Arch Virol (2009) 154:1533–1537
DOI 10.1007/s00705-009-0476-8

BRIEF REPORT

The "human influenza receptor" Neu5Acα2,6Gal is expressed among different taxa of wild birds

Patrik Ellström · Elsa Jourdain · Oskar Gunnarsson · Jonas Waldenström · Björn Olsen

Received: 22 April 2009 / Accepted: 13 July 2009 / Published online: 9 August 2009

Influenza A viruses (IAVs) bind glycoconjugate receptors displaying terminal sialic acids on host cells. The virus hemagglutinin (HA) protein mediates adhesion to the cells via the core structure "Neu5Acα2,3(6)Galβ1,3(4)GlcNAcβ1-" where the linkage between the sialic acid and the galactose can be either α2,3 or α2,6 (reviewed by Suzuki [22]). Adhesion to these structures is a crucial first step in the infection process, and several studies have shown that the linkage between the sialic acids and the galactose is a strong determinant of virus host range [3, 22]. However, a switch in receptor tropism from α2,3- to α2,6-sialylated glycans requires only one amino acid substitution in the HA protein (reviewed by Suzuki [22]).

Most human influenza isolates have tropism for sialic acids linked α2,6 to galactose and cause upper respiratory tract infection, whereas avian virus isolates usually show preferred binding to α2,3-linked sialic acids and preferentially replicate in the intestinal epithelium of birds [4, 20]. Accordingly, α2,6-sialylated glycans have been found in the tracheae of humans [1] and α2,3-sialylated glycans in the intestines of mallards (*Anas plathyrhyncos*), chickens (*Gallus gallus*) and quails (*Coturnix japonica*) [7, 9–11, 23]. Based on these results, the Neu5Acα2,3Gal structure was termed the "avian influenza receptor", whereas the Neu5Acα2,6Gal structure was denoted the "human influenza receptor" [8, 26]. However, poultry isolates with tropism for α2,6-sialylated glycans have been reported [15, 17], and human strains binding to and replicating in quail tissue have been described [9, 23]. Additionally, the presence of both SAα2,6-Gal and SAα2,3-Gal receptors was reported in many tissues of chicken (*Gallus gallus*) and Pekin ducks (*Anas platyrhynchos domesticus*), whereas only SAα2,3-Gal was present in the intestinal epithelial cells of these species [14]. In infection experiments of mallards, highly pathogenic H5N1 viruses showed higher levels of replication in the tracheae of the infected birds compared to the cloacae [21]. In two recent studies comparing cloacal versus oropharyngeal sampling for IAV in wild mallards, oropharyngeal samples accounted for a significant proportion of positive samples [5, 18]. The involvement of tissues other than intestines in IAV infection of birds therefore merits further investigation.

In this study, we determined the expression of Neu5Acα2,6Gal structures in tracheal epithelia of wild birds and domestic chickens using the *Sambucus nigra* agglutinin (SNA) which specifically recognizes α2,6-linked sialic acids [6]. Although other researchers have used the *Maackia amurensis* agglutinin (MAA) to investigate the expression of α2,3-linked sialic acids, we chose to only use SNA because important limitations of MAA lectins have been pointed out ([2, 12, 25], briefly discussed below). For tissue collection, we used naturally succumbed birds (dead from trauma) collected at two Swedish bird rehabilitation

P. Ellström (✉)
Department of Medical Sciences, Clinical Bacteriology, University of Uppsala and Uppsala University Hospital, Uppsala, Sweden
e-mail: patrik.ellstrom@medsci.uu.se

P. Ellström · E. Jourdain · O. Gunnarsson · J. Waldenström · B. Olsen
Section for Zoonotic Ecology and Epidemiology, School of Pure and Applied Natural Sciences, University of Kalmar, Kalmar, Sweden

E. Jourdain
Institut National de la Recherche Agronomique (INRA), UR 346, 63122 Saint Genès Champanelle, France

J. Waldenström · B. Olsen
Section of Infectious Diseases, Department of Medical Sciences, University of Uppsala, Uppsala, Sweden

clinics and at Ottenby Bird Observatory, SE Sweden. The birds were frozen at −80°C shortly after death in order to minimize tissue degradation. Each bird was thawed in the laboratory and the trachea dissected. Tissue quality was examined, and only sections with a preserved tracheal epithelium were included for analysis. In total, nine species were investigated, for which tracheal tissue from two or three individual birds was available (Table 1). Together, these species represent six families and four orders of birds.

Tissues were fixed overnight in 4% paraformaldehyde (Sigma) diluted in Tris-buffered saline (TBS) and afterwards incubated for 24 h in a 15% TBS-sucrose solution (50 mM, pH 7.5). They were then incubated for another 48 h in fresh 15% TBS-sucrose solution (50 mM, pH 7.5) before being embedded in OCT compound (Tissue Tech, Sakura Finetek Europe, Zoeterwoude, NL) and frozen in isopentane on a bed of dry ice. Sections (6 µm) were cut with a cryostat microtome (HM505E, Microm, Heidelberg, GE), mounted on poly-lysine slides (Menzel GmbH & Co, Braunzweig, GE), air dried and stored at −20°C until they were stained the next day. Slides where washed twice in 50 mM TBS and incubated in a moist chamber at room temperature for 1 h with 50 µl digoxigenin (DIG)-labeled SNA (1 µg/ml) (DIG Glycan Differentiation Kit, Roche Applied Science) diluted in 100 µl of buffer (50 mM TBS, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.5). A negative control slide was incubated with buffer only. After several washes, slides were incubated for 1 h at room temperature with a sheep anti-DIG-AP antibody (provided with the glycan differentiation kit) diluted 1/1,000 in 50 µl TBS. After three washing steps, slides were incubated with 50 µl Vulcan fast red (Biocare Medical, Concord, CA), according to the manufacturer's instructions. Staining was stopped by washing twice in demineralised water, and tissues were counterstained with Meyers hematoxylin (Histolab, Gothenburg, Sweden) before dehydration and mounting with Pertex (Histolab, Gothenburg, Sweden). Sections were examined under the microscope (Leica DM IL) by two independent investigators for epithelial expression of sialic acid receptors.

To confirm the specificity of SNA lectin binding to the Neu5Acα2,6Gal structure, control experiments were performed in which tracheal tissues from six birds were depleted of sialic acids by treatment with neuraminidase from *Arthrobacter ureafaciens* (which hydrolyzes sialic acids linked α2,3-, α2,6- and α2,8- to galactose) (Table 2). Consecutive sections were incubated for 24 h at room temperature with 10 mU *A. ureafaciens* neuraminidase (Sigma) in 100 µl of 20 mM HEPES buffer (Sigma) or with Hepes buffer only. After several washes in TBS, tissues were subjected to lectin histochemistry with SNA as described above. Incubations with neuraminidase were performed both at pH 5.5 and 7 for 6 or 24 h with the same results. Tissues from six tracheae of four different bird species were used, and all neuraminidase-treated slides lost SNA staining, whereas the untreated controls were stained strongly by SNA (Table 2; Fig. 1e). For comparison, tracheal epithelia were stained with MAA lectins with or without neuraminidase treatment. Out of five tracheae stained, only one lost the MAA staining after neuraminidase treatment, and this tissue had already low staining in the untreated control (Table 2; Fig. 1f). Other studies have shown that the MAL isolectin (one of the two isolectins included in the MAA preparation from the DIG Glycan Differentiation Kit, Roche Applied Science) can also bind to the unsialylated Galβ1,4GlcNAc structure [2, 12, 25]. This unspecific binding is a likely explanation for our observations and highlights the fact that neuraminidase treatment should always be done when using lectins to study IAV receptors.

We found specific staining with SNA in the tracheal epithelium of all nine species tested (Table 1). Staining was most pronounced along the apical surface of ciliated cells (Fig. 1). Expression of α2,6-linked sialic acids has, to

Table 1 Bird species tested for expression of the Neu5Acα2,6Gal structure in tracheal epithelium as determined by SNA staining

Order	Family	Species (scientific name)	SNA binding
Anseriformes	Anatidae	Mallard (*Anas platyrhynchos*)	+++
Galliformes	Gallidae	Chicken (*Gallus gallus*)	+++
Charadriiformes	Scolopacidae	Dunlin (*Calidris alpina*)	+++
	Laridae	Black-headed gull (*Larus ridibundus*)	++
		Mew gull (*Larus canus*)	++
		Herring gull (*Larus argentatus*)	+++
	Alcidae	Common murre (*Uria aalge*)	+++
Columbiformes	Columbidae	Common wood-pigeon (*Columba palumbus*)	++
		Domestic pigeon (*Columba livia*)	+++

Staining intensity was scored on a scale from – to +++, where − represents no staining; $n = 3$ individual birds per species, except for mallard and chicken, where $n = 2$

Table 2 MAA and SNA staining of tracheal epithelium after neuraminidase treatment

Species (scientific name)	SNA	Neuraminidase SNA	MAA	Neuraminidase MAA
Mallard (*Anas plathyrynchos*)	+[a]	−	+	+
Black-headed gull (*Larus ridibundus*)	+++	−	+++	+++
Black-headed gull (*Larus ridibundus*)	++	−	+	−
Mew gull (*Larus canus*)	+++	−	+++	+++
Mew gull (*Larus canus*)	+++	−	++	+
Domestic pigeon (*Columba livia*)	+++	−	ND[b]	ND

[a] Staining intensity was scored on a scale from − to +++

[b] *ND* not determined

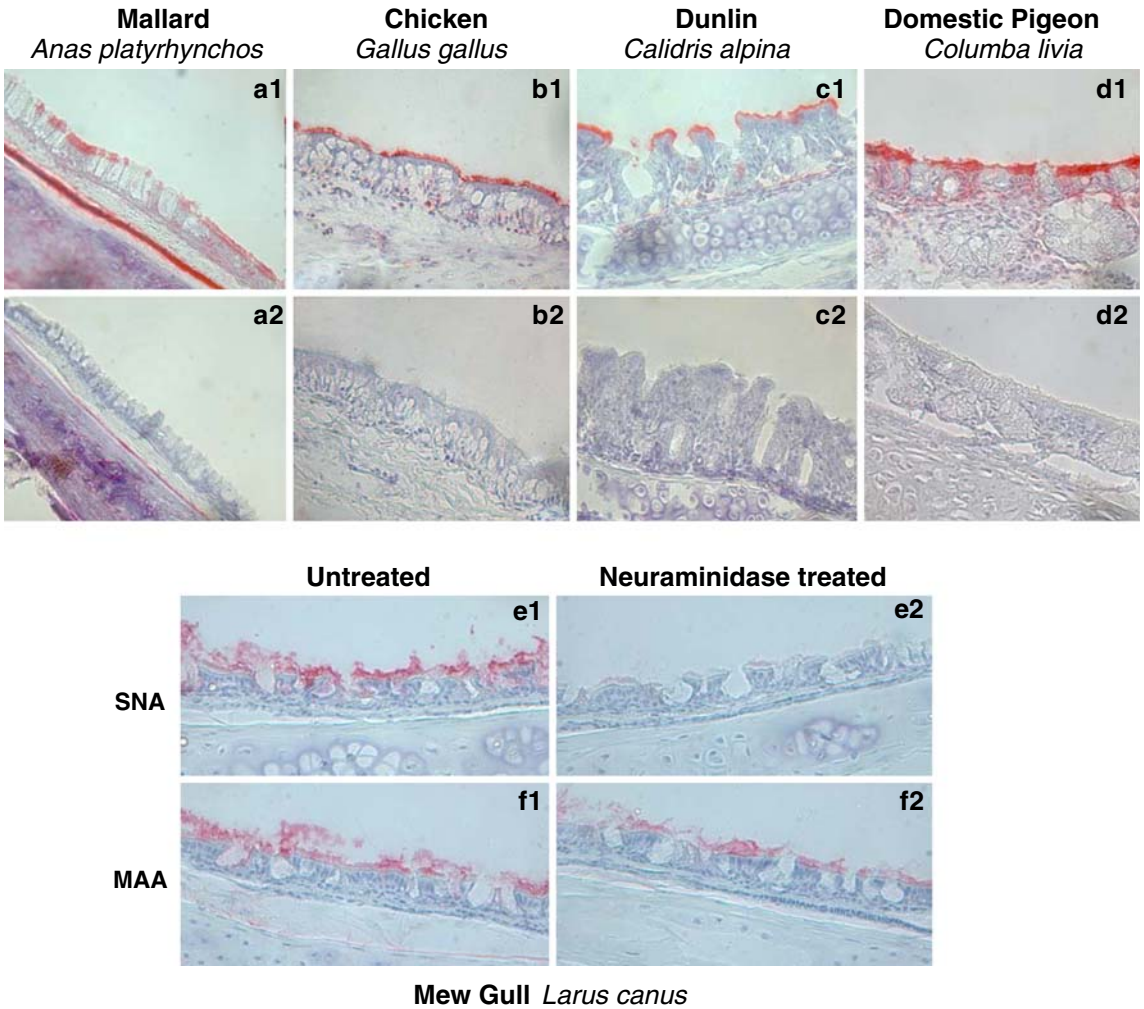

Fig. 1 Lectin histochemistry on avian tracheal tissue sections. Tissues from mallard (**a**), domestic chicken (**b**), dunlin (**c**), domestic pigeon (**d**) and mew gull (**e** and **f**) are shown. Sections were stained with SNA (**a1**–**d1**), or with buffer only (**a2**–**d2**). For specificity assessment, sections were treated with neuraminidase (**e2**, **f2**) or buffer (**e1**, **f1**) before SNA staining (**e**) or MAA staining (**f**)

our knowledge, only been described in the trachea of Mallard/Pekin duck, and in chicken and quail [9, 11, 14, 23]. Our findings extend these observations and suggest that the Neu5Acα2,6Gal structure recognized by SNA is expressed in the trachea of bird species from several different taxonomic orders and genera.

Because binding is only the first step of virus replication, further studies are needed to determine whether IAVs

with tropism for α2,6-linked sialic acids are indeed able to replicate in the trachea of bird species expressing these receptors. Early attempts to infect cultures of tracheal cells with avian influenza viruses showed that mallard and Turkey tracheal epithelium can be infected in vitro [13, 19]. Although Webster and coworkers demonstrated poor viral recovery from mallard tracheas [24], more recent studies detected IAV RNA in oropharyngeal swabs from wild mallards in proportions ranging between 3.7 [18] and 5.1% [5]. The receptor tropism of these avian isolates recovered from oropharyngeal swabs remains to be assessed.

Animal species such as pigs, which express both α2,3- and α2,6-sialylated glycans, are believed to constitute potential mixing vessels for genetic reassortants of human and avian viruses [10, 16]. Detection of both Neu5Acα2,6Gal and Neu5Acα2,3Gal structures on chicken and quail intestinal epithelium [9, 11, 23], and in vitro infection experiments suggest that these bird species might also serve as mixing vessels for human and avian IAVs [9, 23]. Our finding of the Neu5Acα2,6Gal structure in the tracheae of several bird species suggests that bird species other than quail and chicken might also be able to bind both human and avian viruses.

Acknowledgments This work was sponsored by the Framework 6 grant "NewFluBird" from the European Union, The Carl Trygger Foundation, the Swedish Research Council FORMAS (2006-297), The Swedish Research Council (2007-2774) and the French Fondation pour la Recherche Médicale. Birds were provided by Ottenby Bird Observatory, Malmö and Göteborg rehabilitation centers, Swedish Museum of Natural History, Guldfågeln AS and the Swedish Hunters' Association. This is contribution no. 232 from Ottenby Bird Observatory.

References

1. Baum LG, Paulson JC (1990) Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl 40:35–38
2. Brinkman-Van der Linden ECM, Sonnenburg JL, Varki A (2002) Effects of sialic acid substitutions on recognition by *Sambucus nigra* agglutinin and *Maackia amurensis* hemagglutinin. Anal Biochem 303:98–104
3. Chandrasekaran A, Srinivasan A, Raman R, Viswanathan K, Raguram S, Tumpey TM, Sasisekharan V, Sasisekharan R (2008) Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat Biotechnol 26:107–113
4. Connor RJ, Kawaoka Y, Webster RG, Paulson JC (1994) Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205:17–23
5. Ellstrom P, Latorre-Margalef N, Griekspoor P, Waldenstrom J, Olofsson J, Wahlgren J, Olsen B (2008) Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: oropharyngeal versus cloacal swabbing. Vaccine 26:4414–4416
6. Gabius H-J, Gabius S (eds) (1997) Glycosciences, status and perspectives, 1st edn. Weinheim, Chapman & Hall
7. Gambaryan A, Webster R, Matrosovich M (2002) Differences between influenza virus receptors on target cells of duck and chicken. Arch Virol 147:1197–1208
8. Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 79:11533–11536
9. Guo CT, Takahashi N, Yagi H, Kato K, Takahashi T, Yi SQ, Chen Y, Ito T, Otsuki K, Kida H, Kawaoka Y, Hidari KI, Miyamoto D, Suzuki T, Suzuki Y (2007) The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 17:713–724
10. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72:7367–7373
11. Kim JA, Ryu SY, Seo SH (2005) Cells in the respiratory and intestinal tracts of chickens have different proportions of both human and avian influenza virus receptors. J Microbiol 43:366–369
12. Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins. J Biol Chem 266:83–88
13. Kocan AA, Daubney GA, Kocan KM (1978) Structural changes associated with type-A influenza in Mallard duck tracheal organ culture. Avian Dis 22:535–541
14. Kuchipudi SV, Nelli R, White GA, Bain M, Chang KC, Dunham S (2009) Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. J Mol Genet Med 3:143–151
15. Liu M, He S, Walker D, Zhou N, Perez DR, Mo B, Li F, Huang X, Webster RG, Webby RJ (2003) The influenza virus gene pool in a poultry market in South central china. Virology 305:267–275
16. Ma W, Kahn RE, Richt JA (2009) The pig as a mixing vessel for influenza viruses: human and veterinary implications. J Mol Genet Med 3:158–166
17. Matrosovich MN, Krauss S, Webster RG (2001) H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281:156–162
18. Munster VJ, Baas C, Lexmond P, Bestebroer TM, Guldemeester J, Beyer WE, de Wit E, Schutten M, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2009) Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. J Clin Microbiol 47:666–673
19. Robinson JH, Easterday BC (1978) Detection of persisting influenza virus with turkey trachea cultures. Avian Dis 23:354–356
20. Rogers GN, Paulson JC (1983) Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127:361–373
21. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG (2005) Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol 79:11269–11279
22. Suzuki Y (2005) Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull 28:399–408
23. Wan H, Perez DR (2006) Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 346:278–286

24. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG (1978) Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84:268–278
25. Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y (2006) Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 444:378–382
26. Yao L, Korteweg C, Hsueh W, Gu J (2008) Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 22:733–740