Notes for talk on 6th May 2010
HA/NA balance? The evidence

These notes contain a bit more detail than the slides by themselves, and a couple of other studies. The same disclaimer in the on slide 2 applies to these notes!

Introduction (slides 1-5)

We have talked a lot about HA/NA balance before – but have never discussed the evidence that currently exists in the literature for a balance existing. I think that we all currently agree that there must be some degree of balance between the activity of HA and NA as in the extreme case of one or the other being entirely missing, it *usually* cripples viral replication entirely. What remains less clear is the extent of this balance – how much change can be tolerated in either protein before compensation is required by the other.

After writing this, I'm still not convinced about how such a balance would work kinetically, with NA being an enzyme and HA a weak binding protein, but I accept that there are many possible theories that explain how it could work – the problem is finding evidence to support them!

A quick recap - Influenza A and B have two surface glycoproteins, hemagglutinin or HA (glycan receptor binding for cell entry and membrane fusion) and neuraminidase or NA (receptor destroying for virion budding and disaggregation).

Side note of interest: Unlike A and B, Influenza C has a single glycoprotein (HEF) which performs all three functions on its own! If there is balance between HA's receptor binding ability and NA's receptor destroying ability (some sort of co-evolution or compensation after a reassortment event), you would expect to see something similar for HEF glycoprotein. However, you cannot 'reassort' to give totally different binding/destroying activities quickly for HEF as it is single protein, but you might still see coordinated drift between the two different active site regions. There is one (3.2A) crystal structure for HEF but is there any genetic data? Colin tells me that sadly there is no influenza C surveillance so learning more about this now is unlikely.

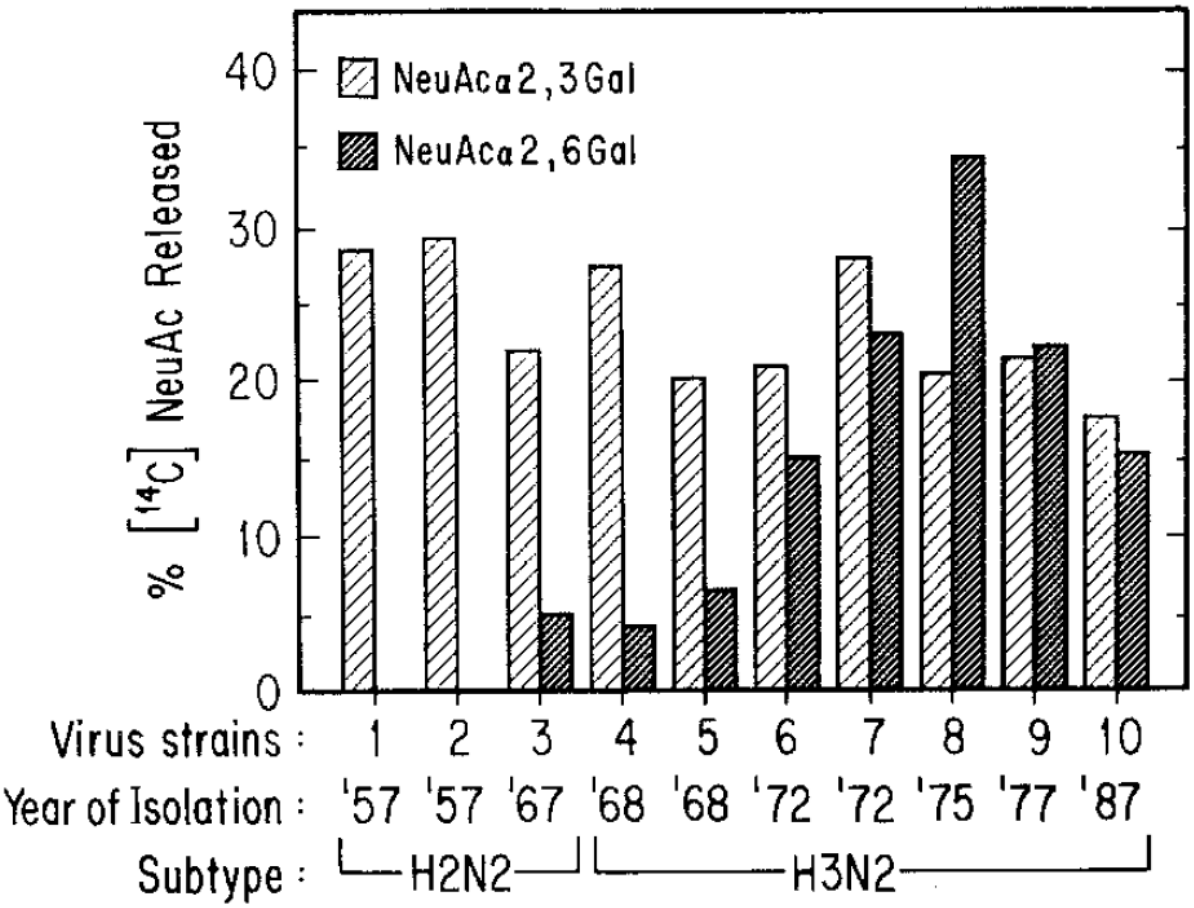
Naturally occurring reassortments (slides 6-8)

An H2N2 pandemic strain emerged in 1957 after reassortment between human (H) and avian (N) viruses became H3N2 (HA2->HA3 and avian NA retained) after another reassortment in 1968. Baum and Paulson (Baum LG, Paulson JC, Virology 1991; 180: 10-15) took 10 human viruses isolated between 1957 and 1987 and tested for NA and HA a(2,3)/a(2,6) linkage specificity:

A/RI/5+/57
A/RI/5-/57
A/Japan/305/57
A/Tokyo/3/67
A/Aichi/2/68
A/Hong Kong/8/68
A/Udorn/307/72
A/Memphis/102/72
A/Victoria/3/75
A/Texas/1/77
A/Los Angeles/2/87

All were grown in eggs.

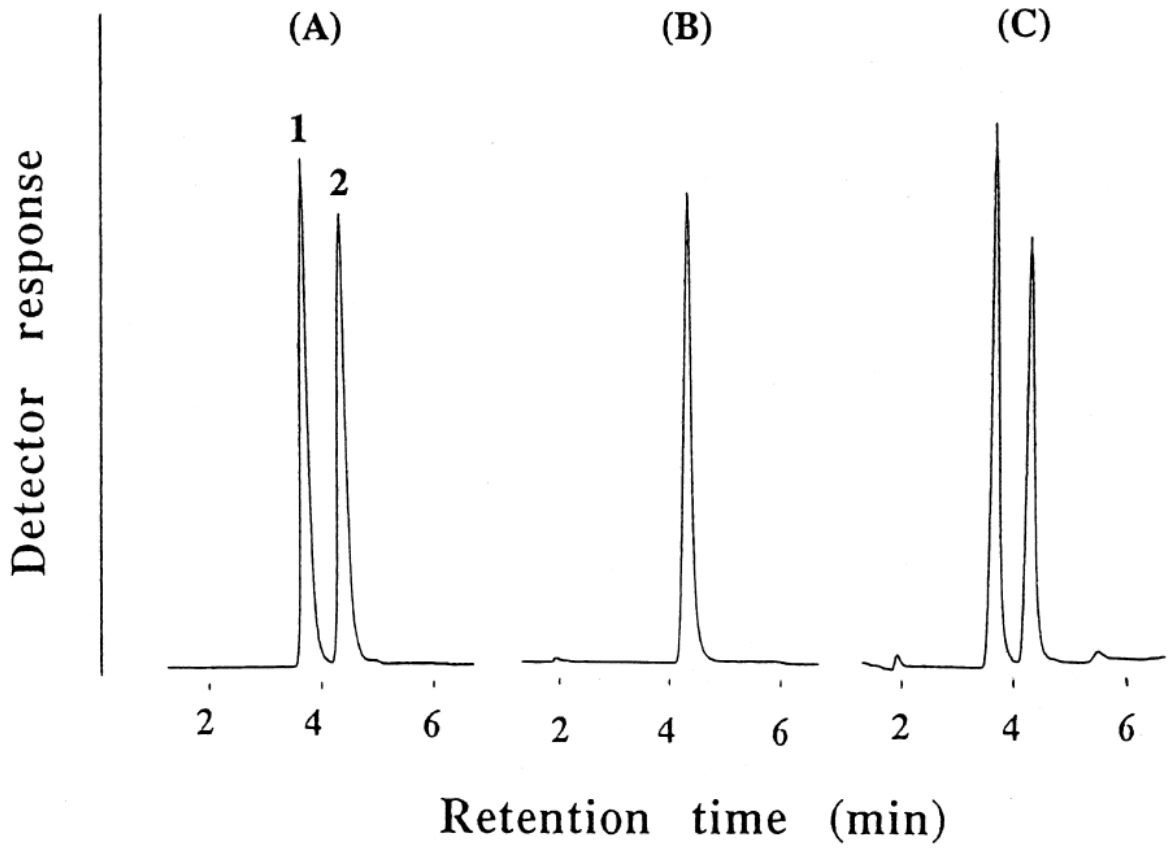
The human H2 and H3 HAs in all strains were found to bind a(2,6), but initially in the early strains the avian N2 showed ONLY a(2,3) destroying activity – correlating with classic avian HA specificity. By 1972, the N2 NAs showed equal a(2,3) and a(2,6) destroying ability. This dual specificity is also seen in H1N1 NAs.



Activity for both is important in human infection as you need to remove a(2,3) to allow virion passage through the mucus layer, and a(2,6) removal is required for efficient escape from host cells and to prevent self aggregation.

To summarise, in 1957 HA bound to a(2,6) + NA bound to a(2,3) ---DRIFTS--> and by 1972 HA still bound to a(2,6) + NA bound to both a(2,3) and a(2,6) i.e. NA acquired changes that allowed it to bind a(2,6).

Another example of this receptor adapted specificity can be seen comparing the NA activity of human and swine isolates (Suzuki T, Horiike G, Yamazaki Y, et al., FEBS Lett 1997;404:192-196). The human NAs investigated were unable to cleave N-glycolyl (Neu5Gc) receptors, but most of the swine NAs could cleave both N-glycolyl and N-acetyl (Neu5Ac) receptors. HPLC was used to show that N-glycolyl receptors are absent in the respiratory tract of humans (the enzyme required to produce them is absent) but both are present in pigs in a roughly 1:1 ratio.



Legend (the pdf text is broken): Chromatograms of DMB derivatives of Neu5Ac and Neu5Gc obtained from tracheal epithelia of humans (B) and pigs (C). A shows a standard mixture of both Neu5Ac (peak 2) and Neu5Gc (peak 1).

Therefore presumably swine HA is often able to bind NeuGc glycans and hence the swine NA needs to be able to cleave them to prevent aggregation and facilitate progeny escape.

An interesting exception to this is A/Swine/Iowa/15/30 which can only cleave Neu5Ac chains. The authors do not offer a hypothesis to explain this observation. Wasn't Swine Iowa a lab release? Maybe that could account for its odd behaviour.

Lab made reassortants (slides 9-12)

There are many possible combinations of H and N subtypes possible, but in natural isolates, some occur very frequently whereas others are rarely or never detected – why is this?

DISCUSSION: actually, pretty much all combinations are seen in birds, and the others have been made in the lab. Some combinations do occur much less frequently than others though, and why this happens is not understood.

Kaverin *et al* (Virology;244:315-321) took NA from the human A/USSR/90/77/H1N1 strain and made reassortants with avian H3/4/10/13 viruses. These new viruses replicated poorly in eggs (virus aggregates were seen) compared to the avian parent viruses.

Here is a specific example: reassortant R2 has HA from A/Duck/Ukraine/1/63/H3N8 and all other genes from A/USSR/90/77/H1N1 making it subtype H3N1. R2 was seen to replicate badly in eggs, with lots of viral aggregation seen. HPLC showed high levels of sialic acids on the reassortant virus particles compared to the avian parent – so the likely explanation for the aggregation in R2 is that the human NA from the USSR strain is not able to effectively remove the a(2,3) receptors from the virus particles. It has the wrong specificity and so the avian HA causes aggregation and inhibits replication.

R2 was serially passaged in chick embryos to produce R2-XXI (21 times) and R2-XIIIa (13 times). These passaged variants do not aggregate and replicate in eggs as well as the avian parent virus. Have they regained NA function, and hence lost surface sialic acid? No! So what has changed?

TABLE 1

The Content of Sialic Acid in Purified Virus Protein

Virus	Subtype	Sialic acid content (pM per 1 μg of virus protein)[a]
A/Duck/Ukraine/1/63	H3N8	0.42 ± 0.12
A/USSR/90/77	H1N1	3.52 ± 0.28
R2	H3N1	3.55 ± 0.40
R2-XXI	H3N1	2.65 ± 0.39
R2-XIIIa	H3N1	2.43 ± 0.25

[a] Mean of four determinations ± standard error. A separate virus preparation was used for each determination.

HA has acquired mutations (many seen but only N248D – note H3 numbering (I think!) - common to both passaged variants) that decrease affinity for SOME a(2,3) receptors, reducing the potential for self aggregation.

TABLE 2

Changes in the Affinity of HA toward Sialic Acid-Containing Substrates Associated with the Loss of Virion Aggregation

	Substrate			
Virus	Fetuin–HRP conjugate	3′-Sialyllactose attached to a polyacrylic acid carrier	α-*N*-Acetyl neuraminic acid	3′-Sialyllactose
R2	0.16 ± 0.02[a]	0.97 ± 0.14	312 ± 43	463 ± 60
R2-XXI	1.17 ± 0.12	19.2 ± 2.2	420 ± 51	770 ± 98
R2-XIIIa	0.95 ± 0.08	14.6 ± 3.0	513 ± 33	460 ± 92

[a] K_{aff} (expressed in μM sialic acid) determined for fetuin conjugate by direct solid-phase enzyme-linked assay (Gambaryan and Matrosovich, 1992) and for SA-polyacrylate, Neu5Ac, and 3′-SL by competition assay (Gambaryan *et al.*, 1997). The data represent means ± standard error. For each substrate four preparations of each virus were assayed.

This observation indicates that you don't need complete desialisation of viral glycoproteins for efficient viral spread - you can module the binding affinity of HA instead to compensate. It is possible that this compensation is not possible for all combinations of HA and NA (you can only reduce HA affinity to a point!), restricting what we see in natural isolates.

NA inhibitor resistance studies (slides 13-16)

NA inhibitor resistant mutants can be generated *in vitro* by passage in cell culture in the presence of an inhibitor such as Zanamivir (ZMR) or Oseltamivir (OTV). Two mechanisms for viruses to escape NA inhibitors have been observed:

(a) make changes to conserved amino acids in the NA binding pocket to lower binding affinity to the specific inhibitor used. Many of these changes can influence the catalytic activity of NA, and although

you can often get such mutants to grow in cell culture, you often compromise their ability to replicate in animal models.

(b) make changes in and around the HA binding pocket (for example T155A, V223I and R229(I/S) – H3 numbering) to reduce receptor affinity, making the virus less reliant on high NA activity. These viruses were able to still bud in the presence of ZMR or OTV, despite their NA being fully sensitive.

TABLE 2. Sequence changes in the NA and HA proteins of resistant viruses[a]

Virus (drug used)	NA amino acid at position:		HA amino acid at position[b]:		
	90	346	155	223	229
NWS/G70C parent	G	N	T	V	R
Variant 1 (4-amino-Neu5Ac2en)	**Q**	N	T	**I**	**I**
Variant 5 (4-amino-Neu5Ac2en and then 4-guanidino-Neu5Ac2en)	G	N	T	V	**S**
Variant 7 (4-guanidino-Neu5Ac2en)	G	**S**	**A**	V	R

[a] Amino acid numbering is based on the N2 NA and the H3 HA. Sequence changes are shown in boldface.
[b] Changes were all in the HA1 chain.

(McKimm-Breschkin JL et al;Antimicrob Agents Cehmother 1996;40:40-46)

These types of mutations lowering HA affinity can even lead to *in vitro* drug **dependant** strains! This is evidenced by an increase in the size and number of plaques when an inhibitor is present (Blick *et al*; Virology 1998;246:95-103). Essentially HA affinity becomes so weak to compensate for the lack of NA activity that without the inhibitor present to block NA, it is unable to bind to the host cells effectively for the initial entry.

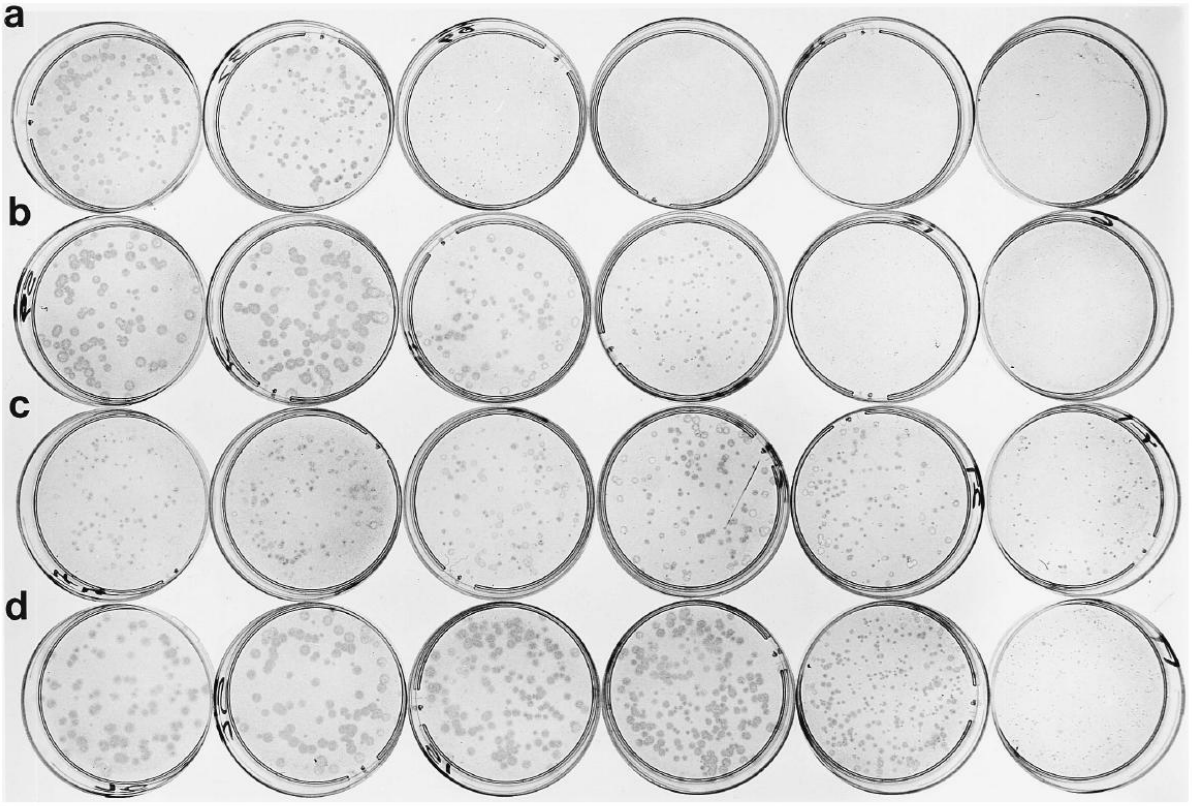


FIG. 1. Plaque inhibition assay with 4-guanidino-Neu5Ac2en in the overlay. Virus was plaqued, from left to right, without inhibitor and with 0.0003, 0.003, 0.03, 0.3, and 3 μg of 4-guanidino-Neu5Ac2en per ml. (a) NWS/G70C; (b) variant 1; (c) variant 5; (d) variant 7.

a shows inhibition, b shows reduced inhibition, c shows some drug dependence and d shows strong drug dependence.

DISCUSSION: not everyone is convinced by this interpretation. The image quality during the talk was also not quite good enough to be sure what is going on. I will forward high quality version to Bjorn to take a look at!

Penn et al (Options for the Control of Influenza III, Brown LE, Hampson AW, Webster RG (eds). Elsevier Science B.V: Amsterdam,1996:735-740) described an HA mutant from a ZMR treated immunocompromised child which was sensitive to ZMR in MDCK cells, but in a mouse model showed resistance. The mutant was seen to bind a(2,6) receptors less strongly than the parent strain, making it less reliant on NA activity to bud, and hence it was ZMR resistant *in vivo*. In contrast, when grown in MDCK cells, the mutant was seen to bind more strongly to a(2,6) receptors than the parent, and so high NA activity would be needed for efficient progeny budding. This made it ZMR sensitive.

Crippling NA (slides 17-18)

Mutants with a totally crippled NA can be created by serial passage in presence of anti-NA serum (NA activity is replaced by bacterial NA during these passages). The NA gene obtains a large deletion in its open reading frame plus a premature stop codon, meaning only the tail and transmembrane domain of the NA are expressed. Hughes et al (J. Virol 2000;74:5206-5212) reported that it is possible to get these mutants to replicate in eggs, mice or MDCK cells by repeated passage and selection for high growth variants. The resulting viruses acquired AA substitutions around the HA binding site that produced a marked drop in receptor binding and significantly increased replication, however, replication was still impaired when compared to the parent virus.

TABLE 2. Hemagglutination activity of parental virus and its NA deletion variants

Virus	ELISA titer	Hemagglutination titer at:		Specific hemagglutination activity (HA_4/ELISA titer)	Ratio of hemagglutination titer at 37°C to that at 4°C (HA_{37}/HA_4)
		4°C (HA_4)	37°C (HA_{37})		
NWS-G70c	6,000	1,600	1,200	0.3	0.75
23ΔNA	2,000	320	320	0.2	1.00
CK2-29	12,000	320[a]	40[a]	0.03	0.13
E17A	5,000	40[a]	<10	0.01	NA[b]
E17E	3,200	<10	<10	<0.003	NA
M18B	6,600	<10	<10	<0.003	NA

[a] Virus did not show complete agglutination.
[b] NA, not available.

TABLE 3. Amino acid changes in NA deletion variants

Virus	Relative receptor-binding affinity[a]	Amino acid changes in the HA by H3 numbering (H1 numbering)[b]								
		96 (89)	132 (128)	135 (132)	140 (137)	141 (138)	145 (142)	193 (190)	205 (202)	220 (217)
NWS-G70c	+++++							S	V	
23ΔNA	+++++	A	T	V	S	H	S	R	M	R
CK2-29	++			A			N			K
E17A	+	T[c]		A			N			
E17E	−		K	A	P		N		V	K
M18B	−			A	P	Q	N			K

[a] Based on hemagglutination data in Table 2. +++++, highest affinity; specific hemagglutinating activity, >0.2; ++, moderate affinity; specific hemagglutinating activity, 0.03; +, weak affinity; specific hemagglutinating activity, 0.01; −, affinity below the sensitivity of the assay.
[b] Changes in the HA1 sequences represent differences between the 23ΔNA and the other viruses, including the parental strain. Blank spaces indicate that the amino acid is identical to that in 23ΔNA.
[c] Substitution A96T creates a potential glycosylation site at Asn-96.

This shows that the compensation possible by changing HA affinity is only partial, probably as you can only decrease HA affinity so far before it becomes unviable.

Varying NA 'stalk' length (slides 19-25)

NA consists of a box-like head containing the active site, and a fibrous 'stalk' region of variable length.

NAs with a short 'stalk' have been shown to be inefficient in releasing and disaggregating progeny virions. This is probably a geometric effect i.e. the active site is not held far enough from the viral membrane and so cannot interact with its substrate efficiently (Els MC, Air GM, Murti KG, *et al* Virology 1985;142:241-247).

Castrucci and Kawaoka (J Virol 1993;67:759-764) created a set of mutants with stalk lengths between 0 and 52 AAs (the wild type A/WSN/33/H1N1 has a stalk length of 24). In MDCK cells, they were all able to replicate at least as well as the parent virus (suggesting NA activity is not so important in this system), but in chicken eggs, the stalk length was well correlated with the efficiency of viral replication, the longer the better.

TABLE 1. Correlation between NA stalk length and virus replication in tissue culture and eggs

Virus	No. of amino acid residues in the NA stalk[a]	Titer in MDCK cells (log_{10} [PFU/ml])	Replication in eggs (log_{10} [EID_{50}[b]/ml])	PFU/EID_{50}
Wild type [WSN(NA15)]	**24**	**7.3**	**5.5**	**63**
SD0	**0**	**7.3**	**—[c]**	**—**
SD9	**15**	**7.0**	**4.5**	**350**
SA14	**38**	**7.0**	**7.0**	**1**
SA28	**52**	**8.1**	**8.5**	**0.4**

[a] Based on assignment of Harley et al. (18).
[b] EID_{50}, 50% egg infective dose.
[c] —, SD0 virus did not grow in eggs, even after inoculation of 10^7 PFU of virus.

Mitnaul et al (J Virol 2000;74:6015-6020) repeatedly passaged the mutant with no stalk in eggs, selecting high growth variants. As with drug resistance, there were two mechanisms seen to allow the recovery of good growth in eggs:
(a) insertions were acquired in the NA the gene derived from NP, PB1 and PB2 genes - thus reconstituting the stalk region.
(b) mutations near the HA binding site were acquired, reducing receptor binding affinity such that high NA activity is no longer required to prevent aggregation and facilitate budding.

TABLE 1. Properties of egg-adapted SD0 strains[a]

Virus	Egg passage at which positive hemagglutination was detected	NA stalk insertion	
		No. of amino acids inserted	Origin of gene insertion
0EA	12	22	PB2
7EA	12	10	PB2
10EA	10	13	PB1
18EA	8	18	PB2
21EA	10	20	NP
2EA	12	0	NA[b]
5EA	8	0	NA
14EA	12	0	NA
19EA	12	0	NA
25EA	10	0	NA

[a] Approximately $10^{7.3}$ PFU of SD0 virus (1 ml) were injected into 10-day-old embryonated chicken eggs. After 2 days of incubation at 35°C, egg allantoic fluid was harvested and tested for viral growth by hemagglutination assays. Viruses were serially passaged by inoculating the eggs with undiluted allantoic fluid until hemagglutination was observed. The passage at which hemagglutination was first observed is shown. Only strains that yielded positive hemagglutination are indicated. The NA stalk insertion was detected by sequencing the NA gene.

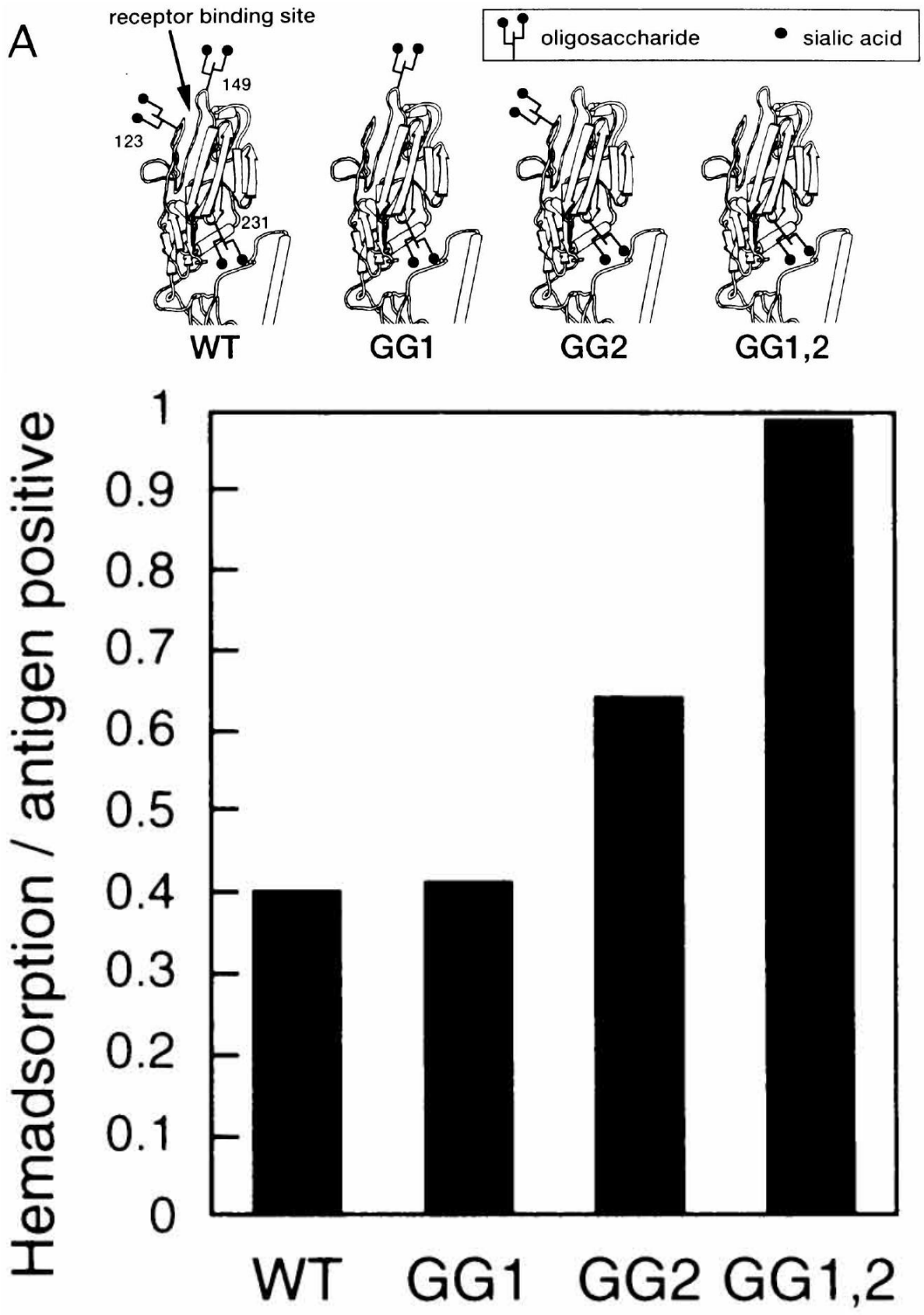
[b] NA, not applicable.

TABLE 3. Mutations in the HA gene of egg-adapted viruses[a]

Virus	NA insertion	Mutation(s) in:	
		HA1	HA2
0EA	YES	95Asn→Asp	72Asn→Lys
21EA	YES	135Val→Ile	
2EA	NO	146Ser→Gly	106Arg→Lys
		262Arg→Lys	

[a] For each adapted strain, HA cDNA was synthesized by reverse transcriptase PCR followed by cDNA sequencing as described in Materials and Methods.

HA binding affinity can also be modulated by glycosylation. It has been shown by Ohuchi et al (J Virol 1997;71:8377-8384) that N-glycans flanking the binding site can dramatically reduce HA-sialic acid affinity. Removing these N-glycans gives strong HA binding.



This effect was confirmed by Wagner et al (J Virol 2000;74:6316-6323) who produced an array of HA mutants with N-glycosylation sites removed from the tip of HA. It was shown that their growth was

dependant only on the NA they were combined with. Using short stalked N1 NA for example caused low growth as the NA is unable to disaggregate and release progeny compared to an N2 NA (with a longer stalk), which showed significantly higher growth efficiency. The same effect was seen in reassortant avian viruses by Baigent et al (Virus Res 2001;79:177-185).

Note: most of these stalk length experiments used lab strains, but there is some evidence from virues normally circulating in wild birds that are transmitted to domestic poultry, that NA deletion and changes in HA glycosylation occur together in nature (Banks J, Speidel ES, Moore E, et al. Arch Virol 2001;146:963-973).