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Gradual adaptation of animal influenza A viruses to human-type sialic acid receptors

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Influenza A viruses (IAVs) originating from animal reservoirs pose continuous threats to human health as demonstrated by the Spanish flu pandemic. Infection starts by attachment to host receptors, a crucial step that is targeted by immunological, prophylactic, and therapeutic intervention. Fine-tuning of virus hemagglutinin binding to host-specific receptor repertoires needs to remain balanced to receptor-destroying neuraminidase (NA) activity and is a key step in host adaptation. It determines NA-dependent virus motility, enabling IAVs to traverse the mucus layer and to bind to, and migrate over, the epithelial cell surface for reaching a location supporting endocytic uptake. Canonical adaptations in enzootic/zoonotic IAVs enhancing human-type receptor binding are well-known, but the context and timespan required for their selection pose many questions. We discuss recent developments, focusing on the dynamic nature of interactions of IAV with the heterogeneous receptor repertoires present in humans and potential intermediate hosts. Potential pre-adaption toward human-type receptor binding in intermediate hosts will be discussed.

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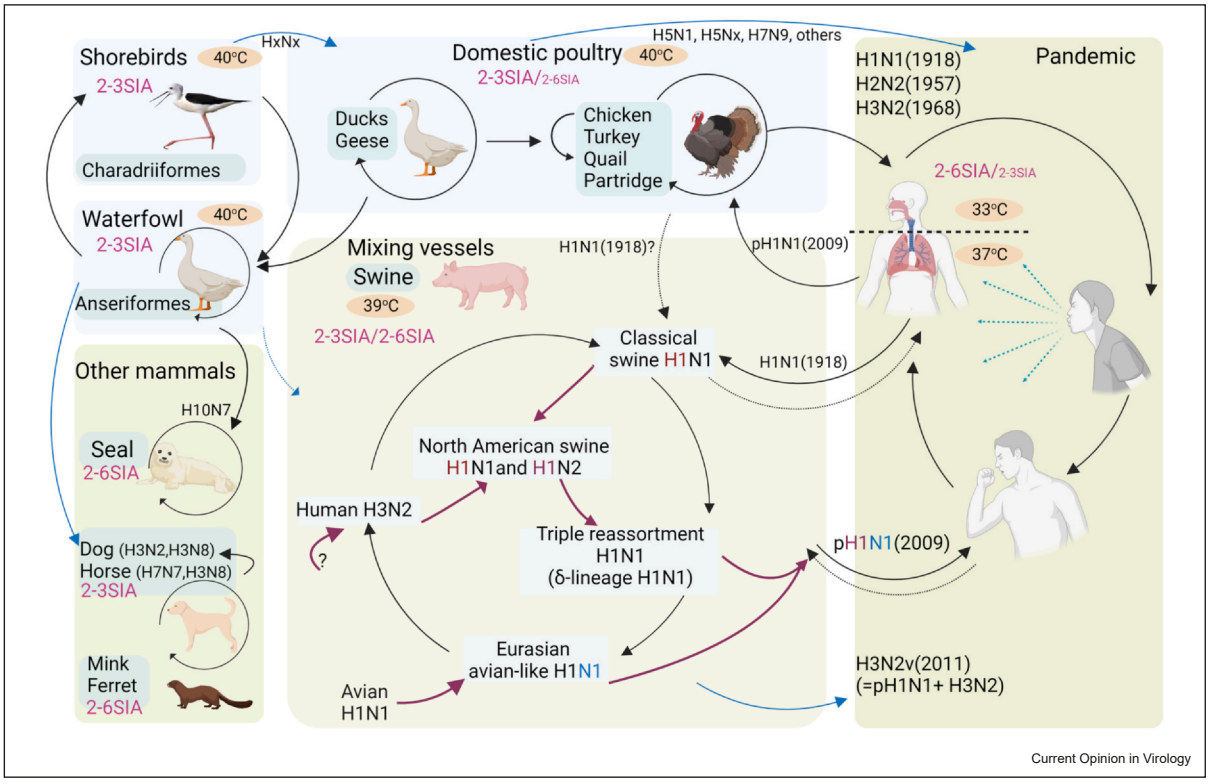
Introduction

Human influenza-A viruses (IAVs) evolve from IAVs circulating in the aquatic bird reservoir. The dichotomy in receptor-binding specificity for avian and human IAVs [1–3] matches the receptor repertoire at their preferred infection sites, that is, the α2–3-linked sialic acid (2–3Sia) enriched the digestive tract (DT) of waterbirds, or the 2–6Sia enriched human upper respiratory tract (URT) [4–6]. Adaptation toward human-type receptor specificity depends on canonical changes in the receptor-binding site (RBS) at the membrane-distal globular domain of the homotrimeric envelope glycoprotein hemagglutinin (HA) [3,7,8]. Specificity-switch mutations are rare in nature, likely requiring coselection of mutations in other viral proteins to become established in human-transmissible strains. Three IAV genotypes (H1N1, H2N2, and H3N2) have adapted to the human URT, causing 4 pandemics in the past 100 years.

Adaptation to human-type receptors is not a mere shift to high affinity for 2–6Sia receptors. It requires fine-tuning of a dynamic binding mode enabling virus motility on heterogeneous receptor surfaces of epithelial cells for reaching yet-unidentified subcellular locations where endocytic uptake is actively signaled. Also, motility in the mucus layer, carrying its own specific Sia repertoire, needs to be warranted [9–11]. Directional motility on receptor surfaces is driven by receptor cleavage by neuraminidase (NA) [11–14] depending on precisely balanced NA activity and specificity and HA binding affinity and specificity [15–22]. Multivalent binding compensates for short-lived individual HA-receptor interactions (K_D~1–20 mM) [23–27] and virus binding is maintained as long as a single HA–Sia interaction is present. Rapidly alternating HA–Sia interactions provide access for NA to temporarily free Sias. Importantly, IAV particles interact with complex receptor repertoires by heteromultivalent binding involving low- and high-affinity interactions, thereby shifting the classical paradigm that only 2–3Sias contribute to IAV binding in waterbirds [28].

Intermediate IAV hosts such as domestic poultry or swine (Figure 1) are involved in zoonotic transmission. Their epithelial mucosa displays the receptor landscapes on which IAV evolution takes place, possibly by posing similar selective constraints on virus evolution as encountered in the human URT [29]. However, the epithelial cell receptor repertoire of intermediate hosts as well as the order and timespan of introduction of

Figure 1



Major transmission routes of IAVs. Species frequently infected by IAV are indicated. Routes of enzootic and zoonotic transfer are indicated. Blue arrows indicated transfer of IAVs that have not yet become established in the new host. For swine IAVs, the three major species that have contributed genome segments (as indicated by purple arrows) to the many IAV strains circulating in swine are indicated (human H3N2, classical swine H1N1, and Eurasian avian-like H1N1). Three important reassortants are indicated (North American swine H1N1 and H1N2, and triple reassortant H1N1). For further details see text.

mutations contributing to the evolution of pandemic IAVs are poorly resolved. The, often non-canonical, substitutions in the RBS of virus isolates from potential intermediate hosts could provide a pre-adaptation to human-type receptors. Receptor binding properties of IAVs isolated from such hosts and humans are therefore compared and mutations in HA possibly resulting in pre-adaptation to human-type receptors are discussed.

Receptor specificity

Structural determinants of HA-binding specificity for 2–3Sias or 2–6Sias have been abundantly reviewed [3,7,30]. In short, whereas the Sia moiety is always bound in near- identical conformation for both linkage types, the 2–6-linkage type imposes a hook-like shape on the glycan, causing subterminal moieties (e.g. Galactoseα1–4N-acetylglucosamine (Galα1–4GlcNAc)) to follow a different track along the HA surface than the spike-like avian-type 2–3Sia receptors that enter the RBS from a different angle. Three structural elements (190-helix, 130-loop, and 220-loop) form the walls of the RBS and canonical substitutions in the 190-helix (E190D for H1N1) and 220-helix (G225D for H1N1, Q226L/G228S for many other genotypes) largely suffice for an avian- to human-type specificity switch. However, this switch is not absolute as early pandemic viruses retain considerable 2–3Sia binding and later seasonal strains can display dual-specific binding [31–33]. Binding affinities are further diversified by variation in subterminal glycan chain structures [34,35], but structural understanding of binding specificity has hardly provided insight into absolute binding affinities for specific structures [30]. On monospecific receptor surfaces, only small differences in K_D for 2–3Sia or 2–6Sia can already result in large differences in multivalent-binding specificity. Multivalent interaction typically increases virus-binding rates from zero to maximal over a small receptor density range, amplifying the effect of small differences in the dissociation constant (K_D) by what is called superselectivity [36–38].

2–3Sias and 2–6Sias coincide at variable ratios and densities depending on host, cell type, and location within the respiratory tract (Tables 1 and 2). Moreover, specific membrane microdomains differing in sialoglycan repertoire [39] result in microheterogeneity of receptor

Table 1

Summary of analyses of the respiratory and digestive tract DT by lectin staining ([86–105]).

			Nasal									Larynx			Trachea				Bronchi			Lung									Digestive tract							
																						Avian lungs				Human lungs													
																											Brochioles				Lumen			Duodenum/jejunum		Ileum/ caeca	Colon				
Order	Species[a]	Structures[b,c,d]	Not specified	Nasal turbinates	Pharynx	Ciliated epithelial cells	Non-ciliated epithelial cells	Olfactory epithelial cells	Bowan gland epithelium	Nasal gland epithelium	Salivary gland epithelium	Not specified	Ciliated epithelial cells	Glands	Not specified	Ciliated epithelial cells	Goblet cells	Mucous gland epithelium	Not specified	Epithelial cells	Glands	Not specified	Air capillary cells	Avian parabronchus	Avian infundibulum	Not specified	Not specified	Ciliated cells	Goblet cells	Alveolar	Pnemocytes	Macrophages	Not specified	Goblet cells	Epithelial lining	Epithelial cells	Not specified	Columnar epithelial cells	Goblet cells	Crypt	Refs
Galliformes	Turkey	2-3Sia				++	-	++	-	++	++		++	+		++/+++	-	+		++			-/+	-/+/+++	++								+					+	-		86-88
		2-6Sia				++	+	++	+	++	++					-/+++	+	+		+++			-	+++									-								
	Pheasant	2-3Sia				++	++	+	+	++	++				+++	-	+	+		+		+	-	+													+				87.88
		2-6Sia				+	+	++	-	+	+				+	++	+	+		+		+	+	-													+				
	Guinea fowl	2-3Sia													+							+															+				87
		2-6Sia													+							+															-				
	Quail	2-3Sia				+	+++	+	++	++	+++					+/+++	+	+		+		+	+	+										+			++	+			87-90
		2-6Sia				+	++	++	++	+	++					+/+++	+	+		+++		+	-	-										-/+			+++	+				
	Chicken	2-3Sia				+++	+	+++	+++	+	++					+	-	++		+++			-	+++														+			5,89-91
		2-6Sia				++	-	+	+	++	+					+/+++	+	+		+++			-	-														+			
Anseriformes	Duck[e]	2-3Sia		+++											+++	+++						+++		+++										- to +++		+++		++	+		5,86,87,90
		2-6Sia		-/+											+++	-/+						-		+++										- to +++		+++					
	Goose	2-3Sia		+++											+++									+++										+++		+++					5.87
		2-6Sia		+/+++											-/+++									-/+++										-		+++					
	Swan	2-3Sia		+++											+++									+++										+/+++		+++					5
		2-6Sia		-											+++									+++										-/+++		+++					
Charadriiformes	Red knot	2-3Sia		+++											+++									+++										-		-					5
		2-6Sia		-											-									+++										-		-					
	Gull	2-3Sia		-/+++											+++									++/+++										+/+++		++/+++					5
		2-6Sia		+++											+++									+++										-/+		-/+					
Mammals	Swine	2-3Sia	-			+		+++		++					-	-/+	-	- to +++	-/+	-/+	-/+						- to +++	-	-	- to ++	-/+++	-		+	+			+++	+++	+	31,67,69,92-97
		2-6Sia	+++			+++		-		+					+++	+++	+++	++/+	+++	+++	+++						+++	+++	+++	+++	++/+++	+/++		+++	+++			+++	+++	++	
	Human	2-3Sia	+		-/+	+++													-/+++	-/+++	-/+++					-	+++			+	-/++	+/++						-	-	-	94,98-100
		2-6Sia	+++		+++	+++													+++	+++	+++					++	+++			+++	+/+++	-/+						-	-	-	
	Dog	2-3Sia			++										+++				+++								+++			+			-/++			-	-			101
		2-6Sia			++										-				-								+			-			+			+	+			
	Equine	2-3Sia	+							+							+				+																			102
		2-6Sia																																						
	Seal	2-3Sia																									+			+										103
		2-6Sia																									+++			+++										
	Ferret	2-3Sia													+	-			+	+/-	+++					-					+									104.105
		2-6Sia														+/+++		+++		+++/-	+++					+++					+++									

[a] Species are included by their common group name of a range of species.
[b] 2-6Sia (SNA, binding Siaα2-6Galβ1-4GlcNAc) and 2-3Sia (MAL I, Siaα2-3Galβ1-4GlcNAc; MAL II, Siaα2-3Galβ1-3GalNAc).
[c] Mal I and II also bind sulfate on the Gal and do not tolerate modifications on the GlcNAc and GalNAc [201]. For conciseness, results have been combined in single 2-6Sia and 2-3Sia columns.
[d] Symbols: - is negative; + is visiable; ++ is moderate; +++ is dominant.
[e] Duck includes non-specified duck, mallard, teal, northern pintail and red head.

Table 2

[a,b] Summary of glycomic analyses of the respiratory tract by mass spectrometry ([106,108]).

			Refs	N-linked glycans				O-linked glycans	specific terminal structures					
				Sia(%)[c]	SIA linkage type[d]	Branch length Sia-$(LN)_n$[e]	Nr of branches (Sialylated/total)[f]	Glycolipids	SLeA/X	Sia-Sulfo[g]	NeuGc	SiacdiNAc[h]	Sda[i]	αGal[j]
Chicken	LRT	trachea	106	36%	2-6>2-3[k]	1>>>2	1/3>1/2>2/2>2/3>>3/3>>1/4		12%	0	0	0,1%	0	0
		lung	106	32%	2-6>2-3	1>>>2>>3	1/2>1/3>2/2>2/3>1/4>1/5> 2/5>2/4>3/3>3/5>3/4		0	0,1%[l]	0	2%	0	0
Human	URT	Naso-pharynx	6	35%	N.D.	1>>>2	1/2~2/2>>>3/3		0	0	0	0	0	0
	LRT	bronchus	6	25%	2-6<<2-3	1>>2 or 3>>> 2 to 7	1/2>>>2/2	Core-1>>>Core2; Sia 8% Di-Sia Core-1<<<Mono-Sia Core-1	0	0	0	0	0	0
		alveoli	107	53%	2-6<2-3	1	1/2~2/2>>>3/3		0,5%	0	0	0	0	0
		Lung	108	76%[m]	2-6>2-3[n]	1>>>2 or 3>>>2 to 21	2/2>>1/2>	Core-1>>>Core2; Sia 96%; 2-6~2-3 Di-Sia Core-1<Mono-Sia Core-1; SLeA/X and 2-8Sia present	max 6%	0	0	0	0	0
								GM3/GD3 are major GSL; Sia 75% Sialylated antennae with 1 to 7 LN 2-6<<<2-3; SLeA/X is present						
			6	38%[o]	2-6<2-3	1>>>2 or 3>>> 2 to 9	1/2>2/2>>>3/3	Core-1>>>Core2; Sia 74% Di-Sia Core-1<Mono-Sia Core-1	0	0	0	0	0	0
Swine	LRT	trachea	67	0.54%	2-6>>2-3	1	1/2~2/2>>1/2		0	0	0,24%	0	0	11%
			64	31%	2-6>>2-3	1	2/2>1/2		0	0	7%	0	0	8%
		lung	67	29%	2-6>2-3	1	1/2>>1/3>1/4	Core-1<Core2; Sia 13% Only mono-Sialylated Core-1 and 2 αGal on Core-2 is abundant (36%)	0	0	14%	0	0	49%
			64	57%	2-6>>>2-3	1	2/2>1/2		0	0	8%	0	0	20%
Minipigs	LRT	lung	67	39%	2-6<<<2-3	1	2/2>1/2>>1/3>>>1/4		0	0	17%	0	0	39%
Ferret	URT	Nasal wash	68	96%	N.D.	1>>>2	2/2>>1/2~3/3		0	0	0	7%	28%	0
		Palate	68	33%	N.D.	1 >>>2	2/2~1/2>>1/3~2/3>>>3/3		0	0	0	0	0,9%	11%
		Turbinate	68	65%	N.D.	1>>>2	2/2>>1/2>>>3/3		0	0	0	0	5,2%	0
	LRT	trachea	68	55%	N.D.	1>>>2 to 5	2/2>>1/2>>1 to 3/3>4/4	Core-1>>>Core2; Sia 17%; Di-Sia Core~Mono-Sia Core-1	0	0	0	14%	0,1%	14%
			68					GM3 is major GSL; Sia 69%; Sialylated antennae: 1 to 3 LN; Sda is present						
		lung	68	77%	2-6>>2-3	1>>>2 or3 >>2 to 6	2/2>>1/2>3/3>>>4/4	Core-1>>>Core2; Sia 82%; Di-Sia Core-1~Mono-Sia Core-1 2-6Sia<<<2-3Sia	0	0	0	0,8%	12%	4%
			68					GM3 is major GSL; Sia 98%; Sialylated antennae: 1 to 3 LN; 2-6Sia<<<2-3Sia; Sda is present						

[a] All data in the table concern N-acetylneuraminic acid. Data on NeuGc for swine are referred to in a separate column.
[b] Symbols: ~ is difference smaller than 1,5-fold; < is 1,5 to 3-fold smaller; < < is 3–10-fold smaller; < < < is more than -10-fold smaller. > / > > / > > > same for larger.
[c] This colum lists the relative abundance of glycans with 1 or more Sia groups attached. It was estimated from the sum of reported relative intensities of m/z peaks of sialylated and nonsialylated glycans.
[d] This column lists the relative abundance of 2–3Sia versus 2–6Sia; it was estimated from the sum of reported relative intensities of m/z peaks of sialylated glycans resistant to sialidase S that specifically cleaves 2–3Sia.
[e] $(LN)_n$ is number of LacNAc repeats $(Gal\beta1–3/4GlcNAc)_n$. A range is indicated as for multi-antennary glycans the number of LN repeats per individual antennae was not determined.
[f] 1/2 refers to a biantennary glycan of which one antenna is sialylated and so on.
[g] Sulfation has been shown to affect receptor specificity for several IAV strains [65] but has not extensively been detected by the glycomic studies mentioned in this table.
[h] LacdiNAc (GalNAcβ1–4GlcNAc) can only be sialylated in α2–6-linkage type.
[i] Sda antigen (Neu5Acα2–3(GalNAcβ1–4)Galβ1–4GlcNAc); the average of all tissue sections together is indicated. No IAVs that can bind this structure have been identified.
[j] The αGal glycotope (Galα1–3 Gal) cannot be sialylated; the average of all tissue sections together is indicated.
[k] In trachea and lung, abundance of 2–3Sia was lower than 2–6Sia on mono- or disialylated N-glycans, but slightly higher on tri- or tetrasialylated N-glycans.
[l] Sulfo or Sia groups on N-glycans from trachea never coincided on the same branch.
[m] Small amounts of phosphorylated glycans were identified in the human lung, which served as ligands for IAV binding [107].
[n] 2–6Sialylated glycans were more abundant on chains with a single LacNAc repeat, whereas 2–3Sialylated glycans were more abundant on chains with multiple LacNAc repeats.
[o] The pediatric lung and bronchus have a greater abundance of 2–3Sia than adult lung and bronchus.

distribution on the cell surface. This will likely affect virus binding and motility and requires a refinement of the simplified view that the strongest binders for a particular virus should be considered as 'the receptor'. Interestingly, but beyond the scope of this review, non-Sia IAV receptors have recently caught attention (reviewed in [40,41]), including glycans phosphorylated at the high-mannose structures of N-linked glycans [42]. Obviously, such receptors cannot be cleaved by NA and will therefore have an effect on virus motility that needs to be investigated.

Heteromultivalent binding determines the receptor repertoire

By specific tuning of a heterogeneous sialoglycan repertoire on a glycoprotein, it was shown that human-type 2–6Sia receptors, by themselves not supporting avian IAV binding, could efficiently enhance binding to a low

density of high-affinity avian-type receptors [28]. Effectively, sialoglycans traditionally considered as 'non-binders' for a specific IAV strain reduce the threshold density required for binding to its, as previously considered, 'real' receptors. Such heteromultivalent binding places the dichotomy in avian *versus* human IAV receptor preference in a different perspective. Proposedly, initial monovalent HA–Sia interactions of sufficient affinity (and therefore frequency and/or duration) enable subsequent multivalent interactions with low-affinity receptors to collectively support high-avidity binding (for a model see [28]). Binding of low-affinity receptors provides opportunities for gradual evolution toward increased affinity for such receptors on heterogeneous receptor surfaces. Understanding the dynamics of heteromultivalent binding urgently requires determination of the individual HA–Sia association and dissociation constants for a range of receptors for different viruses.

Receptor accessibility affects binding avidity

Spatial arrangement of RBSs and cell surface receptors undoubtedly plays a role in multivalent virus binding as the two patterns need to match for optimal interaction. This is exemplified by the receptor preference of seasonal H3N2 strains isolated after 1995 [35,43]. Inefficient binding to mono-antennary sialoglycans was efficiently restored when identical chains are present in a multi-antennary arrangement of N-linked glycans. Modeling showed that the exact length of the chains determines whether simultaneous interactions between two antennae and HA protomers within a trimer can be made [43]. Thus, spatial receptor arrangement may compensate for insufficient monovalent binding affinity by promoting rapid formation of a bivalent interaction. It suggests that bivalent intratrimer interactions display favorable binding kinetics over bivalent intertrimer interactions and emphasizes that low-affinity receptors, when arranged properly, can support IAV binding.

Influenza A virus motility links binding to virus entry

The association of virus particles to high-avidity binding sites represents initial cell surface attachment. Following binding, surface-associated IAV particles extensively explore the cell surface before stalling at spots where specific signaling induces virus entry by endocytosis [44,45]. Virus entry, reviewed elsewhere [40,46,47], can proceed by clathrin-mediated endocytosis (CME) [48,49] *via de novo* formation of clathrin-coated pits at the spot [44] and requires adapters such as epsin1 [49,50] or FBP17 [48]. IAV-binding-induced clustering of sialylated receptors may signal entry by macropinocytosis [51], CME [52], or another dynamin-dependent route [53]. Several membrane proteins involved in entry were identified [40], but whether their functioning requires a specific sialoglycan load is unknown in most cases. Modeling has shown that receptor clustering does not require high binding affinities per binding site and can proceed by multiple low-affinity interactions collectively providing the high avidity that induces clustering and signaling [54]. Thus, high-affinity Sia receptors may not necessarily be required at an IAV entry spot, adding to the versatility of heterogeneous receptor landscapes in supporting IAV entry. Rolling toward entry-competent locations uncouples the SIA-receptor requirements for initial attachment from those present at the entry site.

Directional motility, for example, to reach entry-competent spots, depends on NA activity on Sias — to which access is gained by fast-alternating HA–Sia association/dissociation — and therefore requires fine-tuning of the HA/NA balance. Brownian ratchet-like directional motility, driven by an NA activity-generated Sia gradient, was shown for filamentous IAV particles displaying a patch-like, polarized distribution of NA [11,14]. However, spherical IAV particles displayed directional rolling motility independently of polarized NA distribution [11], a mechanism supported by physical modeling incorporating HA and NA kinetic parameters [55,56]. Rolling efficiency depends on the interplay between HA and NA (12,13,14,32) on a receptor landscape where heteromultivalent interactions may exert effects on virus motility, receptor clustering, and signaling. Expectedly, differences in specificity and affinity for individual glycans, differentially distributed over the surface, will affect the initial site of attachment and subsequent migration to the actual site of endocytosis.

Hemagglutinin/neuraminidase balance adaptation to a new host: gradual or fast evolution?

The HA–NA balance concerns the balance between the activities of HA and NA on the full, highly diverse spectrum of functional and decoy receptors present on cells and mucus [12]. Balanced HA binding and NA activity, preventing irreversible binding to soluble or cell surface decoy receptors, are also deemed necessary to avoid premature virus dissociation from cells. Major adaptations in specificity and/or affinity of avian HA to a human-type receptor repertoire have been well-documented [3,7,30]. Still, mechanistic details of optimal motility and stalling at entry-competent sites are poorly understood in view of an altered HA/NA balance. Readjusting the balance to novel host receptors can potentially take years (discussed for specific virus strains below) and be affected by heteromultivalent binding. Notably, selective forces other than the receptor landscape can also direct HA/NA balance evolution. Amino acids around the RBS, involved in receptor binding, are subject to antigenic drift as a major escape mechanism from protective host antibody responses [57,58]. Also, pH-dependent HA stability is a crucial factor in its

fusogenicity, the other main function of HA. Adaptations in HA to accommodate the different pH stability requirements for fusion of avian *versus* human IAVs may alter HA-binding properties [59]. Thus, after establishment within a new host, continued evolution of the HA/NA balance could be due to drivers other than its adaptation to the receptor repertoire.

Host adaptation of neuraminidase

Surprisingly little is known on host adaptation of NA, for example, on the structural requirements for acquiring specificity for human-type 2–6Sia receptors (for reviews on NA function see [60,61]). The catalytic site is extremely conserved, but extensive variation can be observed in the neighboring 2nd sialic acid-binding site (2SBS). In pandemic IAVs, Sia binding to the 2SBS is rapidly lost. The 2SBS, displaying 2–3Sia-binding specificity, enhances NA activity of avian IAVs by assisting binding to 2–3Sia-decorated receptor surfaces. Forward evolution studies showed that 2SBS–Sia interaction is another factor determining the HA/NA balance for avian viruses [62]. In contrast to human IAVs, the 2SBS is conserved in some swine IAV clades for longer times, suggesting functional importance in those strains. Changes in NA activity have occurred in concert with changes in HA affinity, for example, by functional adaptation of the HA/NA balance of the swine-origin 2009 pandemic H1N1 (pH1N1) to a human-type receptor repertoire by combining a low-affinity HA with a low-activity NA [21]. This virus evolved in swine and acquired, by reassortment, HA of the 1918 H1N1 classical swine lineage and NA from more recent avian IAV ancestry. Variations in the length of the NA stalk, which is frequently observed in poultry-adapted IAVs, may present a species-specific adaptation leading to altered NA activity. Multiple molecular mechanisms causing this activity change have been proposed [61].

As discussed, dual receptor specificity assisting heteromultivalent binding could enable gradual adaptation to human-type receptors. At a low density of avian-type receptors, a gradual increase of 2-6Sia binding affinity could provide a within-host selective advantage by enhancing virus replication. Transmission and spreading of such variants point to the URT as their major site for adaptation. Identifying the glycan identity and distribution along the respiratory tracts of IAV host species is therefore crucial to understand IAV evolution.

Receptor distribution on target tissues of influenza A virus hosts

Correlating mutations in HA or NA to changes in receptor binding (summarized in Table 3) could provide molecular markers for the identification of IAVs pre-adapted to human-type receptor binding. Understanding the temporal and spatial pathways leading to (pre-)adaptation also requires detailed knowledge of the epithelial glycomes on which IAV receptor binding properties evolve. We therefore first summarize structure and distribution of glycans along the respiratory tract of IAV hosts as analyzed by lectin staining (Table 1) and mass spectrometry analysis (Table 2). For an extensive review of the effect of specific glycan structures on receptor binding by virus isolates from different hosts, we refer to [63].

Distribution of α2–3 and α2–6

Semiquantitative lectin staining, unlike mass spectrometry, reveals cell type-specific glycan distribution in tissue sections. Unfortunately, lectin binding mainly distinguishes 2–6Sia (Sambucus nigra lectin SNA) from 2 to 3Sia (Maackia amurensis lectins MAL I and MAL II) without providing detailed knowledge on the underlying glycan structure as revealed by mass spectrometric analysis. In addition, lectin binding is restricted by subterminal glycan modifications and linkage types (see footnotes Table 1). Variations in tissue preparation, staining protocols, individual donors, and donor age inevitably affect comparison of results.

Despite the high 2-3Sia binding specificity of avian IAVs, 2–6Sias are abundant at several places in the avian intestinal tract (ileum and ceca of gull, goose, swan, and red knots and duodenum of ducks) and respiratory tract (nasal turbinate of gull and goose, and trachea of poultry). Glycomic analysis quantitatively confirms the high abundance of 2–6Sia in the trachea and lung of chicken, in that respect resembling human, ferret, and swine. Yet, only H16N3 from gull, H6N6 from duck, and H9N2 and H7N2 from poultry were shown to display considerable 2–6Sia specificity (Table 3). 2-6Sia binding human IAVs were observed to infect turkeys but not other avian species. IAVs originating from waterfowl infect chicken but evolve, at most, only to slightly enhanced 2–6Sia binding despite the abundance of 2–6Sia in chicken trachea. It suggests that adaptation of avian IAVs to 2-6Sia binding specificity does not provide a major advantage for enhanced transmission with a chicken population. For humans and swine, reports of lectin as well glycomics studies differ in the relative distribution of 2–3Sia and 2–6Sia receptors along the respiratory tract. In general, 2–6Sias are dominant in human and swine URT. In the lower respiratory tract (LRT) of swine, 2–6Sias seem more abundant than 2–3Sias. One report shows a gradual decrease of 2–3Sia down along the respiratory tract [64], whereas in humans, 2–6Sia and 2–3Sia seem more equally distributed by lectin staining and glycomics analysis (Table 2). Ferrets mostly display 2–6Sia binding and dogs 2–3Sia binding along their respiratory tracts, matching the specificity of the IAVs isolated from them. Overall, 2–3/2–6Sia distribution along the respiratory tracts of different species does however not fully explain the

Table 3

Binding specificity of IAV strains ([109–200]).

Host		IAV	Year(s)	HA substitutions affecting specificity/avidity					Specificity[m]	Origin	References
				Canonical[a]				Others			
				190	225	226	228				
Human	Pandemic or epidemic	**H1N1**	1918-57; 1977-09	D/N	D/G	Q	G		2-6>>>2-3 to 2-6~2-3[b,c]	Sw or Av	78,109-111
		pH1N1	2009-	D	D	Q	G	T200A, E227A	2-6>2-3 to 2-6>>>2-3	Sw	21,74-77,110,112,113
		H1N2	1988-89; 2001-03	D/N	D/G	Q	G		N.D.	Hu[d]	114-116
		H2N2	1957-68	E	G	Q/L	G/S	K222E	2-6<<2-3 to 2-6>>>2-3[c]	Av/Hu[e]	2,80
		H3N2	1968-	E/D[f]	G	L	S	N193S[g], D63N, D81N,	2-6>>>2-3	Av/Hu[e]	35,42,59,117-120
	Zoonotic	**H1N1v, H3N2v**	1959-	swine consensus[h]					2-6>>2-3	Sw	75,121-123
		H5N1	1997	E	G	Q	G	133Δ+I155T, Q196H, S227N	2-6<2-3 to 2-3	Av	124-126
		H7N9	2013-	E	G	L/Q	G	S138A,G186V,V186I,T221P	2-6<<2-3; 2-6>2-3	Av	127-131
		H9N2	1998-	A/V/T	G	L	G	I155T	2-6<<2-3 to 2-6>>2-3	Av	132-135
	Rare zoonotic transfer	**$H1_{classic}N2v$**	2021	D	N	Q	G	G188D, N189S	2-6>>>2-3 to 2-6>>2-3	Sw	115,122,136
		$H1_{av}N2v$	2021	D	G	Q	G		N.D.	Sw	136
		H3N8	2022	E	G	Q	X[i]		N.D.	Av	137
		H5N6	2014-	E	G	Q	G	T192I	2-3	Av	138
		H6N1	2013	V	G	Q	S	P186L	2-3 to 2-6>2-3	Av	139-143
		H7N2	2003; 2016	E	del	del	del		2-6~2-3	Cat	128.144
		H7N3	2004	E	G	Q	G		2-6<2-3	Av	128
		H7N7	2003	E	G	Q	G		2-6<<<2-3	Av	128
		H10N3	2021	E	G	Q	S		N.D.	Av	145-147
		H10N8	2013-14	E	G	Q	G		2-3	Av	146,148,149
Swine	Endemic	**$H1_{classic}N1$**	1918-[j]	D	D/G/N	Q	G		2-6~2-3[j] to 2-6>>>2-3	Av or Hu	2,75,83
		$H1_{classic}N2$	1980-[j]	D	D/G/N	Q	G		2-6>>>2-3	Av/Hu	83,150,151
		$H1_{av}N1$	1979	D	E/G	Q	G		2-6~2-3 to 2-6>>2-3[c]	Av	2,151-154
		$H1_{av}N2$	2000	D	E	Q	G		N.D.	Av/Hu	
		H3N2	1969-[j]	E/D	G	V/L/I	S		2-6>>>2-3	Av/Hu	151,155,156
		pH1N1	2009-[k]	D	D	Q	G		2-6>>>2-3	Hu	157-160
	Infrequent detection	**H1N7**	1992	E	E	Q	G		N.D.	Hu/Eq	161
		H2N3	2006	E	G	L	G		N.D.	Av/Sw	162
		H3N3	2001	E	G	Q	A		2-6<<2-3	Av	151.163
		H3N8	2005	E	G	Q	G		N.D.	Eq	164
		H4N1	2009	E	G	Q	G		N.D.	Av	165.166
		H4N6	1999/2014	E	G	L	S		2-6>>>2-3	Av	167-170
		H4N8	2011	E	G	Q	G		N.D.	Av	171
		H5N1	2001-	E	G	Q	G		2-3	Av	172
		H5N2	2008/2014-15	E	G	Q	G		N.D.	Av	173
		H5N6	2014-18	E	G	Q	G	K222Q, S227R	N.D.	Av	174
		H6N6	2009-10	E	G	Q	S	A222V	2-6~2-3	Av	175.176
		H9N2	1998-19	A/V/T/E	G	Q/L	G		N.D.	Av	177
Mammals	Dog	**H1N1**	2013	D	E/G	Q	G		2-6>>>2-3 to 2-6~2-3	Sw	178
		H3N2	2006-	E	G	Q	G		2-6<<<2-3	Av	179,180
		H3N8	2003-16	E	G	Q	G		2-3	Eq	181
		H6N1	2015	V	G	Q	S		N.D.	Av	182.183
	Seal	**H3N3**	1992	E	G	Q	G	A138S, R220S	2-6<<<2-3 to 2-6<<2-3	Av	2.184
		H3N8	2011; 2017	E	G	Q	G	A134T	2-3	Av	102,185,186
		H4N6	2002; 2012	E	G	Q	G			Av	184
		H10N7	1980-2021	E	G	Q/L	G		2-6<<2-3 to 2-6>>2-3[c]	Av	187.188
Poultry	Chicken	**H5N1**	1997-	E	G	Q	G	N186K, Q196H	2-6<2-3 to 2-3	Av	124.125
		H5Nx 2.3.4.4	2013-	E	G	Q	G	T160A, K222Q, S227R	2-3 to 2-6~2-3	Av	66,189,190
		H6N1	2000-13	V	G	Q	S		2-6<<<2-3 to 2-6<2-3	Av	139.183
		H6N2	2008-11	E	G	Q	G		2-6<2-3	Av	191
		H6N6	2008-11	E	G	Q	G		2-6<2-3	Av	191
		H7N2	2003	E	G	Q	G		2-6>>2-3	Av	128
		H7N9	2013-	E	G	Q/L	G		2-6<<2-3	Av	127,129-131,167
		H9N2	1966-	A/V/T/E	G	Q/L	G	I155T, I227L	2-6<<2-3 to 2-6>>2-3	Av	132,192-197
		H10N3		E	G	Q	S		2-6<2-3	Av	198
		H10N8		E	G	Q	G		2-6>2-3	Av	27.187
Water fowl	Gull	**H10N7**	2015	E	G	Q	G		2-6<2-3	Av	187
		H16N3	2005	T	G	Q	S	W/Q/K/R222G; A138S	2-6>>2-3	Av	199
	Duck	**H3N2**	2011-14	E	G	Q	G		2-6<2-3	Av	200
		H6N2	2008-11	E	G	Q	G		2-6<2-3	Av	191
		H6N6	2008-11	E	G	Q	G		2-6<2-3 to 2-6>>2-3	Av	191
		H10N6	2010	E	G	Q	G		2-6~2-3	Av	187

[a] Mutations at four canonical positions crucial in causing a shift in binding specificity or affinity as originally identified in H1 (190, 225) or H3 (226, 228). Standard H3 numbering is used throughout. Red is avian IAV consensus, green is human IAV consensus.
[b] 1918 H1N1 isolate A/New York/1/1918 as well as some later seasonal H1N1 isolates display strong 2–3Sia binding.
[c] Evidence of gradual shift to 2–6 specificity.
[d] Several independent reassortment events between seasonal H1N1 and H3N2 have occurred of which the 2001–2003 epidemic is best documented.
[e] Reassortant between avian IAV virus and human H1N1.
[f] E190D is introduced only after 1990.
[g] N193S lowers affinity for 2–6Sia and 2–3Sia but after 1972 mutates back to 193 N.
[h] Swine IAVs identified in human do not seem to differ from consensus sequences of the different swine IAV clades indicating a lack of adaptation to humans.
[i] G228X suggests heterogeneity at position 228.

[j] multiple introductions of human IAV have occurred throughout the years, occasionally contributing to novel reassortant strains that further spread in swine.
[k] pH1N1 frequently transmits to swine, contributing internal gene segments by reassortment and generating novel swine IAV strains.
[l] Increase of 2–3Sia binding due to propagation of initial isolates in eggs cannot be excluded.
[m] Symbols: ~ is difference smaller than 1,5-fold; < is 1,5 to 3-fold smaller; < < is 3–10-fold smaller; < < < is more than -10-fold smaller. > / > > / > > > same.

observed receptor binding specificity of IAV strains isolated from these tissues, suggesting that other factors, such as differences in chain length, branching, and distribution of Sia moieties, could be a factor in binding selectivity.

Glycomic analysis of the respiratory tract

Mass spectrometry analyses of glycans of the respiratory tract of human, swine, ferret, and chicken have been performed, but different sections of the URT and LRT were not uniformly analyzed hampering a direct comparison (Table 2). Analysis of the URT is limited to the nasopharynx of human and nasal wash, palate, and turbinate of ferrets. The URT displays relatively short N-glycans.

Chicken and swine trachea mainly display monosialylated glycans of limited length and branching in contrast to ferrets displaying more, longer, and mostly sialylated branches. The importance of such differences is illustrated by the requirement of such long, multiantennary, sialoglycans for binding of more recent seasonal H3N2 strains [35,42]. Long antennae are present, albeit at low density, in the human lung and bronchus and ferret lung, but absent from swine and chicken. Monosialylated bi- and tri-antennary N-glycans dominate on lungs of swine and chicken, whereas branching is more abundant and bi- and tri- and tetrasialylated glycans are present in lungs of humans and ferrets. Remarkably, 2–6Sia was most abundant on short antennae and 2–3Sia on long antennae in the human lung [6]. The abundant expression of the Sda antigen in ferrets effectively blocks 2–3Sia for binding by IAVs. Also, high levels of α-Gal expression in ferret and swine and of N-glycolylneuraminic acid (NeuGc) in swine will potentially affect IAV binding by reducing functional receptor density. Alveoli of human were shown to display more 2–3Sia than 2–6Sia with similar numbers of Galβ1–3/4GlcNAc (LacNAc) repeats as in the lung.

In conclusion, whereas N-glycome analyses indicate that the distribution of Sia linkage types may seem similar at several places, differences in the fine structures of glycomes may present barriers for interspecies transmission that need to be explored in more detail. Such differences include diversity of glycan termini by, for example, fucosylation at the GlcNAc (sialy lewis A/X (SLeA/X), Table 3) or sulfation at different sites on Gal or GlcNAc. Such modifications are preferentially recognized by specific IAVs, for example, preferred binding of 6-sulfo-sialyl Lewis X by IAVs of poultry [65] or sialyl Lewis X by novel clade 2.3.4.4 H5Nx viruses [66].

The O-glycomes of human [6], swine [67], and ferret [68] are relatively simple. Core 1 structures are in general far more abundant than core 2, except for swine lung. Chains longer than one Galβ1–4GlcNAc repeat were not detected. Sialylation is much more extensive for human and ferret lung, but, in contrast, very low in human bronchus and ferret trachea. Glycosphingolipids (GSL) were only analyzed for the human and ferret lung and ferret trachea. GM3 is the major glycan, but elongated antennae with up to 7 Galβ1–4GlcNAc repeats were identified. Modifications such as SLeA/X and the Sda epitope in ferrets are present. Thus, O-linked glycans and GSLs are abundantly present and could play a (specific) role in IAV binding and entry. More detailed studies are needed to understand their contribution to IAV infection and evolution.

Virus strains adapted to human-type receptor binding

Knowledge on IAV adaptation to humans derives from only four pandemics. Close ancestors remain unidentified, leaving ancestral sequence reconstruction (ASR) the main tool for reconstructing evolutionary histories. Precursors to pH1N1, and potentially other pandemic IAVs, evolved in swine. IAV genotypes from waterfowl occasionally cross the species border but rarely become established (Figure 1). Table 3 displays IAV strains isolated from humans and potential intermediates. Their genotypes, observed binding specificities, and (canonical) HA mutations affecting binding affinity or specificity are listed in Table 3. References are mostly listed in the tables.

Humans

ASR suggests that 1918 H1N1 pandemic virus may be a direct descendant of either pre-1918 swine or avian IAVs that likely have circulated in years before the pandemic [69–73]. Regardless of the exact transmission route, the 1918 virus evolved in swine as classical swine H1N1 while contributing genome segments to novel reassortant swine viruses over the years. One such reassortant, still harboring a descendant of the 1918 H1 segment, was transferred to humans as 2009 pH1N1, replacing the seasonal human H1N1 descendant from 1918. pH1N1 precursors evolved in swine for at least 10 years [74]. By reassortment, the classical swine H1 segment was combined with an N1 segment of an avian IAV introduced in swine around 1979 (Figure 1). Initial

human isolates match the canonical human HA consensus sequence and displayed 2–6SIA specificity [75–77]. The pH1N1 HA/NA balance is characterized by weak binding, in combination with low NA activity [21]. Host species involved in the genesis of H2N2 (1957) and H3N2 (1968) pandemic strains are unknown. Prepandemic reassortment involves genes derived from avian and preceding pandemic strains, possibly by multiple events in different mammalian hosts. Early 1918 H1N1 [78,79] and H2N2 [2,80] isolates displayed dual 2–3/2–6Sia or pronounced 2–3Sia receptor specificity, which not in all cases can be due to mutations proposedly obtained following propagation of initial isolates in eggs. 2–3Sia binding correlated with the presence of a (partial) avian HA consensus sequence, demonstrating that full adaptation to human-type receptors can occur during the initial pandemic phase. Early H3N2 isolates showed the canonical human-type consensus sequence but initially still displayed some 2–3Sia binding [32,33].

Since years, some avian (H5N1, H7N9, and H9N2) or swine (H1N1v, H3N2v) IAVs frequently infect humans without becoming established, despite that swine IAVs and also avian H9N2 display efficient 2–6Sia binding. Remarkably, establishment of human IAVs in swine happens more frequently than *vice versa* [81,82]. The lack of adaptation to human-type receptors of H5N1 and H7N9 (apart from a recent isolate) [3], both replicating at a location (the human LRT) that is unfavorable for transmission, suggests absence of a within-host selective advantage for receptor adaptation. This is in line with the low frequency of pandemics and strongly suggests that pre-adaptation in an intermediate host is a prerequisite. Remarkably, H5N1 and H7N9 hardly have been detected in swine. A range of other avian IAVs, sometimes displaying human-type receptor binding (H6N1, H7N2), have occasionally infected humans, but their pandemic potential remains unclear.

Swine

Swine IAVs contain a large, regionally restricted, diversity of strains due to frequent reassortments driven by repeated introduction of seasonal human IAVs and occasionally avian IAVs [83,84]. Still, genotypes are limited to H1N1, H1N2, and H3N2. Multiple strains deviate from the canonical consensus for 2–6Sia binding, but the consequences for receptor binding specificity are poorly resolved. $H1_{av}N1$ of avian descent (often indicated as European avian-like (EA) H1N1 swine IAV) antigenically differs from human H1N1 and displays dual 2–3/2–6Sia specificity. ASR was used to predict the changes in HA of ancestral viruses, which subsequently were reconstructed in the lab. Receptor specificity studies showed that during ancestral evolution, a stepwise shift from 2-3Sia to 2-6Sia binding has occurred, demonstrating that gradual adaptation to human-type receptors can occur in swine [85].

Other mammals

IAV strains H3N8 and H3N2 became established in dogs and have retained 2–3Sia specificity, but infection with 2-6Sia specific H1N1 originating from swine shows that viruses with 2–6Sia specificity in principle can infect dogs, so far without becoming established. Seals (and whales) are susceptible to infection with avian IAVs and several genotypes have become established. H10N7 was shown to acquire 2-6Sia binding specificity after establishment and represents another example of gradual adaptation to human-type receptors in a mammalian species.

Avian

Relatively few avian IAV genotypes have been detected in poultry. In chicken, nearly all IAVs — except some H7N2, H10N8, and H9N2 isolates — display a strong 2-3Sia binding specificity despite the abundance of 2–6Sia in chicken trachea. From gulls, strains with strong (H16N3) or moderate (H10N7) 2–6Sia specificity were isolated. For H16N3, deviations from the 2-3Sia binding specificity consensus were found. A H10N6 isolate from ducks bound equally well to 2–3Sia and 2–6Sia. Overall, we can conclude that especially H9 and H10 have displayed the ability to become 2-6Sia specific in possible intermediate hosts, suggesting a zoonotic potential.

Concluding remarks

Adaptation to human-type receptor binding is rare in nature, despite the long-standing view that only two substitutions in HA will suffice. Maintaining an HA–NA balance on heterogeneous receptor surfaces involves dynamic heteromultivalent interactions enabling NA-driven motility for reaching, yet enigmatic, entry-competent sites. In addition, adaptation to poorly characterized mucosal decoy receptors, enabling traversal of the mucus layer, is likely another major factor in host species adaptation. The role of virus morphology in infection is largely unknown. Extensive colocalization of 2–6 and 2–3Sias along the respiratory tract of diverse species suggests the importance of additional structural glycan features in determining virus binding and entry specificity. Extended glycomic analyses of specific epithelial cell types and mucus of IAV host species are necessary. Detailed binding specificity of IAVs infecting potential intermediate host needs to be clarified. Especially for swine IAVs, the differences with human IAVs in productive binding (i.e. leading to infection) to human-type receptor surfaces, need to be determined. Also, molecular determinants of NA specificity and activity require further identification to better understand the molecular determinants and evolutionary pathways underlying the mechanisms by which influenza viruses switch hosts.

Conflict of interest statement

Mengying Liu, Frank J. M. van Kuppeveld, Cornelis A. M. de Haan, and Erik de Vries declare no conflict of interest.

Data Availability

No data were used for the research described in the article.

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