

Differences between influenza virus receptors on target cells of duck and chicken

A. Gambaryan¹, R. Webster², and M. Matrosovich^{1,2}

¹M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides,
Russian Academy of Medical Sciences,

P/O Institute of Poliomyelitis, Moscow, Russia

²Department of Virology and Molecular Biology,
St. Jude Children's Research Hospital, Memphis, Tennessee, U.S.A.

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Summary. H5, H7, and H9 subtype influenza viruses in land-based poultry often differ from viruses of wild aquatic birds by deletions in the stalk of the neuraminidase, by the presence of additional carbohydrates on the hemagglutinin, and by occasional changes in the receptor specificity. To test whether these differences could reflect distinctions between the virus receptors in different avian species, we compared the binding of duck, chicken and human influenza viruses to cell membranes and gangliosides from epithelial tissues of duck, chicken and African green monkey. Human viruses bound to cell membranes of monkey and chicken but not to those of duck, suggesting that chicken cells unlike duck cells contain Sia(α 2-6)Gal-terminated receptors recognized by human viruses. Duck virus bound to gangliosides with short sugar chains that were abundant in duck intestine. Human and chicken viruses did not bind to these gangliosides and bound more strongly than duck virus to gangliosides with long sugar chains that were found in chicken intestinal and monkey lung tissues. Our data suggest that the spectrum of sialylglycoconjugates which can serve as influenza virus receptors in chicken is more similar to the spectrum of receptors in the respiratory epithelia of monkey than to that in the epithelial tissues of duck. This notion could explain the recent emergence of avian H9N2 virus lineage with human virus-like receptor specificity and emphasizes the role of the chicken as a potential intermediate host for the transmission of viruses from aquatic birds to humans.

Introduction

The primary natural reservoir of influenza A viruses is wild aquatic birds; all stable lineages of influenza A viruses in other birds and mammals originated

from viruses in this reservoir (reviewed in [1] and [29]). Such lineages emerge rarely because viruses replicate less efficient in a heterologous host species (host-range restriction). Influenza viruses of aquatic birds possess a strict binding preference for Sia(α 2-3)Gal-terminated receptors. This preference may, in addition to many other factors, limit the replication of avian viruses in the respiratory tract of humans, in which Sia(α 2-6)Gal-terminated receptors predominate [4, 11]. Therefore, the alteration of receptor specificity of viruses of aquatic birds in some intermediate host, such as pig, may potentially facilitate their transmission to humans [11, 15].

Recent human infections caused by H5N1 and H9N2 avian viruses [21, 25] suggested that domestic poultry is another potential intermediate host in zoonotic transmission. Turkey and chicken viruses evolve much faster than those of aquatic birds, a finding that suggests virus adaptation to land-based avian species after transmission from aquatic birds [24]. Surface glycoproteins of turkey and chicken viruses often differ from those of duck (*Anseriformes* spp.) viruses by the presence of additional carbohydrates on the hemagglutinin and by deletions in the stalk of the neuraminidases, these features affect receptor-binding and receptor-destroying activities of the virus ([2, 14] and references therein). Particularly intriguing are the preferential binding of H9N2 viruses recently isolated from poultry in south-eastern Asia to Sia(α 2-6)Gal-terminated receptors, their poor binding to Sia(α 2-3)Gal-containing receptors, and mutations in their NA hemadsorbing site [16], characteristics that are typical of human influenza viruses and have not been previously observed among avian viruses.

Structural and functional distinctions between HAs and NAs of viruses from wild aquatic birds and those from land-based domestic birds suggest that a spectrum of sialic acid-containing receptors, or of inhibitors of influenza viruses in different avian species are not identical. In this study, we addressed this hypothesis by comparing influenza virus receptors on target cells of mallard duck, the typical host among aquatic birds, with those on target cells of chicken, the typical host among domestic poultry. In addition, receptors in both avian hosts were compared with those of tracheal and lung epithelial cells of African green monkey (*Cercopithecus aethiops*). This primate species is susceptible to human influenza viruses [19]; therefore, we assumed that sialylglycoconjugate receptors on monkey target cells are similar to those on human target cells.

Materials and methods

Viruses

All avian influenza A viruses and human virus A/Singapore/1/57 (H2N2) were from the repository of St. Jude Children's Research Hospital. These viruses were grown in 9-day-old to 10-day-old chicken eggs. The highly pathogenic H5N1 and H7N1 viruses were inactivated by treatment with 0.05% β -propiolactone [14]. Human non-egg-adapted H1N1, H3N2, and type B virus strains were kindly provided by J.S. Robertson, National Institute for Biological Standards and Control, UK and were propagated solely in MDCK cells. The allantoic and culture fluids were clarified by low-speed centrifugation; the viruses were pelleted by

high-speed centrifugation, suspended in 0.1 M Tris buffer (pH 7.2) containing 50% glycerol, and stored at -20°C .

Preparation of plasma membranes

Freshly killed adult chickens (*Gallus gallus*) and mallard ducks (*Anas platyrhynchos*) were purchased from a live bird market in Moscow, Russia. Tracheal and lung tissues of African green monkey (*Cercopithecus aethiops*) were provided by the Department of Standardization and Control of Poliomyelitis Vaccine, M.P. Chumakov Institute of Polyomyelitis, Moscow. Epithelial cells and their plasma membranes were prepared as described previously [8]. In brief, epithelial cells were gently scratched off the tissues with a spatula, purified by repetitive centrifugation in 50% Percoll, and disrupted by hypotonic shock (0.01 M Tris buffer pH 7.3, 15 min on ice) and homogenization with a Dounce homogenizer. Nuclei and cellular debris were removed by low-speed centrifugation (1000 g, 5 min) and plasma membranes were pelleted by high-speed centrifugation (150,000 g, 1.5 h). The pellets were suspended in Tris-buffered saline containing 0.5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride, dispersed by ultrasound treatment, and stored in aliquots at -20°C .

Preparation of total gangliosides

Total lipids were extracted from freshly prepared cells with 20 volumes of chloroform/methanol solution (2:1) followed by extraction with 20 volumes of ethanol/water solution (4:1). Each extraction was performed at 60°C over the course of 2 h. Gangliosides were isolated from combined lipid extracts by two consecutive partitions in chloroform/methanol/water [6].

Thin-layer chromatography (TLC)

Analytical TLC of total gangliosides was performed by using HPTLC silica gel 60 plates with a concentrating zone (Merck, Germany) in a solution of chloroform/methanol/20 mM CaCl_2 , (120:85:20) as a mobile phase [22]. Resorcinol was used to stain sialic acids [27]. Chromatographs were scanned and processed using Adobe Photoshop 5.0 software. Total gangliosides were fractionated by preparative TLC on Polygram Sil G plates (Macherey-Nagel, Germany) in the same solvent system. The bands were visualized by iodine staining, and all visible spots and sections between spots were scratched off and extracted with 50% methanol at 37°C .

Binding assays

1. *Virus attachment to plasma membranes and to ganglioside fractions* (Figs. 1 and 3) was assayed by the microplate adsorption method as previously described [8, 12]. In brief, the wells of 96-well polyvinyl chloride microplates (Costar; USA) were coated with either membranes or ganglioside fractions, and nonspecific binding sites were blocked by incubation with 0.02% solution of bovine serum albumin (BSA) in PBS at 4°C for 2 h. Virus suspensions in reaction buffer (RB; 0.1% BSA in PBS) with hemagglutination titers ranging from 32 to 64 were incubated in the microplate wells at 4°C for 1 h. Unbound virus was removed by washing the plates with 0.05% solution of tween-80 in PBS. We measured the amount of bound virus by evaluating its ability to bind a standard peroxidase-labeled fetuin preparation; the binding values were expressed in terms of peroxidase activity (absorbency of the oxidized o-phenylenediamine substrate at 490 nm).

2. *Quantitative assay of the virus binding affinity for gangliosides* (Table 1) was performed as previously described [8]. In brief, serial two-fold dilutions of the virus in RB

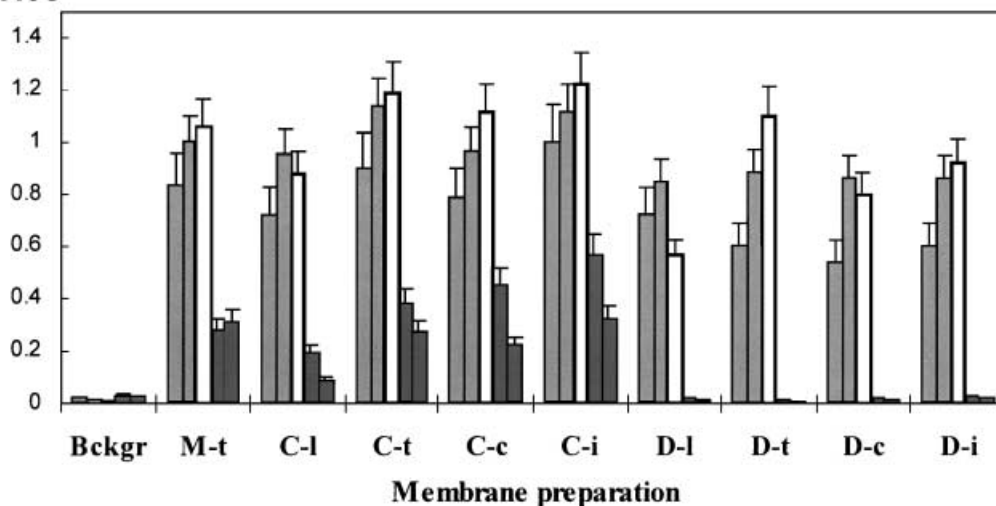
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Fig. 1. Binding of duck influenza viruses (grey bars), chicken influenza virus (open bar), and human influenza viruses (dark bars) to plasma membranes of epithelial cells from duck (*D*), chicken (*C*), and monkey (*M*). The membranes from epithelial cells of trachea (*t*), lung (*l*), crop (*c*), and intestine (*i*) were allowed to adhere to the wells of plastic 96-well microplates and were then incubated with the virus suspensions. Uncoated wells (*Bckgr*) served as controls for background binding. Viruses (bars from left to right) included the following strains: 1. A/Pintail duck/Primorie/695/76 (H2N3), 2. A/Duck/Czechoslovakia/56 (H4N6), 3. A/Chicken/FPV/Rostok/34 (H7N1), 4. A/Chr/157/83-MDCK (H1N1), 5. A/NIB/44/90-MDCK (H3N2). Each bar is a mean \pm SD, $n = 2$

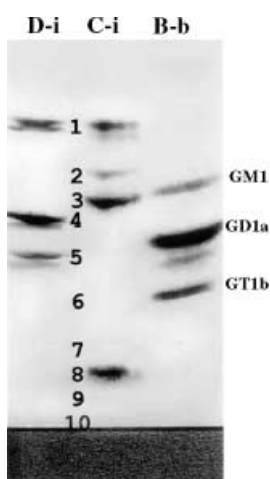


Fig. 2. Thin-layer chromatogram of gangliosides isolated from epithelial cells of duck intestine (*D-i*) and chicken intestine (*C-i*) and of the bovine brain gangliosides (*B-b*), which served as the standard. Gangliosides were stained with resorcinol after separation. The numbers designate chromatographic zones that were isolated by preparative TLC

were incubated in the ganglioside-coated BSA-blocked wells. The virus binding was quantified by using peroxidase-labeled fetuin, and the data were subjected to Scatchard analysis. As a positive control for the maximal virus binding, the non-specific virus attachment to the plastic wells in the assay conditions was measured. The wells without coating were used, the blocking step was omitted, and no BSA was present in the incubation mixture, all other

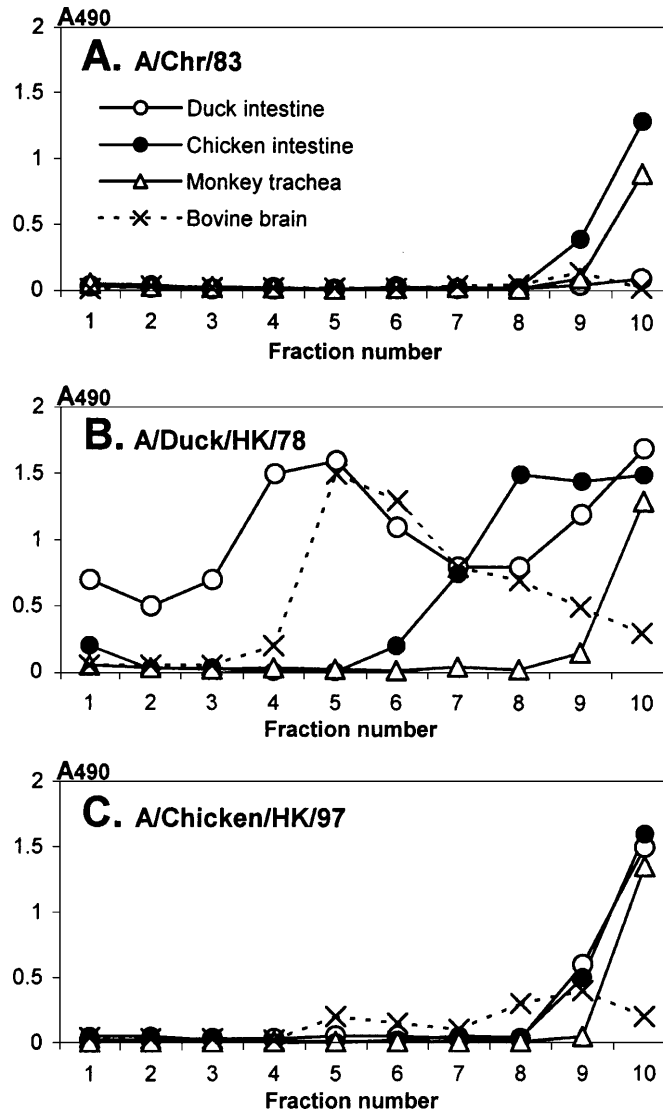


Fig. 3. Binding of **A** human influenza virus A/Chr/157/83-MDCK (H1N1), **B** duck influenza virus A/Duck/Hong Kong/308/78 (H5N3), and **C** chicken influenza virus A/Chicken/Hong Kong/786/97 (H5N1) to gangliosides from epithelial cells of duck intestine (○), chicken intestine (●), monkey trachea (△), and bovine brain (×). Total gangliosides were fractionated by preparative TLC, the fractions were allowed to adhere to the wells of plastic microplates, and the virus binding was assayed as described in Materials and Methods. The fraction numbers correspond to the chromatographic zones shown in the Fig. 2. Standard bovine brain gangliosides were located in the following fractions: GM1 in fraction 3, GD1a in fraction 5, and GT1b in fraction 6. SD values were in the range from 3 to 15% of the mean of two replicates at each experimental point (not shown)

steps of the assay were the same as described above. Apparent binding affinity constants of virus attachment to gangliosides and to plastic were determined from the slopes of the corresponding Scatchard plots. The affinity constant of the nonspecific adsorption of the virus to the plastic was arbitrarily established as 100%, and the relative virus affinity for gangliosides

Table 1. Binding of influenza viruses to complex gangliosides from epithelial cells of duck intestine (D-i), chicken intestine (C-i), and monkey lung (M-l)

Virus		Relative affinity (%) ^a		
		D-i	C-i	M-l
Duck				
Duck/Alberta/35/76	H1N1	19 ± 6	20 ± 4	20 ± 7
Black duck/New Jersey/1580/78	H2N3	25 ± 4	24 ± 4	24 ± 3
Duck/Buryatia/652/88	H3N8	32 ± 4	33 ± 3	38 ± 4
Duck/Czechoslovakia/56	H4N6	41 ± 6	41 ± 1	41 ± 7
Duck/Hong Kong/308/78	H5N3	41 ± 5	42 ± 6	42 ± 2
Duck/Alberta/60/76	H12N5	30 ± 6	24 ± 5	30 ± 4
Chicken				
Chicken/Hong Kong/220/97	H5N1	39 ± 6	57 ± 2	77 ± 9
Chicken/Hong Kong/728/97	H5N1	41 ± 6	60 ± 7	71 ± 6
Chicken/Hong Kong/786/97	H5N1	32 ± 10	52 ± 10	61 ± 6
Chicken/FPV/Rostok/34	H7N1	30 ± 4	49 ± 9	51 ± 4
Chicken virus isolates from humans				
Hong Kong/156/97	H5N1	44 ± 16	62 ± 7	63 ± 11
Hong Kong/481/97	H5N1	40 ± 3	62 ± 10	71 ± 6
Hong Kong/485/97	H5N1	51 ± 9	70 ± 6	69 ± 7
Human				
Chr/157/83-MDCK	H1N1	< 5	81 ± 6	82 ± 11
Singapore/1/57	H2N2	< 5	62 ± 12	75 ± 21
NIB/26/90-MDCK	H3N2	< 5	81 ± 5	83 ± 18
B/NIB/48/90-MDCK	Type B	< 5	95 ± 14	120 ± 22

^aThe relative binding affinity is expressed as a percentage of the binding affinity for the positive control (non-specific binding to plastic); higher values of relative affinity reflect stronger binding to the receptor. Standard deviations from the mean of 2 replicates are shown

was calculated with respect to this control. Each set of experiments for the figures and the table presented here was repeated on two to four occasions with similar results. Data were averaged from the same set of experiments.

Results and discussion

Chicken and monkey epithelial cells, unlike duck epithelial cells, contain Sia(α2-6)Gal-terminated receptors

To assess presentation of the two major natural sialyloligosaccharide determinants, Sia(α2-3)Gal and Sia(α2-6)Gal, on the target cells of different virus hosts, we utilized influenza virus strains with a strict selective binding to one or the other determinant. Thus, two duck virus strains, A/Pintail duck/Primorie/695/76 (H2N3) and A/Duck/Czechoslovakia/56 (H4N6), and the chicken virus A/Chicken/FPV/

Rostok/34 (H7N1) were previously shown to bind to Sia(α 2-3)Gal-terminated glycoconjugate receptors but essentially did not bind to Sia(α 2-6)Gal-terminated receptors [7, 14]. Two human virus strains used, A/Chr/157/83 (H1N1) and A/NIB/44/90 (H3N2), were clinical isolates propagated in MDCK cells and lacking egg-adaptation mutations in their HAs [23]. Previous studies revealed that these viruses have a strict binding preference for Sia(α 2-6)Gal determinants and do not bind to receptors with Sia(α 2-3)Gal moieties [7, 9]. Using these five viruses as Sia-Gal linkage-specific lectins; we evaluated their ability to bind to epithelial cell membranes of mallard duck, chicken and African green monkey (Fig. 1). Duck viruses and a chicken virus bound to all membranes. These data indicated that all of the tested cell preparations were abundant in Sia(α 2-3)Gal-containing glycoconjugates.

The human viruses bound to membrane receptors of the monkey epithelium preparation and those of all of the chicken epithelium preparations with similar efficiency, but they did not bind to membrane receptors neither in duck intestine, nor in any of the other duck preparations. This binding pattern indicates that Sia(α 2-6)Gal moieties are present in a variety of chicken epithelial tissues but in neither of the duck tissues. The lack of binding of human viruses to duck tissues supports and extends the previous finding of Ito et al. [11] who reported that the duck intestinal epithelium was not stained with *Sambucus nigra* lectin, which specifically recognizes Sia(α 2-6)Gal moieties.

Ducks are one of the primary species of wild aquatic birds responsible for perpetuating influenza viruses in nature [1]. The lack of Sia(α 2-6)Gal-containing receptors in the enteric and respiratory tracts of ducks may explain why influenza viruses of wild aquatic birds maintain their receptor preference for Sia(α 2-3)Gal-containing receptors [3, 7, 13]. The linkage specificity of sialylglycoconjugates in avian species other than duck was addressed in only one previous study that revealed the expression of Sia(α 2-6)Gal moieties on endothelial and epithelial cells of embryonic chicken lung and liver [5]. However, given the known tissue-specific and developmentally regulated expression of sialylglycoconjugates, this finding could not be unambiguously extrapolated on the respiratory and intestinal tissues of adult chicken. Our results indicate that Sia(α 2-6)Gal moieties are present on a variety of epithelial cells of adult chicken as well.

We recently described an unusual receptor specificity of poultry H9N2 viruses from southeastern Asia [16]. These viruses, unlike any other avian influenza viruses previously studied, displayed a human virus-like preferential binding to Sia(α 2-6)Gal moieties, and yet replicated efficiently in and were transmitted to various species of poultry including chickens. This finding appears to contradict those that have indicated that avian influenza viruses preferentially bind to Sia(α 2-3)Gal-linked receptors, and that viruses with human virus-like receptor specificity cannot replicate in the intestinal tract of ducks [18, 28]. The data of Feldmann et al. [5] and especially our present finding of Sia(α 2-6)Gal-linked receptors in respiratory and intestinal epithelial tissues of adult chicken resolve this apparent discrepancy. Furthermore, these findings suggest that the adaptation of duck viruses in chickens and possibly in other land-based birds could cause the

occasional emergence of viruses with an enhanced ability to bind to Sia(α 2-6)Gal-terminated receptors and a concomitantly enhanced propensity for transmission to humans.

The ganglioside receptors of chicken and monkey epithelial cells differ from those of duck epithelial cells

The receptor recognition by influenza viruses is not limited to the binding to terminal Sia-Gal moieties and can be significantly affected by the structure of more distant parts of the receptor molecules (reviewed in [20] and [26]). To further characterize potential differences between virus receptors in target tissues of duck and chicken, we compared the virus-binding activity of gangliosides isolated from intestinal epithelial cells of these species. Ganglioside extracts were first analyzed by using high-performance thin-layer chromatography (TLC) with resorcinol staining. The chromatographic patterns of gangliosides in chicken and duck cells differed significantly (Fig. 2), for example, chicken preparation unlike duck preparation contained ganglioside species with the mobility similar to that of GM1 from bovine brain and did not contain ganglioside with the mobility of GD1a. These differences were reproducible in replicate samples prepared from fresh avian tissues on different days.

The total gangliosides from duck and chicken intestines were separated by using preparative TLC, and isolated fractions were tested for their ability to support the binding of the human, duck, and chicken influenza viruses (Fig. 3). Total gangliosides isolated from monkey trachea and commercial preparation of bovine brain gangliosides were used for a comparison in these experiments. The human influenza virus did not bind to any of the ganglioside fractions from the duck cells but did bind to the slowest-moving gangliosides (fractions 9 and 10) from chicken and monkey tissues (Fig. 3A). These gangliosides migrated much slower than the GT1b ganglioside from bovine brain, a finding that indicates the high length or complexity of their sugar chains. Because of the small quantity of these gangliosides in the total ganglioside mixture, they could not be detected by resorcinol staining (Fig. 2), and therefore appeared to have a relatively high virus-binding capacity. The pattern of human virus binding to gangliosides from chicken and monkey cells resembles that of human influenza virus recognition of gangliosides from human tissues reported by Miller-Podraza et al. [17]. In that study, a human influenza virus did not bind to relatively short gangliosides (as many as seven monosaccharides per molecule of ceramide) but did bind strongly to minor slower moving Sia(α 2-6)Gal-containing molecules with eight and more saccharides per ceramide residue. Thus, our data indicate that chicken and monkey tissues, similar to human tissues, contain complex gangliosides that bear Sia(α 2-6)Gal moieties.

The duck virus bound to gangliosides of various lengths and complexity (Fig. 3B). In addition to binding to the most complex but less abundant gangliosides (fractions 9 and 10) from duck, chicken, and monkey, the duck virus also bound to gangliosides from bovine brain, and to most of the ganglioside fractions from duck intestine, including the one with the highest chromatographic

mobility, presumably GM3. Pretreatment of any ganglioside fraction with *Vibrio cholerae* NA completely abolished binding of the virus from any host species (data not shown); this result confirmed the essential role of sialic acid in binding. In contrast to duck gangliosides, chicken gangliosides and monkey gangliosides with sugar chains of low to intermediate length (fractions 1–5) were not bound by duck virus. In particular, the duck virus did not bind the major ganglioside species from chicken intestine (fraction 3, Fig. 2) that demonstrated chromatographic mobility comparable to that of the GM1 standard. Thus, chicken and monkey gangliosides are different from duck gangliosides not only by the presence of Sia(α 2-6)Gal residues in the former gangliosides but also by the structure of their sugar cores.

The chicken virus differed markedly from the duck virus; the chicken virus did not bind to short gangliosides from any tissue and demonstrated much weaker binding to bovine brain gangliosides (Fig. 3C). Similar to the human virus, the chicken virus bound to the most complex gangliosides from chicken and monkey tissues. However, chicken and human viruses differed in their binding to complex gangliosides from duck intestine (compare Fig. 3A and 3C): the human virus did not bind to the duck gangliosides because it did not recognize the Sia(α 2-3)Gal-containing gangliosides.

The relative roles of ganglioside receptors and sialylglycoprotein receptors in influenza virus infection remains unclear. Because of their size and accessibility on the cell surface, glycoprotein receptors may serve as the point for the initial attachment of influenza viruses, whereas viral binding to gangliosides may be important for the fusion process, because this binding would bring the viral membrane and cell membrane into close proximity (discussed in references 10 and 20). With the assumption that gangliosides are essential for the influenza virus infection, our data on the differences in the structure of gangliosides in chicken cells and those in duck cells suggest that these differences may be responsible for the alteration of the receptor specificity of aquatic bird viruses in chickens.

Compared with the duck virus, chicken and human viruses bind more strongly to complex gangliosides from chicken and monkey tissues

Because chicken cells are relatively deficient in ganglioside receptors with short sugar chains that are recognized by duck viruses (Fig. 3B), the adaptation of duck viruses to chicken cells may require the virus to undergo alterations so that it is able to more efficiently bind to ganglioside receptors with longer or more complex sugar chains. To test this hypothesis, we compared the affinity of duck, chicken, and human viruses for preparations of complex gangliosides. Fractions 8 through 10 of gangliosides isolated from duck, chicken, and monkey epithelial cells by preparative TLC were pooled, adsorbed in the wells of plastic microplates, and tested for the ability to bind to influenza viruses from different host species (Table 1). None of the six duck viruses representing different HA subtypes discriminated between complex gangliosides from different host species; all of the duck viruses bound to duck, chicken, and monkey gangliosides with similar affinities. In contrast and in support of our hypothesis, chicken H5N1

and H7N1 viruses and the H5N1 viruses that were isolated during an outbreak of chicken influenza in humans in Hong Kong displayed a binding affinity for chicken gangliosides that was greater than their affinity for duck gangliosides.

Furthermore, the affinity of chicken viruses for monkey gangliosides was also greater than that for duck gangliosides and was comparable to the binding affinity of human influenza A and B viruses for monkey gangliosides. Because of the potential similarity between ganglioside receptors in monkey and those in humans, this finding may indicate that chicken viruses bind better than duck viruses to ganglioside receptors on human respiratory cells. This feature could lower restriction on replication of chicken viruses in humans as compared with duck viruses.

The molecular basis of the increased affinity of chicken viruses for gangliosides with long sugar chains is not clear. One could speculate that additional carbohydrates on the HA globular head and deletions in the NA stalk, which are the typical changes separating chicken viruses from their precursors in aquatic birds [2, 14], deepen the sialic acid binding sites of both glycoproteins with respect to the surface of the virion. This effect in turn may facilitate the recognition of longer receptor moieties by the chicken virus.

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Author's address: Dr. M. Matrosovich, Institute of Virology, Philipps University, Robert-Koch Str. 17, D-5037 Marburg, Germany; e-mail: Mikhail.Matrosovich@med.uni-marburg.de