Recognition of N-Glycoly neuraminic Acid Linked to Galactose by the α2,3 Linkage Is Associated with Intestinal Replication of Influenza A Virus in Ducks

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The hemagglutinin (HA) of H3 human influenza viruses does not support viral replication in duck intestine despite its avian origin. A Leu-to-Glu mutation at position 226 and a Ser-to-Gly mutation at position 228 in the HA of human A/Udorn/307/72 (H3N2) permit a reassortant virus [human Udorn HA, with all other genes from A/mallard/New York/6750/78 (H2N2)] to replicate in ducks. To understand the molecular basis of this change in host range restriction, we investigated the receptor specificity of duck influenza viruses as well as of human-duck virus reassortants. The results indicate that the recognition of a glycoconjugate moiety possessing N-glycol neuraminic acid (NeuGc) linked to galactose by the α2,3 linkage (NeuGcα2,3Gal) is associated with viral replication in duck intestine. Immunofluorescence assays with NeuGcα2,3Gal-specific antiserum detected this moiety on the intestinal epithelium of duck colonic crypts. Such recognition, together with biochemical evidence of NeuGc in crypt cells, correlated exactly with the ability of the virus to replicate in duck colon. These results suggest that recognition of the NeuGcα2,3-Gal moiety plays an important role in the entero tropism of avian influenza viruses.

Influenza A viruses have been isolated from a variety of animals, including humans, pigs, horses, sea mammals, and birds (37). Each of the different antigenic subtypes of influenza A viruses (H1 to H15 and N1 to N9) has been isolated from wild aquatic birds (7, 27, 37), which appear to serve as the reservoir for all influenza A viruses that infect mammals (37). Despite their common origin, influenza A viruses do not replicate indiscriminately across animal species but rather show a clear pattern of host range restriction. For example, in experimental infection of nonhuman primates, avian influenza viruses replicate poorly (1, 20, 28), while human viruses replicate well and cause influenza symptoms (20, 28); the titers of avian viruses isolated from A/mallard/New York/6750/78 (H2N2) differ by 2 logs in nonhuman primates (19).

However, the molecular mechanism of these restrictive effects remains unknown. Although all influenza viruses recognize oligosaccharide-containing terminal sialic acid, the receptor specificity of the HA differs: most avian influenza viruses preferentially bind to the sialic acid-α2,3-galactose (SAα2,3Gal) linkage, while human influenza viruses favor the SAα2,6Gal linkage on cell surface sialyloligosaccharides (3, 24, 25). Couceiro et al. (4) reported the presence of SAα2,6Gal but not of SAα2,3Gal sialyloligosaccharides on the surface of epithelial cells from human trachea. It was recently shown that the epithelial cells of duck intestine contain SAα2,3Gal but not SAα2,6Gal sialyloligosaccharides (13). Thus, the host range of influenza A viruses may correspond to the presence or absence of certain sialic acid-galactose linkages in host animals, although it is highly likely that this phenomenon is controlled by multiple host and viral genes.

Sialic acid is a generic term for a nine-carbon, acidic amino sugar (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid) whose amino group is replaced with either an N-acetyl or N-glycolyl group, yielding N-acetyl neuraminic (NeuAc) or N-glycoly neuraminic (NeuGc) acid, respectively. The hydroxyl groups can be replaced by acetyl, lactoyl, methyl, sulfate, or phosphate residues. The distribution of specific sialic acids differs among animal species. For example, cells from cows, horses, and pigs express both NeuAc and NeuGc (16, 18, 22, 23), but human cells do not express NeuGc (15, 16). Influenza viruses differ in their recognition of NeuAc, NeuGc, and 9-O-Ac-NeuAc (6), suggesting that it is not only the type of sialic...
acetyl-galactose linkage but also the sialic acid species that contributes to the host range restrictions of influenza A viruses.

Influenza viruses isolated from various animal species differentially agglutinate erythrocytes from different animals (11). For example, although all influenza viruses agglutinate human and chicken erythrocytes, duck but not human influenza viruses agglutinate horse erythrocytes (12). Chicken and human erythrocytes both possess SA2,6Gal, SA2,3Gal, and only NeuAc, while horse erythrocytes contain primarily NeuGe and SA2,3Gal (12). These findings suggest that duck viruses recognize NeuGc2,3Gal molecules, but experimental support for such an association is lacking.

Human influenza A viruses do not replicate in ducks (14, 36). This host restriction appears to reside in the receptor specificity of the HA: a reassortant virus containing only the HA gene from a human A/Uporn/307/72 (H3N2) virus and all remaining genes from A/mallard/New York/6750/78 (Mal/NY) (H2N2) and its host specificity for this molecule. The human A/Udorn/307/72 (H3N2) virus (Fig. 3). The pooled monoclonal antibodies to NeuGc (16), the antiserum did react with NeuGc-containing duck jejunum (Fig. 2C) and cecum (Fig. 2B) were also positive (though to a lesser extent), while those of duodenum (Fig. 2D) were not, suggesting that much higher concentrations of NeuGc are present in the lower intestine of ducks.

Additional experiments sought to establish the presence of NeuGc in duck intestine chemically. Epithelial cell fractions obtained by EDTA treatment (10) were hydrolyzed with sulfuric acid and analyzed by liquid chromatography (5, 32). The presence of NeuAc and NeuGc in this sample was determined with authentic sialic acid standards. The molar ratio of NeuAc and NeuGc in this sample was 98:2 (data not shown). These findings suggest that epithelial cells in duck intestine contain NeuGc2,3Gal, albeit as a minor species. This limited amount of NeuGc among the total sialic acid content in duck intestine seems reasonable, since only crypt cells reacted with antiseraum to NeuGc2,3Gal.

Preferential replication of avian influenza virus in the crypt cells of duck intestine. To determine whether the presence of NeuGc in crypt cells of duck intestine is important for viral replication, we performed immunofluorescence assays of thin sections of duck colon infected with A/duck/Hokkaido/5/77 (H3N2) virus (Fig. 3). The pooled monoclonal antibodies to the NS1 protein detected viral antigens mainly in the crypt cells, in accord with a previous finding (14). Thus, NeuGc
Localization is associated with the efficient replication of duck viruses.

In the present study, we found that recognition of NeuGc\(^{a2,3}\)Gal is associated with the efficient replication of influenza viruses in duck intestine. The HAs of all duck viruses tested (with the exception of A/duck/Ukraine/1/63) recognized this moiety, while those of human viruses did not. The R3 virus, which efficiently recognized NeuAc\(^{a2,3}\)Gal but not NeuGc\(^{a2,3}\)Gal, failed to replicate in duck intestine, whereas the R2 mutant, which efficiently recognized both NeuAc\(^{a2,3}\)Gal and NeuGc\(^{a2,3}\)Gal,

**FIG. 1.** Comparison of receptor specificity among duck, human, and reassortant influenza viruses. To determine the receptor specificity of the viruses, we relied on a TLC-virus binding assay using lacto-series gangliosides containing the type I sugar chain, i.e., NeuAc\(^{a2,6}\) lactotetraosyl ceramide \([\text{II}^{6}\text{(NeuAc)LacCer}]\), NeuAc\(^{a2,3}\) lactotetraosyl ceramide \([\text{II}^{3}\text{(NeuAc)LacCer}]\), and NeuGc\(^{a2,3}\) lactotetraosyl ceramide \([\text{II}^{3}\text{(NeuGc)LacCer}]\), as described previously (33). Each ganglioside (1 nmol) was applied to a silica gel (Polygram Sil G plate; Nagel) that was developed in chloroform–methanol–12 mM MgCl\(_2\) (5/4/1, vol/vol/vol) and dried. After being blocked with phosphate-buffered saline supplemented with 1% egg albumin and 1% polyvinylpyrrolidone (solution A) at room temperature for 2 h, the plate was incubated with purified virus (2 hemagglutinating units) suspended in phosphate-buffered saline at 4°C for 2 h. After being washed with phosphate-buffered saline, the plate was blocked with solution A and incubated with a pool of 11 monoclonal antibodies at 4°C for 2 h. After being washed with phosphate-buffered saline and blocked again with solution A, the plate was incubated at 4°C for 2 h with horseradish peroxidase-conjugated protein A and then incubated with the substrate solution (0.1 M citrate buffer [pH 6.0]–3% 4-chloro-1-naphthol in methanol–3% aqueous H\(_2\)O\(_2\) [5/1/0.01, vol/vol/vol]) at room temperature for 20 min. The binding activity of the virus was determined by scanning the stained chromatogram at 629 nm with a TLC scanner (CSR-9000; Shimazu, Kyoto, Japan). The results were recorded as the relative binding reactivity of virus with gangliosides, with the highest reactivity set at 100.

(A) Receptor specificities of Udorn, Mal/NY, and reassortant viruses possessing a mutation(s) in the Udorn HA; (B) receptor specificities of other avian viruses.
and NeuGcα2,3Gal, replicated well (8). These findings indicate that the recognition of NeuAc linked to galactose by the α2,3 linkage is not sufficient to support influenza A virus replication in duck intestine. Rather, NeuGc recognition also appears to be essential. In accord with the receptor specificity of the HA and viral replication in ducks, SAα2,3Gal but not SAα2,6Gal is predominant (13) and NeuGcα2,3Gal was found in the crypt cells of duck colon epithelium (Fig. 2), the exclusive site of viral replication (Fig. 3). However, duck viruses can recognize NeuAcα2,3Gal, which is also present in duck

![Immunodetection of the NeuGcα2,3Gal moiety in duck intestine.](image_url)
FIG. 3. Presence of viral antigen in epithelial cells in the colon crypts of a duck infected with A/duck/Hokkaido/5/77 (H3N2), 4 days after inoculation. One-day-old mallards (A. platyrhynchos platyrhynchos) were purchased from Ridgeway Hatcheries Inc. and used at 6 to 12 weeks of age. Ducks were orally inoculated with 0.5 ml of allantoic fluid containing 10^9.5 EID₅₀ of virus. NS1 protein in virus-infected cells was detected with a pool of monoclonal antibodies to this protein. Magnification, ×300.

intestine. Why do other intestinal cells likely possessing NeuAcα2,3Gal fail to support viral replication? One possibility is that molecules containing NeuGcα2,3Gal serve as functional receptors, while those possessing NeuAcα2,3Gal do not. Collectively, our data indicate the importance of both the sialic acid species and the type of linkage between sialic acid and galactose in establishing the host range of influenza viruses. Additional support for this hypothesis comes from a recent study in which we show that the recognition of NeuGc is also critical for influenza virus replication in horses (unpublished data).

H3 human viruses isolated in 1968 recognize only NeuAc, whereas those isolated after 1972 recognize both NeuAc and NeuGc (6). The biologic significance of this change is unknown. Since human cells lack NeuGc (15, 16), the acquisition of NeuGc specificity cannot be attributed to the adaptation of the virus in humans. In light of the present finding that recognition of NeuGcα2,3Gal is associated with viral replication in ducks and that the HA of human H3 viruses was introduced from wild birds, we suggest that the human virus isolated in 1968 lost its ability to recognize NeuGc to accommodate its change in linkage specificity from α2,3 (avian type) to α2,6 (human type) or perhaps because of other (unknown) requirements for adaptation to a new environment (i.e., human cells). During subsequent replication in humans, the virus may have undergone further amino acid changes to escape immunologic pressures, rendering it capable of NeuGc recognition.

This report appears to be the first to document the presence of NeuGc in avian species. This sialic acid has not been identified in normal human and chicken tissues (15, 16) but is commonly found in cows, horses, and pigs (16, 18, 22, 23). Hence, equine and swine influenza viruses would be expected to recognize both NeuGc and NeuAc, a prediction supported by previous findings (6, 32).

Higa et al. (6) reported that two duck viruses, including A/duck/Ukraine/1/63, failed to recognize NeuGcα2,3Gal on enzymatically modified erythrocytes, while two others reacted. The majority of duck viruses in our study recognized NeuGcα2,3Gal. One exception was the A/duck/Ukraine/1/63 virus, which bound to this glycoconjugate only weakly and failed to replicate well in duck intestine, further emphasizing the essential role of NeuGcα2,3Gal recognition in the successful replication of influenza A viruses in ducks.

Couceiro et al. (4) showed that in addition to the predominance of the NeuAcα2,6Gal linkage in human tracheal epithelial cells, human bronchial mucin, which contains sialic acid primarily with the NeuAcα2,3Gal linkage (17), can potently inhibit the binding of NeuAcα2,3Gal-recognizing viruses to tracheal sections. This finding suggests that a combination of a missing receptor moiety and the presence of receptor analog inhibitors could protect humans from infection by avian influenza viruses. Such strategies may well prove useful in the influenza armamentarium but are not likely to provide universal protection, as demonstrated by the direct transmission of H5N1 avian influenza viruses from birds to humans during the recent outbreak in Hong Kong (2, 31). By contrast, previous studies demonstrate strict restriction of a virus possessing the human virus HA in duck intestine (8). These findings suggest that the extent of host range restriction controlled by the HA appears to depend on the combination of host animal species and virus.

In this study, we focused on the receptor specificity of the HA. However, the genes of influenza A viruses encoding internal proteins in addition to the HA and NA may also play roles in host range restriction. For example, the NP and M genes can attenuate avian influenza virus infection in squirrel monkeys (34), and depending on the human influenza viruses used to prepare reassortants with avian viruses, a combination of polymerase genes may affect viral replication in this host animal (29, 30). Further studies are needed to fully delineate the contribution of these gene products to host range restriction.

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