 6.2 (UK25), 6.0 (UK43), and 5.7 (UK49). Receptor specificity was confirmed by hemagglutination titrations with derivatized erythrocytes, and results are shown in Table I.

[b] Hemagglutination titers toward resialylated SAα2,3Gal (α2,3) and SAα2,6Gal (α2,6) erythrocytes were assessed for each virus preparation as described in Table I. All preparations were concentrated 100-fold prior to assay.

[c] Seed stocks were diluted as shown and grown on MDCK monolayers in the presence of trypsin (20 μg/ml) at 34 °C. Virus was harvested at optimum times for each infectious dose, as determined by visual assessment of the cytopathic effect.

[d] Appropriate dilutions of virus seed stocks were injected (0.2 ml) into the allantoic cavity of 10-day-old chicken embryos and incubated 20 h at 37 °C.

TABLE III

Time course of adaptation by Ukraine variants during growth in ovo

Variant[a]	Receptor specificity after growth in eggs[b]							
	1 day		2 day		3 day		4 day	
	α2,3	α2,6	α2,3	α2,6	α2,3	α2,6	α2,3	α2,6
	HA titer							
UK19	128	4					256	0
UK25	1024	16					1024	0
UK43	0	0	128	32	128	16	256	0
UK49	0	2	128	256	1024	128	128	0

[a] The allantoic cavity of 10-day-old chicken embryos were injected (0.1 ml) with 10 pfu/egg of virus and incubated at 37 °C.

[b] Hemagglutination of SAα2,3Gal (α2,3)- or SAα2,6Gal (α2,6)-derivatized cells by concentrates (100 ×) of egg grown virus was examined as described in Table I. Results are expressed as reciprocals of the highest dilution of virus that resulted in agglutination (0 = <2).

At the lower infectious doses (10–100 pfu/egg), the SAα2,6Gal clones grew poorly in eggs, and of the virus that was present (after only 1 day of growth), there was no evidence for the specificity changes that occurred at the higher doses (Table II). However, incubation of eggs inoculated with SAα2,6Gal-specific variants at 10 pfu/egg for 2, 3, and 4 days also revealed evidence of selection on the basis of receptor binding (Table III). The same pattern of adaptation by the SAα2,6Gal-specific variants was also observed when plaque eluates were used as the source of inoculum (data not shown). These observations suggested that the wild type virus with the SAα2,3Gal-binding phenotype grew more rapidly *in ovo* than the SAα2,6Gal variants (Tables II and III) and that selection could occur simply by the wild type virus outgrowing and variants.

Absence of Receptor Shift when Other Receptor Variants of the H3 Serotype Are Grown in Ovo—A change in receptor specificity upon transfer from MDCK cell culture to growth *in ovo* is not a phenomenon observed with all SAα2,6Gal-specific viruses of the H3 serotype. Indeed, numerous SAα2,6Gal-specific human isolates have been routinely passaged *in ovo* with retention of receptor specificity (4, 7). To illustrate this, Table IV compares the receptor specificities of the SAα2,6Gal- and SAα2,3Gal-specific A/duck/Ukraine/1/63 viruses with the corresponding receptor specific viruses derived from the human virus, A/Memphis/102/72 (8), after growth in MDCK cells or eggs. The SAα2,3Gal-specific variants of A/duck/Ukraine/1/63 and A/Memphis/102/72 both retain the specificity of MDCK grown parents as indicated by agglutination of erythrocytes bearing SAα2,3Gal sequences. As reported above for the UK43 and UK49 clones (Tables II and III), three other SAα2,6Gal-specific variants of A/duck/Ukraine/1/63 grew in MDCK cultures, with retention of specificity, but revert to the SAα2,3Gal-specific wild type when grown in the allantoic cavity of fertile eggs. In contrast, SA2,6Gal-specific clones of the human virus A/Memphis/102/72 retain their specificity for the SAα2,6Gal linkage when grown in either MDCK cell culture or the embryonated egg.

Differences in Receptor Binding between Human and Avian SAα2,6Gal-specific Variants—Since human isolates of the H3 subtype specific for SAα2,6Gal determinants readily replicate in eggs and the A/duck/Ukraine/1/63 variants specific for SAα2,6Gal sequences do not, attempts were made to identify possible differences in receptor binding between them. Asialo human erythrocytes were modified to contain terminal SAα2,6Gal linkages of two naturally occurring sialic acids *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic

TABLE IV

Comparison of receptor specificities for human and avian receptor variants bearing the H3 hemagglutinin after growth in ovo

Original specificity	Parent virus	Clone	Host[a]	Specificity[b]	
				SAα2,3Gal	SAα2,6Gal
				HA titer	
SAα2,3Gal	duck/Ukraine	UK6	MDCK	64	0
			Egg	16	0
		UK48	MDCK	64	0
			Egg	32	0
		UK62	MDCK	128	0
	Memphis	M1/HS8	MDCK	64	0
			Egg	128	0
		M2/2	MDCK	64	0
			Egg	32	0
SAα2,6Gal	duck/Ukraine	UK3	MDCK	0	128
			Egg	32	0
		UK16	MDCK	0	64
			Egg	16	0
		UK50	MDCK	0	32
			EGG	256	0
	Memphis	M1/5	MDCK	0	64
			Egg	0	1024
		M1/HS10	MDCK	0	64
			Egg	0	128

[a] Virus (MDCK cell lysates) was grown either in MDCK cells or 10-day fertile eggs as described under "Materials and Methods."

[b] Receptor specificity was determined by agglutination of derivatized erythrocytes as described in the legend to Table I.

TABLE V

Agglutination of erythrocytes derivatized to contain different sialic acids in the α2,6 Gal linkage

Virus variant	Sialic acid[a]	
	NeuAc	NeuGc
	HA titer	
A/duck/Ukraine/1/63		
UK3	128	0
UK16	64	0
UK43	64	0
UK49	64	0
UK50	32	0
A/Memphis/102/72		
M1/5	64	32
M1/HS9	128	128
M1/HS10	64	32

[a] The donor substrates CMP-NeuAc and CMP-NeuGc were prepared by published procedures (23, 24) with modifications (H. Higa and J. Paulson, manuscript in preparation). Asialo erythrocytes were resialylated as described under "Materials and Methods" using other 39.6 milliunits (NeuAc) or 18.7 milliunits (NeuGc) of purified α2,6Galβ1,4GlcNAc rat liver sialyltransferase. The amount of sialic acid incorporated, expressed as nmol/ml of packed erythrocytes, was 40.7 nmol/ml for NeuAcα2,6Gal cells and 50.9 for NeuGcα2,6Gal cells. Hemagglutination assays were performed as described under "Materials and Methods" using clarified MDCK cell lysates, and results are expressed as the reciprocal of the maximum dilution required to give agglutination (0 = <2).

acid (NeuGc). As shown in Table V, while the human isolates agglutinated erythrocytes containing either NeuAc or NeuGc, the A/duck/Ukraine/1/63 variants only agglutinated cells containing NeuAc. Analysis by thin layer chromatography (19) of sialidase-sensitive sialic acid present on the chorioallantoic membrane of 11-day-old fertile hens' eggs, however, did not reveal any NeuGc; only NeuAc was present (data not shown). Thus, while differential binding of human and avian SAα2,6Gal-specific variants to NeuGc containing receptors does not provide an explanation for differences in egg selection, functional variation in the receptor binding properties of their hemagglutinins can be demonstrated.

DISCUSSION

We have reported previously that influenza viruses of the H3 subtype which recognize sialic acid in different linkages can be obtained by passaging wild type virus in the presence of inhibitors of agglutination (7, 8). Specifically, Hong Kong influenza viruses isolated from humans and grown in the absence of inhibitor recognize sialic acid in the α2,6 linkage to galactose on oligosaccharide side chains, and receptor variants which recognize sialic acid linked α2,3 to the penultimate residue can be isolated by growing virus in the presence of nonimmune horse serum, a source of α_2-macroglobulin which is a potent inhibitor of virus bearing SAα2,6Gal-specific hemagglutinins. In this paper, we demonstrate that the reverse selection can also be made. Using the H3 virus A/duck/Ukraine/1/63 which recognizes the SAα2,3Gal linkage, variants which specifically recognize SAα2,6Gal linkages can be obtained by a procedure, fundamentally different from previous selection procedures, involving multiple cycles of adsorption to and elution from enzymatically modified erythrocytes. Nucleotide sequence analysis of the genes for the hemagglutinins of the wild type A/duck/Ukraine/1/63 virus and the variants indicate that the only change in sequence leads to the substitution of a glutamine at residue 226 in the SAα2,3Gal-specific hemagglutinin for a leucine in the SAα2,6Gal-specific molecule. These observations confirm our previous finding that residue 226 is a component of the sialic acid receptor binding pocket and emphasize its importance in determining the receptor specificity of the H3 hemagglutinin molecule.

Unlike the H3 viruses isolated from humans, however, and the variants derived from them, which all replicated efficiently in fertile hens' eggs, the SAα2,6Gal-specific variants of A/duck/Ukraine/1/63 grew very poorly in eggs, and the virus recovered from such infections had reverted to the SAα2,3Gal-specific phenotype. These observations provide direct evidence for the host-mediated selection, by growth in eggs, of viruses with modified receptor specificity. Since allantoic fluid does not inhibit hemagglutination by either the wild type or variant viruses (data not shown), the results suggest that membrane receptors which interact with the hemagglutinins of the virus are involved in the selection of viruses with appropriate specificity. The potential for cell-surface receptors to mediate selection of receptor-specific viruses has been demonstrated using MDCK cells enzymatically modified to carry either SAα2,6Gal or SAα2,3Gal linkages.[3] However, since hemagglutinins of influenza viruses of human origin with specificity for SAα2,6Gal sequences readily bind to receptors present in the egg, the apparent restricted binding of the SAα2,6Gal-specific A/duck/Ukraine/1/63 implies that a component of the receptor molecule in addition to sialic acid linkage is involved in determining the precise specificity of the virus-receptor interaction. This conclusion is supported by our observation that the SAα2,6Gal-specific isolates of A/Memphis/102/72 and A/duck/Ukraine/1/63 differ in their ability to agglutinate derivatized erythrocytes containing *N*-glycolylneuraminic acid. The molecular basis of this additional specificity is unknown. However, by comparing the amino acid sequences of the SAα2,6Gal-specific hemagglutinins from human and avian isolates and examining the locations of differing residues in the three-dimensional structure of the X-31 hemagglutinin (13), it is apparent that the amino acid differences at positions 137, 155, 158, 189, 193, 227, and 228 in or near the receptor pocket could individually or in combination impose the additional receptor requirements.

Finally, the observation that cell-surface receptors can mediate selection of receptor variants provides a partial explanation for the apparent changes in receptor binding properties which can occur during adaptation of influenza viruses to growth in different hosts. It is in this regard particularly relevant to the suggestion that an avian virus antigenically related to A/duck/Ukraine/1/63 was the progenitor of the virus responsible for the Hong Kong influenza pandemic of 1968 (20, 21).

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Note Added in Proof—A recent report relevant to work presented here has examined reassortant viruses of A/duck/Ukraine/1/63 containing a human virus H3 hemagglutinin (27). Results suggest that changes in amino acids 226 and 228 can alter the tissue tropism of the virus in ducks.

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