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Soluble trimeric hemagglutinins to study receptor binding and immunogenic properties

Oplosbare trimere hemagglutinines voor het bestuderen van receptor binding en immunogene eigenschappen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 21 juni 2012 des middags te 12.45 uur.

door

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geboren op 26 mei 1983 te Naarden

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Contents

Chapter 1

1

General introduction

The highly contagious, acute respiratory illness known as influenza appears to have afflicted humans since ancient times. The sudden appearances of epidemics of respiratory disease that persist for a few weeks and then disappear are sufficiently characteristic to permit identification of a number of major epidemics in the distant past (Noble, 1982). While influenza has killed millions throughout the centuries, the 1918-1919 pandemic was particularly severe. This "Spanish influenza" not only killed between 20 and 50 million people worldwide, but may even have altered the course of the First World War (Crosby, 1976).

Influenza viruses belong, together with the Thogoto virus and Isavirus, to the family Orthomyxoviridae. They have been classified into genera A, B and C based on the antigenic differences between their matrix (M1) proteins and nucleoproteins (NP) (Ruigrok, 1998). Influenza A viruses (IAV) are further divided on the basis of the antigenicity of their two envelope glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) protein. Sixteen HA subtypes (or serotypes; H1-16) and nine NA subtypes (or serotypes; N1-9) of IAV have been identified (Fouchier et al., 2005), all of which exist in the wild water fowl reservoir (Webster et al., 1992). Some IAVs are also able to infect a wide range of mammalian species including pigs, horses and humans. The nomenclature of IAV is based on genus, host of origin (not mentioned for humans), geographical isolation site, strain number, year of isolation and a description of the antigenic subtype, in this specific order. So for example A/California/04/09/H1N1 is a human IAV, sampled in California, with strain number 04, in 2009 of H1N1 subtype.

IAV genome and particle

The genome of IAV consists of 8 single stranded, negative sense RNA segments. The 8 segments are numbered 1 to 8 and range in size from 890 to 2341 nucleotides. All coding sequences are flanked by highly conserved untranslated regions (Steinhauer and Skehel, 2002). The 8 segments' code for 10-11 viral proteins (Table 1). The viral RNA genome (vRNA) is packaged by the NP and bound to the polymerase complex, which consists of the basic polymerase 1 and 2 (PB1 and PB2) and the acidic polymerase (PA). Together, vRNA, NP, PB1, PB2 and PA form the ribonucleoprotein complex (RNP). The RNPs are enveloped by a lipid bilayer derived from the host cell in which the virus is grown. The M1 protein, which interacts with the RNPs, underlies the envelope. The envelope contains three integral membrane proteins: i) the glycoprotein HA, which is responsible for virus-cell attachment and fusion, ii) the NA protein, which is the receptor-destroying enzyme essential for release of the virus from the host cell, and iii) the matrix protein 2 (M2), which functions as an ion channel (Figure 1A) . IAV virions are about 80 to 120nm in diameter for spherical particles. Spherical particles are typically found in the case of laboratory-adapted strains (Calder et al., 2010). Field strains on the other hand often occur as filamentous particles, measuring up to several μm in length.

IAV life cycle

The IAV life cycle can be divided into several steps, as depicted in Figure 1B.

1. Attachment. The IAV life cycle starts with the attachment of HA to its sialic acid (SIA) receptor (Skehel and Wiley, 2000). SIA is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a 9-carbon backbone. SIAs are terminal carbohydrate residues found on N- and O-glycosylated proteins and glycolipids (Figure 1B) . SIAs attached to the penultimate galactose via a α2-3 or α2-6 linkage may serve as receptors for IAV.

2. Entry and uncoating. After receptor binding, the virus is endocytosed into the target cell. While

Segment	Protein	Function
1	PB2	Polymerase
2	PB1 / PB1-F2	Polymerase / Induction of Apoptosis
3	PA	Polymerase
4	HA	Receptor Binding & Fusion
5	NP	vRNA packaging
6	NA	Sialidase
7	M1 / M2	Matrix Protein / Ion Channel
8	NS1 / NS2	IFN antagonist / Nuclear Export

Table 1. An overview of the 8 gene segments of IAV. The proteins encoded and their functions are indicated

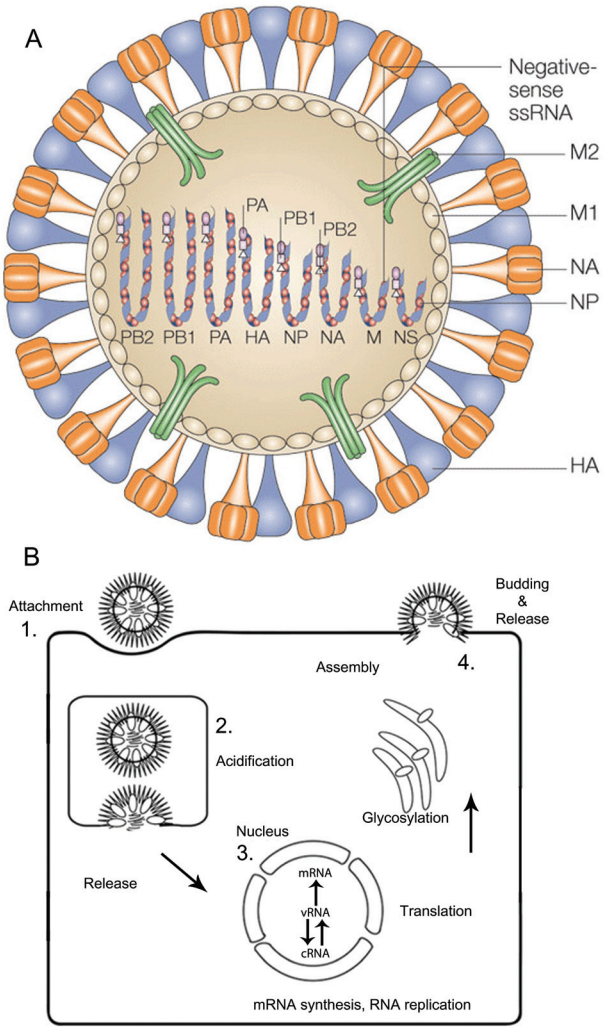

Figure 1. (A) Influenza A virus particle, (taken from (Nelson and Holmes, 2007)). (B) The replication cycle of influenza A virus (adapted from (Pinto and Lamb, 2006)). See text for details.

clathrin-mediated endocytosis has been identified as an entry route for IAV (Matlin et al., 1981; Mercer, Schelhaas, and Helenius, 2010; Rust et al., 2004), other endocytic routes may be used as well (Sieczkarski and Whittaker, 2002). More recently macropinocytosis was identified as an alternative IAV entry route (de Vries et al., 2011). After internalization, IAVs are transported to late endosomes, where the acidic environment (pH ~5) triggers HA conformational changes that mediate fusion of the viral and cellular membranes. In addition, acidification of the IAV particle, which depends on the M2 protein, is required for virion disassembly and subsequent delivery of the RNPs to the cytoplasm. (Skehel et al., 2001).

3. Replication and Transcription. After cytoplasmic release, the RNPs are transported to the nucleus, which is the subcellular location of IAV genome replication and transcription. vRNA genomes are the templates for subgenomic messenger RNA (mRNA) and full-length copy RNA (cRNA) (Neumann et al., 2004). Capped RNA fragments cleaved from the 5'end of cellular RNAs serve as primers for mRNA synthesis. cRNAs serve as a template for the synthesis of new vRNAs.

4. Assembly and release. vRNA genomes associate with NP and the polymerase proteins, thereby forming the RNPs. The RNPs associate with M1, which in turn allows binding of the viral NS2 protein and nuclear export. RNP-M1 complexes are subsequently transported further to the plasma membrane. The envelope proteins HA, NA and M2 are co-translationally inserted into the membrane of the endoplasmic reticulum (ER), after which they are processed by enzymes of the ER and Golgi and transported to the cell surface. RNP-M1 complexes bud through plasma membrane domains containing HA, NA and M2 (Chen et al., 2007). After budding, the enzymatic action of the NA protein is required for release of the progeny virions (Rossman and Lamb, 2011).

IAV in animals

IAVs constantly circulate in many animal hosts, including birds and mammals (Figure 2) (Medina and Garcia-Sastre, 2011; Olsen et al., 2006). Wild waterfowl species are the natural hosts for IAV and form the virus reservoir in nature. In addition, IAV is found in a wide range of domestic birds including turkey, chicken and ducks. The disease signs associated with IAV infections in avian species vary considerably with the strain of virus. Infections with most strains are asymptomatic. However, a few strains produce a systemic infection with death occurring within 1 week. These latter highly pathogenic (HP) viruses contain HA proteins of the H5 or H7 subtype, which contain a multibasic cleavage site that can be cleaved by furin-like proteases. The outbreaks of HP H5N1 and H7N7 viruses that affected both poultry and people have increased the level of awareness (de Jong et al., 2009; Shortridge et al., 2000). The HP H5N1 virus that emerged in Southern China in 1996 has continued to circulate in domestic poultry to date. Hundreds of people have been infected, of which more than 60% succumbed to the infection (Gambotto et al., 2008). IAVs have also been isolated from several mammalian species including horses and pigs (Myers, Olsen, and Gray, 2007). Sporadically, natural infections are also observed in seals, dogs and cats (Harder and Vahlenkamp, 2010; Kuiken et al., 2004; Lang, Gagnon, and Geraci, 1981).

IAV in humans

In humans, IAV is the cause of seasonal epidemics and occasional pandemics of influenza. The yearly epidemics are caused by human IAVs that continuously evolve thereby constantly generating new antigenic variants (antigenic drift) that partially overcome the pre-existing immunity in humans. The occasional pandemics are caused by suddenly appearing IAVs to which most humans have no immunity. These pandemic viruses have been associated with a change in HA subtype (antigenic shift) resulting from an exchange of gene segments between different IAV subtypes (reassortment) (Bouvier and Palese, 2008). However, as shown by the new pandemic H1N1 virus, a pandemic not necessarily depends on the introduction of a virus with a HA subtype that is new to the human

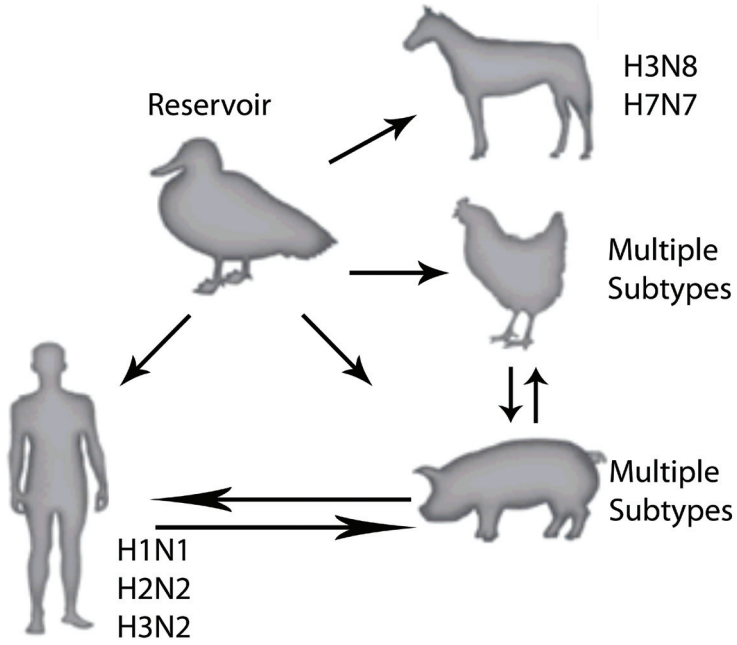

Figure 2. The natural hosts of influenza A virus.

population; the new pandemic H1N1 virus simply replaced the seasonal H1N1 virus (Klenk, Garten, and Matrosovich, 2011).

Although IAV has probably infected humans for centuries, the first scientifically documented pandemic was the Spanish flu in 1918. This devastating pandemic took the lives of an estimated 20 to 50 million people worldwide. Reconstruction of the virus from archival patient tissues allowed analysis of its extraordinary pathogenic properties. As no human pre-1918 IAV sequences are currently available, the origin of the pandemic virus, the timing of its emergence in humans, whether an intermediate host was involved, and whether the virus was a reassortant virus all remain unresolved (Sheng et al., 2011). After the pandemic, the H1N1 virus was maintained in the human and swine population.

In 1957, the H1N1 virus was replaced by a H2N2 virus. This latter virus caused the so-called Asian influenza pandemic. The H2N2 virus was derived from the previous H1N1 virus that had acquired HA and NA genes of subtypes H2 and N2, respectively, as well as its PB1 gene from avian viruses by reassortment (Scholtissek et al., 1978). With 1 million deaths worldwide this pandemic virus was significantly less pathogenic than the 1918 virus. In 1968 the H3N2 IAV, which caused the Hong Kong influenza pandemic, was first isolated. This virus again was the result of gene reassortment between the previous (H2N2) and avian viruses providing the HA and PB1 gene. (Kawaoka, Krauss, and Webster, 1989). As for H2N2 pandemic, the H3N2 pandemic caused approximately 1 million deaths worldwide. In 1977, the H1N1 virus reappeared in the human population, possibly resulting from the release of a laboratory strain (Young, Desselberger, and Palese, 1979). Since then H1N1 and H3N2 viruses "co-existed" as seasonal viruses in the human population (Klenk, Garten, and Matrosovich, 2011; Taubenberger and Kash, 2010). In 2009 a new pandemic H1N1 virus emerged. This virus, which swept around the world in six months, was designated as swine origin new pandemic H1N1 virus. The new pandemic virus probably resulted from the reassortment of different swine viruses as detailed elsewhere (Dawood et al., 2009; Smith et al., 2009). Disease caused by this virus has mostly been mild, with a mortality rate comparable to previous seasonal viruses.

It is thought that transmission and circulation of avian viruses in pigs may adapt these viruses for efficient replication and transmission in humans by facilitating the acquisition of mutations in HA that are needed for recognition of human receptors (Ito et al., 1998). In addition, mutations in the IAV polymerase may facilitate such transmissions, indirectly, by adapting the IAV replication machinery to human cells (Mehle and Doudna, 2009; Neumann and Kawaoka, 2006). Alternatively, pigs serve as a mixing vessel in which different viruses reassort to give rise to new human viruses. Either way, the specificity of the interaction of HA with SIA receptors largely explains the host range of IAVs. Thus, viruses that infect humans bind preferentially to SIA linked to the penultimate galactose in an α2–6 configuration, whereas avian viruses prefer binding to SIA with α2–3 linkages.

The HA protein

The HA attachment and fusion protein is a type I transmembrane glycoprotein that after synthesis and folding will interact to form homotrimers. The precursor HA0 protein is cleaved into HA1 and HA2 subunits by trypsin like proteases; HA proteins containing a multibasic cleavage site are additionally recognized by furin-like proteases (Steinhauer, 1999). HA1, which forms the head of the protein, contains the receptor-binding site (RBS). HA2, which forms the stem, contains the fusion peptide at its N terminus and two heptad repeat regions, which are central to the complex conformational changes leading to fusion between the virus and host cell membranes (Figure 3) (Ha et al., 2001; Wilson, Skehel, and Wiley, 1981).

The HA protein is synthesized as a glycosylated polypeptide. In the ER, a 14-sugar $Glc_3Man_9GlcNAc_2$ glycan tree is cotranslationally linked to Asn residues that reside within the Asn-X-Thr/Ser N-glycosylation consensus sequence (Stanley, Schachter, and Taniguchi, 2009) (Figure 4). In the ER, glycosidases and mannosidases trim the glycan tree down to $Man_8GlcNAc_2$. Further processing of the oligosaccharide side chains in the Golgi by several glycosidases and glycan transferases gives rise to a variety of oligosaccharide side chains. These modifications may be cell, tissue and species dependent. The number of potential N-glycosylation sites in HA ranges from 2 to 7 (Vigerust et al., 2007), with the glycosylation sites in the stem region being highly conserved (Das et al., 2010).

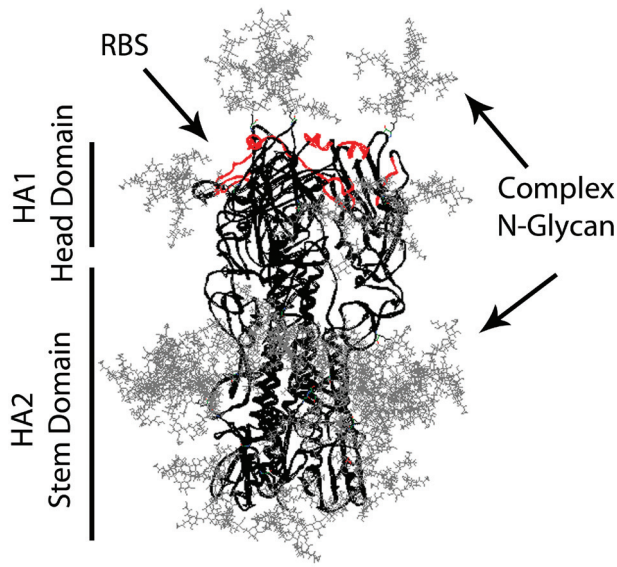

Figure 3. X-ray crystal structure of a H5 trimer (A/Vietnam/1203/04) (Stevens et al., 2006c). N-Linked glycans are shown using glyprot (Bohne-Lang and von der Lieth, 2005). The HA1 and HA2 domains are indicated. The receptor binding domain (RBS) sits on top of the molecule and is indicated in red.

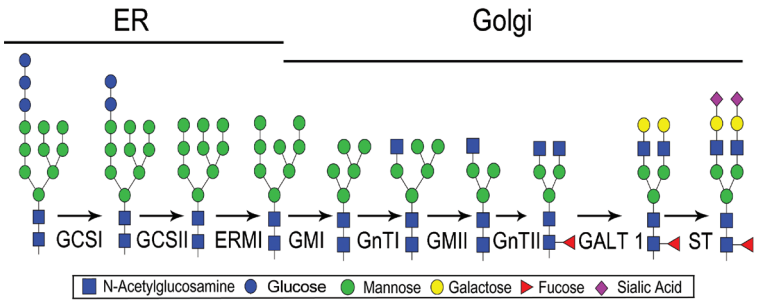

Figure 4. The glycosylation process in the ER and Golgi. After transfer of the 14-sugar $Glc_3Man_9GlcNAc_2$ glycan tree to the protein, glycosidases in the ER remove the three glucose residues (α-glucosidase I [GCSI] & α-glucosidase II [GCSII]), while the ER mannosidase I (ER-M I) removes one mannose residue. Additional mannose residues are removed in the cis compartment of the Golgi, catalyzed by Golgi-α-mannosidase I (GM I) until $Man_5GlcNAc_2$ is generated. After addition of one N-acetylglucosamine, by N-acetylglucosaminyl transferase I (GnT I), mannose residues are removed by Golgi α-mannosidase II (GM II). Further processing occurs by the action of several tranferases that add fucose or galactose (e.g. GALTI). SIAs are terminal modifications for which SIA transferases (ST) are responsible (Bertozzi and Rabuka, 2009; Kornfeld and Kornfeld, 1985).

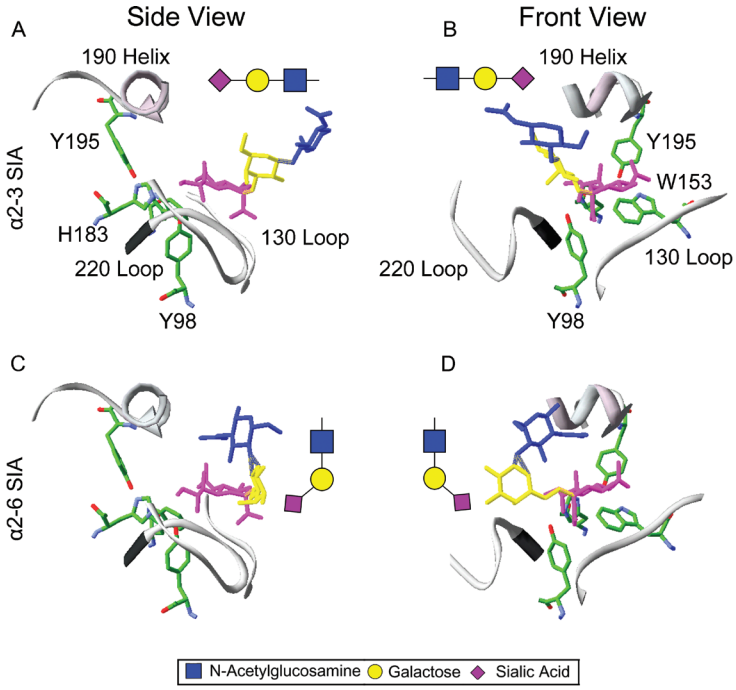

Figure 5. Structural model of the receptor binding site (RBS) in complex with a sialyl glycan. X-ray crystal structure of a trimeric H1 Hemagglutinin (A/Puerto Rico/8/30), in complex with an α2-3 (PDB: 1RVX) or α2-6 (PDB: 1RVT) SIA (Gamblin et al., 2004). The three structural elements (130 loop, 190 helix, 220 loop) of the RBS are indicated as well as the four conserved amino acids that form the base of the RBS. The side chains of the amino acids are shown in a stick representation (gray, carbon; red, oxygen; blue, nitrogen). SIA is shown in pink, the penultimate galactose in yellow, and the N-Acetylglucosamine in blue. Side (A) and front (B) view of H1 in complex with a α2-3 sialoside. Side (C) and front (D) view of H1 in complex with a α2-6 sialoside.

N-linked glycosylation is essential for proper folding, oligomerization and transport of the protein (Hanson et al., 2009). In addition, HA oligosaccharides may block antigenic sites (Munk et al., 1992; Skehel et al., 1984) and may affect proteolytic activation (Ohuchi, Ohuchi, and Matsumoto, 1999). Glycosylation of HA may also affect receptor binding as loss of N-glycan sites neighbouring the receptor binding site was shown to result in increased affinity of HA for its receptor (Ohuchi et al., 1997).

HA-receptor interactions

In 1983 the first X-ray crystal structure of HA was solved for H3 of A/Hong Kong/68/H3N2 (Wilson, Skehel, and Wiley, 1981). Since then, the X-ray crystal structure of several other HA subtypes has been solved as well, some of them in complex with SIA-containing receptors (Eisen et al., 1997; Ha et al., 2001; Ha et al., 2003; Russell et al., 2004; Stevens et al., 2006c; Yang et al., 2010). These structures provided detailed insights into the RBS of HA. The RBS has at its base four conserved amino acids (Y98, W153, H183, and Y195; H3 numbering), while the edges are formed by three structural elements, an α-helix composed by residues 190-198 (the 190-helix) and two loop structures formed by residues 133-138 (the 130-loop) and 220-229 (the 220-loop) (Skehel and Wiley, 2000). A subset of amino acids mediates binding to either α2-3- or α2-6-linked SIAs. For H1, glutamic acid and glycine residues at position 190 and 225, respectively, will confer binding to α2-3 sialosides (avian receptors). Aspartic acids at these positions will enable binding to α2-6-linked SIAs (human receptors) (Glaser et al., 2005; Tumpey et al., 2007) (Figure 5). For H2 and H3, glutamine and glycine residue at position 226 and 228, respectively, result in binding to avian receptors. Mutation of these residues to a leucine (position 226) and serine (position 228) will mediate a shift from avian to human specificity (Matrosovich et al., 2000; Stevens et al., 2006c). The same mutations also allow binding of H5 to human SIA receptors, although for this HA subtype a complete shift in receptor specificity is not observed (Stevens et al., 2006c).

A variety of assays has been used to study HA-receptor interactions. Hemagglutination assays, which measure the ability of intact viruses to agglutinate red blood cells, are used to estimate the number of virus particles. Hemagglutination inhibition assays are used to detect and quantify antibodies or receptor analogues that inhibit agglutination of red blood cells. As erythrocytes from different species are biased in their expression of either α2-3 or α2-6 linked SIA, hemagglutination may also be used to assay the specificity of SIA-receptor binding. The specificity of such assays is improved by prior removal of all endogenous SIAs followed by specific resialylation of the erythrocytes with either α2-3- or α2-6-linked SIA (Paulson and Rogers, 1987). However, while these assays reflect the overall affinity of viruses for α2-3- or α2-6-linked SIA receptors, they do not distinguish between different glycan classes and modifications of sialylated glycans.

Also solid-phase binding assays have been developed to study IAV glycan interactions (Baenziger and Fiete, 1979; Gambaryan and Matrosovich, 1992). In such a set up, the viruses are immobilized on microtiter plates, after which their binding to various (multivalent) sialylated glycans is evaluated (Gambaryan and Matrosovich, 1992; Matrosovich et al., 2000). With these assays researchers showed that binding of HA to specific glycans not only depends on the linkage of SIA to the penultimate galactose, but that also the linkage type of the galactose to the GlcNac may be of importance. For example, IAVs from galliform host species preferentially recognise α2-3 linked SIA attached to type 2 chain glycans (Gal(β1→4)GlcNAc), while IAVs from anseriformes preferred type I chain glycans (Gal(β1→3)GlcNAc) containing α2-3 linked SIAs (Gambaryan et al., 2005; Gambaryan et al., 2008). The major drawback of the solid phase binding assays is their relative low throughput and, in view of bio safety, that they are optimized for screening with whole viruses.

More recently, glycan microarray technology has been developed, which may be used to profile

the glycan specificity of a diverse range of glycan binding proteins (Blixt et al., 2004). Such arrays are created using standard robotic printing technology using amine-functionalized glycans in combination with amino-reactive glass. Binding of lectins to these arrays is monitored using fluorescently labelled antibodies, followed by image acquisition by confocal scanners (Blixt et al., 2004). The glycan arrays of the Consortium for Functional Glycomics (CFG) contains a continuously increasing number of structurally defined glycan targets that not only differ in glycan structure but also in backbone. With these arrays, one can rapidly assess virus receptor specificity and the potential emergence of human receptor-adapted viruses (Stevens et al., 2006a; Stevens et al., 2006b).

The glycan array technology is useful for the analysis of receptor binding of both whole viruses and recombinant HA (Stevens et al., 2006a). As the affinity of HA for its receptor is very low (mM range), the high valency of the virus particles results in high avidity binding (Chandrasekaran et al., 2008). However, the use of viruses also has some disadvantages as the binding of virus particles may be influenced by the activity of the viral NA, while biosafety aspects also may play a role. In addition, the growth of viruses in cell culture or eggs may result in adaptive mutations. As an alternative, recombinant HA proteins have been produced in a baculovirus expression system. In this approach, the HA ectodomain is genetically fused to a carboxy-terminal trimerization sequence (foldon) and a His tag. Pre-complexation of HA with fluorescent primary and/or secondary antibodies is needed to provide the increased valency needed to obtain detectable signals (Srinivasan et al., 2008; Wei et al., 2008).

IAV vaccines

Vaccination against influenza virus is an effective way to prevent and restrain disease. Current seasonal influenza virus vaccines consist of H1N1, an H3N2 and influenza B virus components. Most influenza virus vaccines used in the United States and Europe consist of embryonated chicken egg-grown and formaldehyde-inactivated preparations. After purification, the preparations are chemically disrupted with non-ionic detergents, resulting in split virion vaccines. In case of subunit vaccines, the split virion vaccines are purified further to remove the internal proteins leaving only HA and NA. Obviously, the manufacturing of these vaccines depends on the availability of embryonated eggs and vaccine seed strains. Thus, the selected variants need to be selected for high yields in eggs by serial passage or by reassortment with other high-yield strains. Furthermore, large numbers of eggs are needed, as in general the vaccine dose for adults corresponds approximately to the amount of purified virus obtained from 1 egg (15 μg of HA for each of the 3 HAs). Although these inactivated vaccines have demonstrated their effectiveness, the manufacturing of these vaccines is time-consuming. Furthermore, egg contaminants are a problem for part of the vaccine recipients, while the availability of eggs can become variable in a pandemic setting. In addition to the inactivated vaccines, also one cold-adapted attenuated live viral vaccine (Flumist) has been approved for use to prevent influenza. Also for this vaccine, the regularly updated vaccine strains are generated via reassortment with viruses closely related to the actually circulating ones. The vaccine strains, which are also grown on eggs, carry 6 nonsurface protein genes derived from the cold-adapted master strains and the HA and NA genes, from circulating A and B strains. They are administered by nasal spray.

Despite the efficacy of the approved influenza vaccines, there is room for new developments, including the use of new adjuvants (Vogel, 2000), and the use of reverse genetics to modify vaccine strains to increase their ease of manufacturing and handling (O'Neill and Donis, 2009). Reverse genetics also allowed the generation of alternative live attenuated viral vaccines, lacking the NS1 (Richt and Garcia-Sastre, 2009). In addition, alternative methods for the production of influenza vaccines are needed in order to replace the out-dated egg-based manufacturing process. However, these cell-based vaccines also require the generation of high yielding viruses either via reassortment

or reverse genetics (Cox, 2005).

As protection against influenza virus infection and disease correlates with serum anti-HA antibody levels (Osterhaus, Fouchier, and Rimmelzwaan, 2011), the use of heterologous vaccine vectors that express HA genes may also be an attractive approach to vaccinate against influenza (Dudek and Knipe, 2006). For example, effective immune responses have been elicited against influenza by using modified vaccinia virus Ankara (MVA) and adenovirus as vaccine vectors (Gao et al., 2006; Sutter et al., 1994). Alternatively, the use of recombinant HA proteins may also provide an appealing vaccination approach. Recombinant HA antigens can be produced using safe, quality controlled and scalable conditions without the need for virus cultivation. Furthermore, adverse reactions may be limited as the HA preparations can be highly purified an do not contain egg contaminants. In addition, recombinant HA proteins can be manufactured with short lead time, allowing an accelerated response to emerging influenza strains.

These recombinant HA proteins may be produced using different expression systems. Although recombinant proteins expressed in E. coli (Aguilar-Yanez et al., 2010) or plants (Shoji et al., 2009) were shown to induce protective immunity, expression of HA in higher eukaryotes is expected to result in superior immunogens; proper folding and trimerization of HA is known to require multiple posttranslational modifications such as glycosylation and disulfide bonding (Hanson et al., 2009). Promising results have been obtained with recombinant full-length HA proteins that are expressed with the baculovirus expression system in insect cells (Cox, Patriarca, and Treanor, 2008), even though purification of the membrane protein may affect the native structure. Furthermore as this vaccine is not adjuvanted, a relatively high amount of protein is required for protection.

Outline of the thesis

As described above, the IAV HA protein is not only the major IAV immunogen, to which antibody levels correlate with protection against infection and disease, but is also the most important determinant of virus tropism because of its role in SIA receptor binding. However, many questions remain unsolved; particularly with respect to the receptor fine specificity of HA proteins and the molecular determinants thereof. Solid phase binding assays and glycan array technology appear to be the methods of choice to study the interaction of HA with sialylated receptors in detail. In view of the apparent disadvantages of using virus preparations for such assays, we explored the feasibility of studying IAV-receptor interactions using a recombinant protein approach based on the expression of soluble trimeric HA proteins in either insect or mammalian cells. In addition, we studied to what extent HA-receptor binding is affected by the particular system used to express the recombinant soluble trimeric HA proteins, as the different cell types produce glycoproteins that carry different oligosaccharide side chains (Chapter 2).

Not much is known about the adaptations required for swine IAVs to enable efficient spread in the human population. As these mutations are likely to involve changes in the avidity and/or specificity with which different sialylated receptors are recognized by HA, we studied the receptor-binding properties of different recombinant H1 proteins derived from swine, new pandemic swine-origin and seasonal IAVs (Chapter 3).

The current IAV vaccines have several drawbacks as detailed above, which may be partly overcome by the use of recombinant proteins. In view of the efficient expression of the recombinant soluble HA proteins and the ease of their purification, we sought to determine the potential of our recombinant protein preparations to protect against influenza virus infection and disease using different animal models, including chickens, mice, pigs, and ferrets (Chapters 4, 5 and 6). In addition, we studied whether the addition of recombinant soluble oligomeric NA proteins had a beneficial effect on the

protective efficacy of our vaccine preparations (Chapter 6). Finally, we analyzed to what extent the HA-specific antibody response is affected by the expression system used to produce the HA preparations, and thus by the glycosylation state of the recombinant proteins (Chapter 7). Chapter 8 provides a summarizing discussion, in which the main results of the research described in this thesis are discussed in a broader perspective and suggestions are made for future research.

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The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity.

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Abstract

In this study we evaluated the receptor binding properties of recombinant soluble hemagglutinin (HA) trimers (subtype H2 and H7) produced in insect S2 cells, human HEK293T or HEK293S GnTI(-) cells, which produce proteins with paucimannose, complex or high-mannose N-linked glycans, respectively. The results show that HA proteins that only differ in their glycosylation status possess different receptor fine specificities. HEK293T cell-produced HA displayed a very narrow receptor specificity. However, when treated with neuraminidase this HA was able to bind more glycans with similar specificity as HEK293S GnTI(-) cell-produced HA. Insect cell-produced HA demonstrated decreased receptor specificity. As a consequence, differences in HA fine receptor specificities could not be observed with the insect cell-, but were readily detected with the HEK293S GnTI(-) cell-produced HAs.

Introduction

Influenza A viruses are enveloped, negative-strand RNA viruses with a segmented genome. They cause acute viral disease that affects a large variety of animal species, including humans, pigs, horses, and birds. Influenza A virus classification is based on the antigenic properties of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). All subtypes of HA (H1-H16) have been identified in avian influenza A viruses. Birds are therefore considered to be the reservoir from which all influenza A viruses in other species originate (Webster, et al., 1992) The HA protein is the most important determinant of virulence and host switching as it binds to sialic acid-containing cell surface receptors on epithelial cells (de, Munster et al., 2009; Shinya, et al., 2006; yora-Talavera, et al., 2009; Nicholls, et al., 2008). While avian viruses preferentially bind to sialic acids attached to the vicinal galactose via a α2-3 linkage (Connor, et al., 1994b), human viruses prefer the α2-6 linkage (Rogers, et al., 1985; Rogers & D'Souza, 1989).

HA is a homotrimeric type I transmembrane protein with an ectodomain composed of a globular head and a stem region (Ha, et al., 2001; Wilson, et al., 1981a). The sialic acid moieties of the viral receptors bind to a shallow depression at the top of the HA protein (Chandrasekaran, et al., 2008a; Stevens, et al., 2004; Wilson, et al., 1981b). HA receptor-binding affinity was found to be affected by oligosaccharide linkages adjacent to the receptor binding site (Ohuchi, et al., 1997b; Ohuchi, et al., 1997c; Gunther, et al., 1993). In addition, a recent study demonstrated that enzymatic truncation of the N-glycan structures on HA increased receptor binding affinity, while decreasing specificity towards disparate sialic acid ligands (Wang, et al., 2009e).

Cross-species transmission is an important aspect of the epidemiology of influenza A viruses. Although the requirements for and mechanisms of influenza A viruses to cross the species barrier are still poorly understood mutations in HA are known to be critical (Tumpey, et al., 2007b). At the present time, the specificity of HA-receptor interactions are most conveniently analyzed using glycan array technology (Stevens, et al., 2006c). Glycan array analyses can either be performed using intact virus preparations or recombinant soluble HA proteins. Intact virus preparations have obvious biosafety issues when dealing with unknown viruses, while the growth of viruses in cell culture or eggs may result in adaptive mutations in HA. Recombinant soluble proteins therefore provide an attractive alternative (Stevens, et al., 2006b). The production of soluble HA proteins, the trimeric state of which is essential for effective binding to sialic acid moieties can be relatively easily achieved once the sequence of the particular viral genome has been determined.

With our research we aim to determine HA-receptor interactions for various HAs, using recombinant soluble HA trimers. These trimers can be produced in different cell types, such as insect cells, by using among others the baculovirus expression system (Chandrasekaran, et al., 2008c), or mammalian cells (Wei, et al., 2008e). These different cell types produce, however, glycoproteins that carry different oligosaccharide side chains. Although insect cells are able to produce N-linked glycoproteins, the cells fail to elongate the trimmed N-glycan precursors to produce complex terminally galactosidated and/or sialylated N-glycans that are produced in mammalian cells (Kost, et al., 2005). Rather, the insect cells produce glycoproteins that carry paucimannose N-glycans, containing three mannoses. As differences in the glycosylation status of the recombinant HAs might interfere with HA-receptor interactions, we first specifically investigated this aspect. Thus, we produced soluble avian influenza A virus H2 and H7 trimers, which are predicted to contain 7 and 4 glycan side chains, respectively, using insect and mammalian cell expression systems. Two types of mammalian cells were used: HEK293T and HEK293S GnTI(-) cells (Reeves, et al., 2002b). While the former produce glycoproteins carrying complex N-glycans, glycoproteins produced in the latter contain high mannose glycans as these cells lack a functional N-Acetylglucosaminyltransferase I. The purified HA trimers were subsequently analyzed for their ability to bind sialic acid receptors using different

assays. The results show that HA-receptor interactions depend on the specific expression system used. HA trimers produced in the insect cells bind sialic acid ligands with decreased specificity when compared to the trimers produced in the HEK293S GnTI(-) cells. HEK293T cell-produced HA displayed the most narrow receptor specificity, which broadened after treatment of the protein with neuraminidase to resemble the specificity of the HEK293S GnTI(-) cell-produced HA. Differences in receptor specificity between H2 and H7 proteins, which are derived from influenza A viruses from different species of birds, could be demonstrated using recombinant HA proteins produced in HEK293S GnTI(-) cells, but not with the HA proteins made in the insect cells.

Results

Expression, purification, and characterization of the HA trimers.

As we aim to study HA-receptor interactions for a large panel of recombinant HAs, we first analyzed to what extent the specific expression system used would affect this interaction, by using H2[N8](A/Herring Gull/DE/677/88) as a model. In order to express a soluble, trimeric HA ectodomain in either insect or mammalian cells, the H2 ectodomain-coding sequence was first cloned into the appropriate expression vectors. In both plasmids used, the HA-sequence was preceded by a signal peptide-encoding sequence and followed by sequences coding for the GCN4 isoleucine-zipper trimerizaton motif (Harbury, et al., 1993a) and the Strep-tag II, the latter for purification purposes (Fig. 1A). Expression of the HA ectodomain was achieved by transient transfection of the appropriate plasmid into either HEK293T cells or HEK293S GnTI(-) cells or by the generation of stably-transformed Drosophila Schneider S2 cells. HA proteins secreted into the culture media were purified using the Strep-tag technology and subjected to gel electrophoresis followed by western blotting using an antibody directed against Strep-tag II (Fig. 1B). The results show that the HA ectodomains differ in their electrophoretic mobility depending on the expression system used. The HA protein migrated as a discrete band after expression in the insect cells or in the HEK293S GnTI(-) cells, with the insect cell-produced HA migrating slightly faster. This result is in agreement with the expected oligosacharide modifications of the proteins, as glycoproteins expressed in the GnTI(-) or the insect cells will be homogenously modified by high mannose or paucimannose N-glycans, respectively. In contrast, the HA protein produced in the HEK293T cells, which appeared as a fuzzy band, migrating slightly slower in the gel consistent with its more heterogeneous and complex N-linked glycans. However, when the HA proteins derived from the different expression systems were treated with N-Glycosidase F (PNGase F) to remove all N-linked glycans, they ran at the same position in the gel in agreement with the HA proteins, expressed in the different cells, having identical protein backbones and only differing in their N-linked glycosylation. The positions of the glycan side chains were modeled into the structure of a homologous H2 trimer (A/duck/Ontario/77; Protein Data Bank ID 2WR3) (Liu, et al., 2009), which showed that 1 N-linked glycan is present at the tip of H2 (Fig. 1E).

Next, the oligomerization state of the different HA proteins was analyzed by gel filtration column chromatography (Fig. 1C). In all three cases the bulk of the HA protein eluted with the velocity of an oligomer, presumably a trimer, while only a minor fraction was found as aggregates in the void volume. The mammalian cell-derived HA oligomers were larger than those from the insect cells, which is in agreement with their different extent of N-glycosylation, although the increase in size was greater than expected. Similar differences in gel filtration column chromatography for HAs produced in either mammalian or insect cells were also reported by Wei and coworkers (Wei, et al., 2008d). To confirm their trimeric nature, the HA preparations were subjected to blue-native gel electrophoresis followed by western blotting (Fig. 1D). When the insect-derived HA protein was heat-denatured prior to electrophoresis, three protein species appeared which very likely correspond to HA monomers, dimers and trimers, of which the fastest migrating monomeric form was most

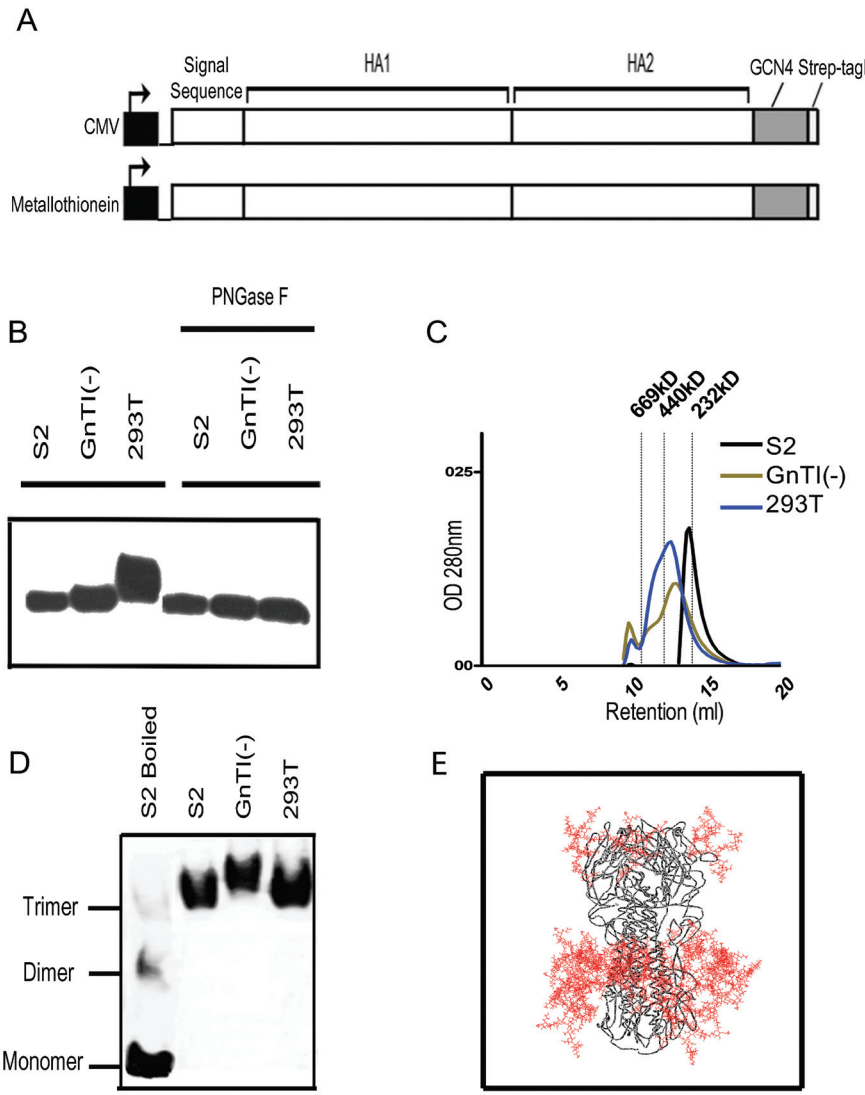


Figure 1. Expression of recombinant trimeric HA proteins. (A) Schematic representation of the HA expression cassettes used. The HA ectodomain encoding sequence was cloned in frame with DNA sequences coding for a signal sequence, the GCN4 isoleucine zipper trimerization motif and the Strep-tag II under the control of either a CMV or a methallothionein promoter for expression in mammalian or insect cells, respectively. (B) HA ectodomains expressed in insect (S2), HEK293S GnTI(-) cells [GnTI(-)] or HEK293T (293T) cells and purified from the culture media were analyzed by SDS-PAGE followed by western blotting. The recombinant proteins were detected using a mouse anti-Strep-tag antibody. When indicated samples were treated with PNGase F prior to electrophoresis. (C) Analysis of purified recombinant HA proteins by gel filtration. The elution profiles of the different HA proteins using a Superdex200GL 10-300 column are shown. The elution of a 232kDa catalase control is indicated by the dotted line. (D) Blue native-PAGE analysis of the recombinant HA proteins. The position in the gel of the momomeric, dimeric and trimeric ectodomain species observed after heating of the HA sample prior to electrophoresis is indicated. HAs subjected to gel electrophoresis without prior heating migrated at the position of the trimer. (E) Ribbon representation of the structure of H2 (A/dk/Ontario/77; Protein Data Bank ID 2WR3), N-linked glycans are displayed in red. All N-glycans are modeled by GlyProt (Bohne-Lang & von der Lieth, 2005.), while the graphics are generated by Swiss-PdbViewer (http://spdbv.vital-it.ch/).

abundant. When the HA preparations were analyzed directly, i.e. without heating, the major part of the protein migrated at the position of the trimer. In conclusion, soluble trimeric HA ectodomains, differing only in their oligosaccharide modification, were expressed in the three expression systems and easily purified.

Biological activity of the HA ectodomain trimers

The biological activity of the different HA preparations was studied using hemagglutination and solid phase-binding assays. These studies were performed with recombinant HA proteins pre-complexed with anti-Strep-tag antibody as detailed in the Materials and Methods. The results of the hemagglutination assay are shown in Fig. 2A. Clearly, the HA protein produced in the HEK293T cells was much less capable of hemagglutinating chicken red blood cells than the insect cell-derived (Fig 2A) and the HEK293S GnTI(-)-produced HA protein. No hemagglutination was observed when the red blood cells had been treated with Vibrio Cholera derived neuraminidase (VCNA), when the HA proteins had not been pre-complexed with the antibody directed against Strep-tag II, or after addition of only the antibody (data not shown). HA proteins were also evaluated in a bovine fetuin solid phase-binding assay. Fetuin is a blood glycoprotein that contains 3 N-linked and 3 O-linked sialylated glycan side-chains. Binding of HA was measured by means of the horseradish peroxidase (HRP) conjugated to the anti-Strep-tag II antibody as detailed in the Material and Methods. No binding was observed when the HA proteins had been produced without a GCN4-trimerization tag, when the proteins had not been precomplexed with antibody directed against the purification tag, when antibody was applied alone (data not shown), or when fetuin had been treated with VCNA (Fig. 2B). All trimeric HA proteins demonstrated a concentration dependent binding to fetuin. The HA proteins derived from the insect or the HEK293S GnTI(-) cells displayed a similar, much higher activity than the HA protein produced in the HEK 293T cells. Essentially similar results were obtained when the HA proteins were used to bind to wells coated with either human A549 cells or chicken DF-1 cells (Fig. 2C and D). The reduced ability of the HEK293T cell-produced HA protein to bind to sialylated substrates is in agreement with previous studies (Ohuchi, et al., 1995c; Wei, et al., 2008c; Uhlendorff, et al., 2009) which showed that removal of sialic acids from the HA protein is important for efficient substrate binding. As shown in Fig. 2E, this was also the case in our experimental set up. When the HA protein produced in the HEK293T cells was treated with VCNA prior to elution from the Strep-Tactin beads it acquired the ability to bind to sialylated substrates with an efficiency comparable to that of HA protein synthesized in the HEK293S GnTI(-) cells (Fig. 2E and data not shown). Our results demonstrate that HA trimers produced in all three expression systems are biologically active.

Sialic acid receptor binding specifcities of the different HA trimers: Glycan array analysis

HA proteins produced in the different expression systems were characterized for their oligosaccharide binding specificity by performing glycan array analyses in collaboration with the Consortium for Functional Glycomics. To this end, the different HA trimers, precomplexed with mouse antibodies directed against the Strep-tag II (2:1 molar ratio), were subjected to an array containing approximately 400 different glycans. Glycan binding by the HA trimers was monitored using a FITC-conjugated secondary anti-mouse IgG antibody. The glycan array data, including the glycan structures, can be accessed via the website of the Consortium for functional Glycomics (http://www.functionalglycomics.org/static/index.shtml; see the legends of Fig. 4 and 5 for direct links to the raw data). A summary of the results is shown in Fig. 3. The HA protein produced in the HEK293T cells exhibited considerably less glycan binding activity than its counterparts produced in the other expression systems (compare Fig 3A with 3B and 3C and see Fig. 3E), consistent with our earlier binding assays (Fig. 2.). This 293T cell-produced HA protein showed a preference for glycan structures containing 2 sialic acids. In addition to these glycans the HA trimer produced in the HEK293S GnTI(-) cells recognized many other glycans containing α2-3-linked sialic acids (Fig. 3B

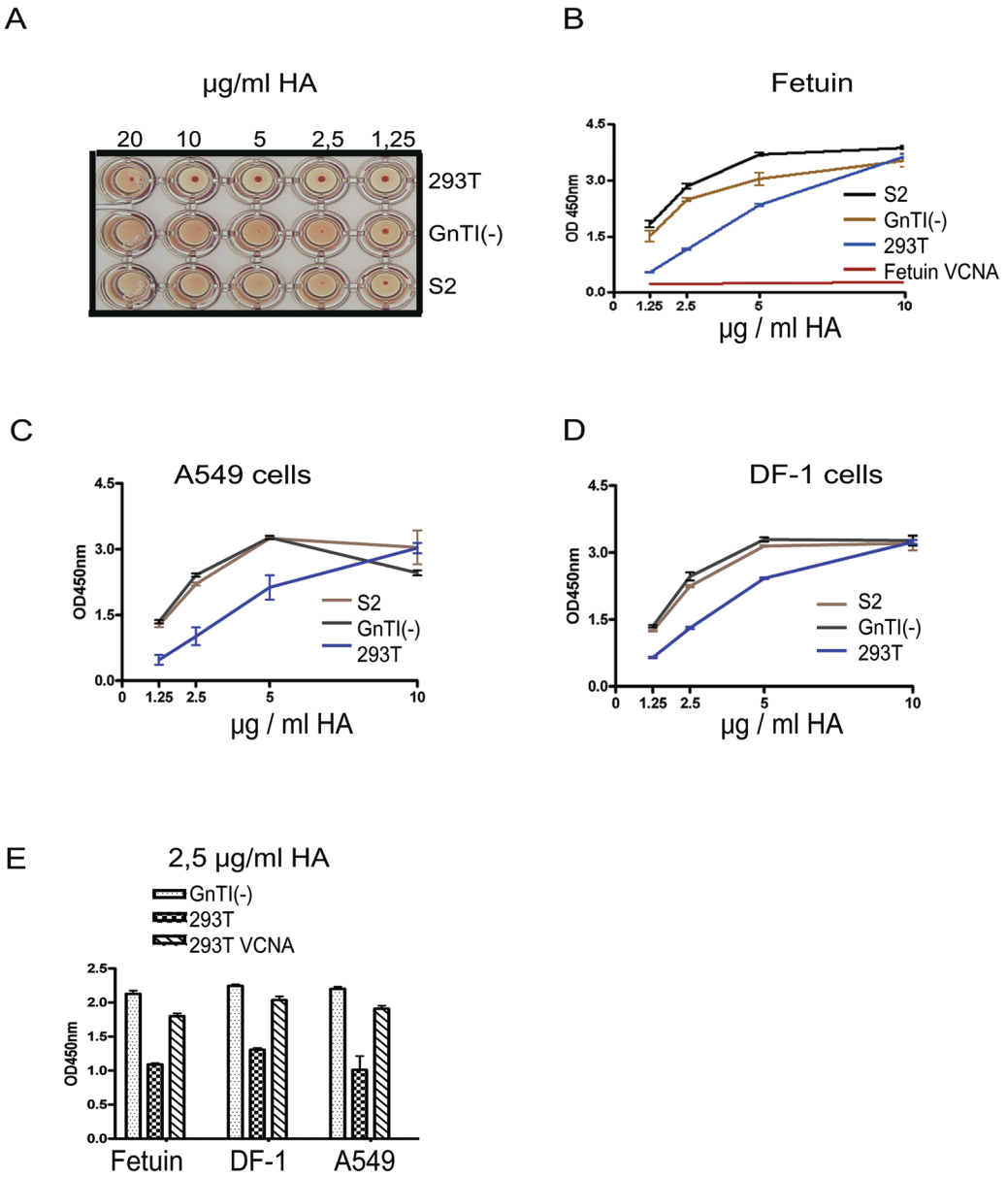


Figure 2. Binding of recombinant trimeric HA proteins to various sialic acid containing substrates. Recombinant soluble HA trimers were complexed with a HRP-conjugated, mouse antibody directed against the Strep-tag prior to their application in the different binding assays. (A) Hemagglutination of chicken red blood cells by HA protein expressed in insect (S2), HEK293S GnTI(-) [GnTI(-)] or HEK293T (293T) cells is shown at different HA concentrations as indicated. Binding of the HAs to fetuin (B, E), fetuin treated with VCNA (B), A549 cells (C, E) or DF-1 (D, E) cells was detected using TMB substrate by reading the OD at 450nm. When indicated recombinant HA protein expressed in HEK293T cells was treated with VCNA prior to the binding assay (E).

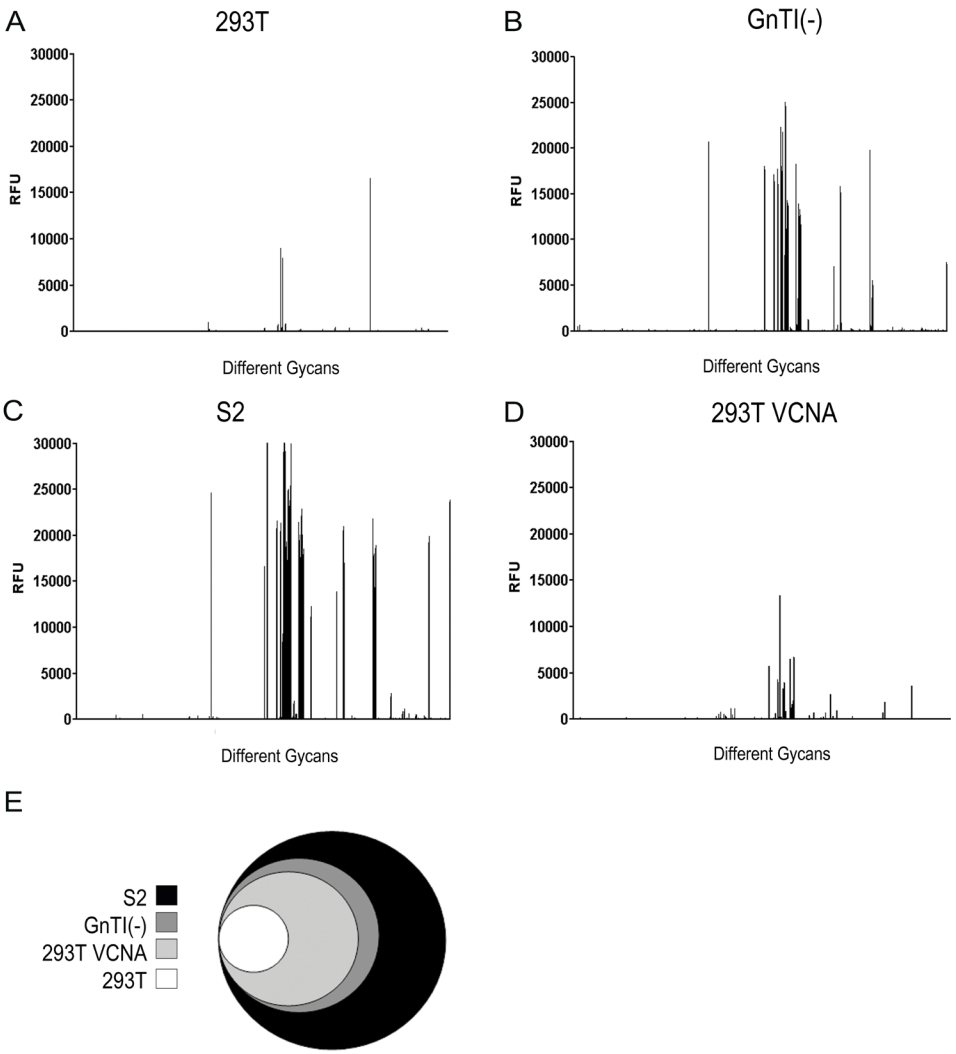


Figure 3. Glycan Array Analysis of HA trimers produced in the different expression systems. Glycan array analyses are shown for the HA proteins produced in HEK293T cells (293T; A and D), HEK293S GnTI(-) cells [GnTI(-); B] and insect cells (S2; C). (E) The HA protein was treated with VCNA prior to the analysis (293T VCNA). Glycan array 4.0 (A, B, and C) or array 4.1(D) was used. The raw data and the glycan structures can be accessed at the following websites:
A; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2622,
B; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2621,
C; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2623,
D;http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2725.
Binding of HA to different glycans was measured in relative fluorescence units (RFU) as indicated on the Y-axis. The order of the different glycans arranged on the X-axis of graphs A-D is identical. (E) A Venn-diagram demonstrates the overlaps observed in the different HA-receptor binding profiles. The surfaces of each of the ovals correspond with the number of glycans bound by the different HAs.

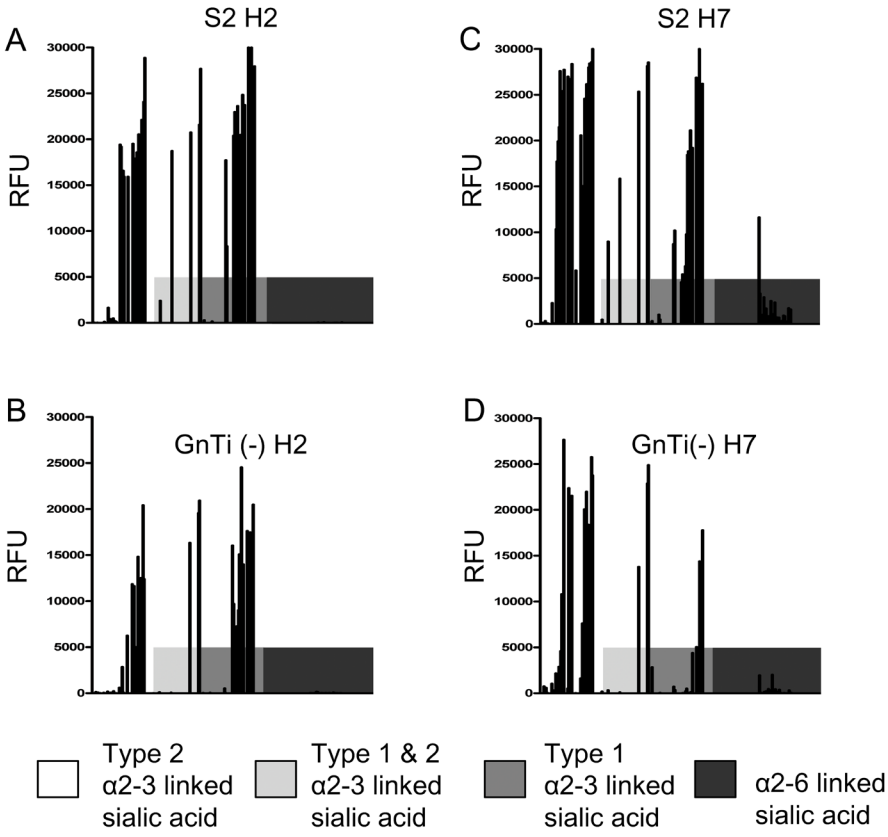
and Fig. 3E), as did the HA trimer produced in the insect cells. This latter trimer additionally bound to a variety of other glycans containing α2-3-linked sialic acids, not recognized by the HEK293S GnTI(-) derived HA. (Fig. 3C and 3E).

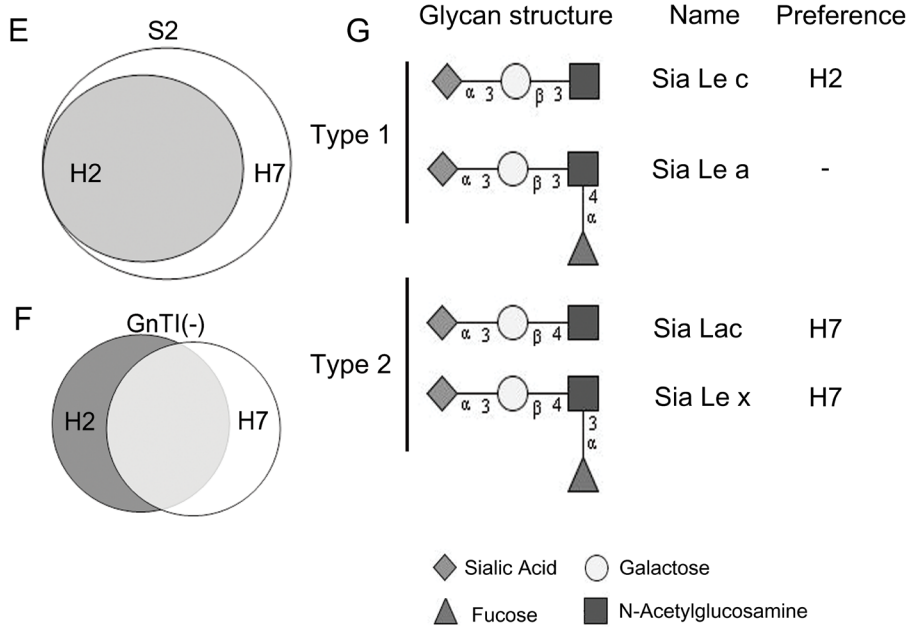
To investigate the contribution of the terminal sialic acids to the binding of complex glycosylated HA trimers, HA protein produced in HEK293T cells was treated with VCNA before applying it to the glycan array. The results are shown in Fig. 3D and E. Although generally lower signal intensities were measured for the VCNA-treated HA trimers than for the HEK293S GnTI(-) derived trimers, almost all glycan structures bound by the latter HA were also bound by the desialydated HA (Fig. 3E). In summary, our results show that trimeric HA proteins that are produced in different expression systems and hence differ only in the length of their N-glycans exhibit different receptor fine specificities. The specificity of HA-receptor binding appears to decrease with the HA protein itself carrying shorter glycan side chains (S2-HA < HEK293S GnTI(-)-HA < HEK293T-HA). HA produced in the HEK293S GnTI(-)-cells demonstrated a specificity similar to that of the VCNA-treated HA protein produced in the HEK293T cells.

Receptor binding specificities of different HA subtypes.
Finally, we studied whether HA proteins produced in the different expression systems are suitable to distinguish between HA-receptor binding fine specificities. Therefore, we compared the glycan array binding profile of the H2[N8] (A/Herring Gull/DE/677/88), which we used so far, with that of an other HA subtype derived from an influenza A virus isolated from a different bird species: H7[N2] (A/turkey/NY/4450-5/94). Also this HA protein carries a N-glycan at the tip of the molecule. H2 and H7 are expected to have similar but different receptor fine specificities. The soluble trimeric H7 ectodomain was expressed in HEK293S GnTI(-) and insect S2 cells and purified as described above. The trimeric nature of the soluble ectodomain was confirmed by gel filtration and blue native gel electrophoresis, while its ability to bind sialylated substrates was confirmed by solid phase binding assays (data not shown). Next, glycan array binding profiles were generated, the detailed results of which can be found on the website of the Consortium for functional Glycomics (http://www.functionalglycomics.org/static/index.shtml). The results are compiled in Fig. 4 and 5. As for the H2 protein, more sialic acid-containing ligands were recognized by the H7 protein produced in the insect cells than by that derived from the HEK293S GnTI(-) cells (compare Fig. 4A with B and C with D).

Comparison of the receptor fine specificities of the two different HA proteins revealed that all glycans recognized by H2 were also bound by H7, provided the proteins had been produced in the insect cells (compare Fig. 4A with C and see Fig. 4E). However, when produced in the HEK293S GnTI(-) cells, the two proteins had clearly different receptor fine specificities, though many ligands were still bound by both HAs (compare Fig. 4B with D and see Fig. 4F). In general, it appeared that H2 produced in HEK293S GnTI(-) cells preferred binding to type 1 chain glycans [Gal(β1→3)GlcNAc] containing α2-3-linked sialic acids, except when these glycans were fucosylated, while HEK293S GnTI(-) cell-produced H7 preferred binding to α2-3-sialylated type 2 chain glycans [Gal(β1→4)GlcNAc]. The core glycan structure of type 1 and 2 chain glycans, containing α2-3 linked sialic acid, is shown in Fig. 4G.

This observation was confirmed by comparing the relative binding strength of H2 and H7 produced in HEK293S GnTI(-) cells to type 1 and type 2 glycans. To this end the ratio of the fluorescence intensities of H2 and H7 (measured in RFU), were determined and plotted as in Fig. 5A. The results show that type 1 chain glycans were preferentially bound by H2 (value > 1), while H7 preferred binding to type 2 chain glycans (value < 1). When the same procedure was applied to the insect cell derived HA proteins carrying paucimannose glycans (Fig. 5B), the large majority of the type 1


A
S2 H2
RFU
30000
25000
20000
15000
10000
5000
0
C
S2 H7
B
GnTi (-) H2
D
GnTi(-) H7
Type 2
α2-3 linked
sialic acid
Type 1 & 2
α2-3 linked
sialic acid
Type 1
α2-3 linked
sialic acid
α2-6 linked
sialic acid


E
S2
H2
H7
F
GnTI(-)
H2
H7
G
Glycan structure
Name
Preference
Type 1
Sia Le c
H2
Sia Le a
-
Type 2
Sia Lac
H7
Sia Le x
H7
α 3
β 3
β 4
4
3
α
Sialic Acid
Galactose
Fucose
N-Acetylglucosamine

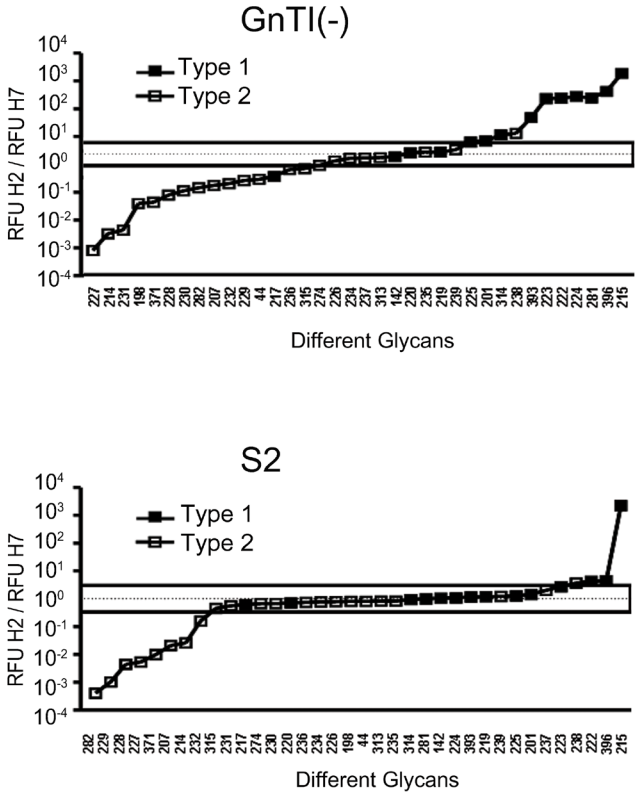


Figure 5. Relative binding of H2 and H7 to type 1 and type 2 chain glycans.
The ratio of the relative fluorescence units (RFU), which are a measure for the binding of the HAs to the different sugars, of H2 and H7 for type 1 and 2 chain glycans (on the x-axis) is plotted. Only those type 1 and type 2 chain glycans were included that showed significant binding by either insect cell-produced H2 or H7 (> 4,000 RFU). Type 1 and 2 chain glycans are indicated by black and white boxes, respectively. A ratio higher than 1 indicates preferred binding by H2, while a ratio smaller than 1 indicates preferred binding by H7. The large rectangles mark the ratios ranging between values 3 and 0.33. The dotted line indicates a ratio of 1. The ratios are shown for HA proteins expressed in HEK293S GnTI(-) (A) or insect S2 (B) cells.

Figure 4. Fine specificity analysis of insect and mammalian cell produced HA proteins.
Glycan array analyses are shown for the H2 (A and B) and H7 (C and D) proteins produced in insect cells (S2; A and C) or HEK293S GnTI(-) cells [GnTI(-); B and D]. The raw data for H7 and the structures of the bound glycans can be accessed at the following websites: C; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2618 and D; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2620. Graphs A-D show binding of HA to sialidated glycans on Glycan array 4.0 (in relative fluorescence units [RFU]). On the X-axis the sialidated glycans are grouped according to the α2-3 or α2-6 linkage of the sialic acid to the vicinal galactose. Glycans containing α2-3 linked sialic acids are divided further into type 1 or type 2 chain glycans. Glycans that contain both type 1 and type 2 chains are also indicated. (E-F) Venn diagrams demonstrate the overlap observed in the HA-glycan array 4.0 binding profiles of H2 and H7 expressed either in insect S2 cells (E) or in HEK293S GnTI(-) cells (F). (G) Schematic structures of the type I chain glycans Sialyl Lewis c (Sia Le c) and Siayl Lewis a (Sia Le a) and the type II chain glycans Sialyl Lewis x (Sia Le x) and Sialyl lactosamine (Sia Lac) are shown. H2 or H7 indicates the preferred binding of these HAs to these different sugars, while – indicates the absence of HA binding

or 2 chain glycans appeared to be bound by H2 and H7 with similar efficiency. However, as only a single concentration of the HAs was applied to the glycan arrays, we can not excluded that at lower concentrations similar differences in receptor fine specificities can be observed for the insect cell-produced H2 and H7. In summary, differences in receptor fine specificities between different HAs could most readily be observed with recombinant soluble trimeric HA ectodomains produced in HEK293S GnTI(-) cells.

Discussion

One of the key factors in the epidemiology of influenza A viruses is their adaptability to different sialic acid receptors occurring at the cell surfaces of different species. It is now well established that this adaptive potential most often is caused by subtle mutations in the viral glycoproteins, providing the virus with improved binding properties in its new host. Much less is known about the possible influence on these binding properties of the glycans attached to the viral surface glycoproteins, though previous reports support the significance of such effects (Ohuchi, et al., 1995b; Wang, et al., 2009d; Wei, et al., 2008b). Influenza A virus HAs, which constitute the receptor binding structures, generally carry some 2 to 8 oligosaccharides side chains distributed across the trimeric ectodomain complex (Vigerust, et al., 2007), some of them located close to the sialic acid receptor binding surface depression. With the aim to study the influence of the HA glycosylation states on receptor binding and to develop tools for convenient analysis of binding specificities of different HAs, we established expression systems for the production of soluble, variously glycosylated trimeric HAs. The resulting HAs, identical in their protein structure but differing in their glycan make-up, allowed us to compare the receptor binding specificities of HAs with 4 different glycosylation states.

The HA proteins produced in HEK293T cells demonstrated only very limited receptor binding as demonstrated in the hemagglutination assay, solid phase binding assays and the glycan array profiling. In agreement with previous studies (Ohuchi, et al., 1995a; Wang, et al., 2009c; Wei, et al., 2008a), removal of sialic acids from the HA protein itself was important for efficient receptor binding. The sialic acid-containing oligosaccharide may interfere with the accessibility of the receptor binding site by steric hindrance or by electrostatic repulsion. Alternatively, HA-bound sialic acid may completely fill the receptor pocket of another HA molecule (HA-HA interaction). Indeed, both the H2 and the H7 subtypes used in this study carry an N-linked glycan at their tip, which may be bound to or interfere with the accessibility of the receptor binding site of the neighboring monomer. Either way, the HA proteins containing sialylated N-glycans were only able to bind to di-sialylated glycan structures (Fig. 3 and data not shown). Apparently, only di-sialylated glycan structures are able to obviate the steric hindrance or the electric repulsion caused by the sialylated glycans on HA itself, or alternatively to effectively compete with the HA-glycans for binding with the receptor pocket.

Influenza A viruses normally contain HA proteins that carry complex glycans that are desialylated by the viral neuraminidase. To mimic this HA glycosylation status, we removed the sialic acids from the HA protein produced in HEK293T cells. This desialylated HA protein revealed a similar receptor specificity as when the HA protein was produced in the HEK293S GnTI(-)-cells. These observations are consistent with a recent study (Wang, et al., 2009a), which demonstrated that the association constants and calculated free energy changes of H5, expressed either in HEK293S GnTI(-) or in HEK293T cells followed by neuraminidase treatment, were very similar when binding to sialylated substrates. From these results we assume that HA produced in the HEK293S GnTI(-) cells display similar receptor specificity as HA expressed in virus-infected cells, although desialylated HA probably mimics the natural situation most.

Our results show that receptor binding specificity of influenza A virus HA is not only affected by the presence or absence of sialylated glycans, but also by glycan length. HAs produced in the insect

cells, which carry paucimannose glycans exhibited decreased receptor specificity, when compared to the proteins expressed in the HEK293S GnTI(-) cells, which contain high mannose oligosaccharides. Consistent with our results, enzymatic trimming of N-glycan structures on HA was shown to increase receptor affinity, while decreasing the specificity (Wang, et al., 2009b). Complete elimination of the glycan side chains either by treatment with PNGase F or by disruption of the N-linked glycosylation consensus sequence increased the affinity of HA for its receptor to such an extent that release by neuraminidase was severely impeded (Ohuchi, et al., 1997a). The different HAs used in these studies (Ohuchi, et al., 1997a; Wang, et al., 2009; this study) all carry N-linked glycosylation-sites at the top of the HA molecule. Therefore, the length of the HA glycans is likely to also affect the receptor binding specificity of other HAs with N-linked side chains at their tip.

The decreased specificity of the insect cell-produced HA probably makes this protein less suitable to study receptor fine specificities than HA produced in the GnTI-lacking cells. This notion was supported by comparing the glycan array binding profiles of H2 and H7 derived from influenza A virus from herring gull and turkey, respectively. These HAs were expected to exhibit comparable, but different receptor binding specificities. While both HAs demonstrated clear preference for binding to sialic acids attached to the vicinal galactose via a α2-3 linkage (Neu5Acα2-3Gal), regardless of the expression system used, differences in fine receptor specificity could not be observed with the insect cell-, but were readily detected with the HEK293S GnTI(-) cell-produced HA.

The comparison of the H2 and H7 glycan array profiles demonstrated that binding of HA is not only determined by the linkage of the terminal sialic acid to the vicinal galactose, but is also affected by the linkage of the galactose to N-acetylglucosamine. Thus, while H2, derived from a herring gull-virus, preferred binding to type I chain glycans (Neu5Acα2-3Galβ1-3GlcNAc), H7, derived from a turkey-virus, more readily bound to type 2 chain glycans (Neu5Acα2-3Galβ1-4GlcNAc). Furthermore, H2 did not bind fucosylated type I chain glycans, which might be explained by steric hindrance of the fucose moiety. Our results are consistent with the results of Gambaryan and coworkers who showed that influenza viruses isolated from distinct species of aquatic and terrestrial birds may differ in their fine receptor-binding specificity by recognizing the structure of the inner parts of the Neu5Acα2-3Gal-terminating receptors (Gambaryan, et al., 2005; Gambaryan, et al., 2008).

Successful cross-species transmission of influenza A virus usually involves alterations in the HA receptor specificity, allowing the virus to replicate efficiently within and spread efficiently between its new host. (Chandrasekaran, et al., 2008b; Connor, et al., 1994a; Kuiken, et al., 2006). Glycan array technologies may aid in the surveillance of animal viruses by rapidly assessing HA receptor specificity in fine detail (Childs, et al., 2009; Tumpey, et al., 2007a). The production of recombinant HAs may be very useful for these analyses, as it obviates the use of potentially dangerous viruses, while in addition receptor adaptation of cell culture- or egg-grown viruses is prevented (Wang, et al., 2006; Widjaja, et al., 2006). In this study we evaluated the effect of producing recombinant HAs in different expression systems on receptor binding specificity. Our results show that the expression system used, and hence the glycosylation status of the recombinant protein, affects the receptor fine specificity of HA.

Material and Methods

Genes and expression vectors. pCIneo plasmid containing full-length HA of A/Herring Gull/DE/677/88 H2 (GenBank accession no. AAA43096) and A/Turkey/NY/4450/94 H7 (GenBank accession no. AAD26925) were a kind gift of Dr D.L. Suarez (Lee, et al., 2006) Based on H3 numbering (Wilson, et al.), cDNAs corresponding to residues 18 to 523 were produced by PCR, isolated and cloned into the pGEMTeasy vector (Promega, USA). The HA ectodomain encoding

cDNAs were subsequently cloned into the pCD5 vector for efficient expression in mammalian cells (Zeng, et al., 2008) or into the pMT-Bip vector (Invitrogen) for expression in Schneider S2 cells (Kim, et al., 2009). Both vectors were modified such that the HA-encoding cDNAs were cloned in frame with DNA sequences coding for a signal sequence, an artificial GCN4 isoleucine zipper trimerization motif (RMKQIEDKIEEIESKQKKIENEIARIKK) (Harbury, et al., 1993b)and the Strep-tag II (WSHPQFEK; IBA, Germany).

Protein expression and purification. pCD5 expression vectors containing the HA ectodomain-encoding sequence were transfected into HEK293T and HEK293S GnTI(-)cells (Reeves, et al., 2002a) using polyethyleneimine I (PEI) in a 1:5 ratio (µg DNA: µg PEI). At 6 h post transfection, the transfection mixture was replaced by 293 SFM II expression medium (Invitrogen), supplemented with sodium bicarbonate (3.7 g/liter), glucose (2.0 g/liter), Primatone RL-UF (3.0 g/liter), penicillin (100 units/ml), Streptomycin (100 µg/ml), glutaMAX (Gibco), and 1,5% DMSO. Tissue culture supernatants were harvested 5–6 days post transfection. HA proteins were purified using Strep-Tactin sepharose beads according to the manufacturer's instructions (IBA, Germany). When indicated, HA trimers bound to Strep-Tactin beads were treated with VCNA for 3hrs at 37°C (2µU/ml), followed by three washing steps prior to elution of the protein from the beads. Drosophila Schneider S2 cells were co-transfected with pMT-Bip vector encoding the HA ectodomain and pCoBlast using Cellfectin in a 19:1 ratio, and stable cell lines were selected according to manufacturer's protocols (Invitrogen), using blasticidine selection. Cells were kept under blasticidine pressure and cultured in serum-free Insect Xpress Medium (Lonza, Belgium) at 28ºC. Protein expression was induced with CuSO4 (500µM). Culture supernatants were harvested after 10 days. HA proteins were purified as described above. HA protein expression and purification was confirmed by SDS-PAGE followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). When indicated, the HA proteins were treated with PNGase F (NEB) prior to electrophoresis according to the manufacturer's procedures in order to remove N-linked oligosaccharides. Oligomerization status of the HA proteins was determined by analyzing the elution profile using a Superdex200GL 10-300 column (GE Healthcare) and by blue native-PAGE analysis.

Biological characterization of recombinant HAs. Functionality of the HAs was assessed using different solid phase assays as well as by analyzing the ability of the recombinant HAs to hemagglutinate chicken erythrocytes. For the fetuin binding assay, 100µg/ml fetuin per well was used to coat 96-well nunc maxisorp plates. Recombinant HA was pre-complexed with HRP-linked anti-Strep-tag antibody (2:1 molar ratio) for 30 min at 0 ºC prior to incubation of limiting dilutions on the fetuin-coated plates (60 min, room temperature [RT]). HA-binding was subsequently detected using tetramethylbenzidine substrate (BioFX) in an ELISA reader (EL-808 [BioTEK]), reading the OD at 450nm. In the CellELISA assays, essentially the same protocol was used, with the exception that DF-1 and A549 (10^5 cells per well) were cultured in a 96 well plate, for 16 h at 37ºC and fixed with 4% paraformaldehyde solution in Phosphate Buffered Saline (PBS) rather than coating the wells with fetuin. The hemagglutination assay was performed according to standard methods, briefly; 2-fold dilutions of the HA-anti-Strep-tag antibody complex were incubated with 0.5% chicken erythrocytes for 30 min, at RT.

Glycan array analysis. Microarrays were printed as described (Blixt, et al., 2004b). The glycan array analysis of the HA proteins was performed as previously described (Stevens, et al., 2006a). Briefly 200 µg/ml recombinant HA was pre-complexed with HRP-linked anti-Strep-tag antibody (2:1 molar ratio) for 30 min at 0 ºC prior to incubation for 60 min on the microarray slide under a microscope cover-glass in a humidified chamber at RT. Microarray slides were subsequently washed by successive rinses in PBS with 0.05%Tween and overlaid with a AlexaFluor 488 anti-Mouse Ab. After repeated washes with PBS with 0.05%Tween, PBS, and deionized water the slides were

immediately subjected to imaging as described (Blixt, et al., 2004a).

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Only two residues are responsible for the dramatic difference in receptor binding between swine and new pandemic H1 hemagglutinin

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Abstract

In view of its critical role in influenza A virus (IAV) tropism and pathogenesis we evaluated the receptor binding properties of hemagglutinin (HA) proteins of the closely related swine and new pandemic human IAVs. We generated recombinant soluble trimeric H1 ectodomains of several IAVs and analyzed their sialic acid binding properties using fetuin-binding and glycan array analysis. The results show that closely related swine and new pandemic H1 proteins differ dramatically in their ability to bind these receptors. While new pandemic H1 protein exhibited hardly any binding, swine H1 bound efficiently to a number of α2-6-linked sialyl glycans. The responsible amino acids were identified by analyzing chimeric H1 proteins and by performing systematic site-directed mutagenesis of swine and new pandemic human H1 proteins. The difference was found to map to residues at position 200 and 227. While substitution of either residue significantly affected the binding phenotype, substitution of both was found to act synergistically and reverse the phenotype almost completely. Modeling of the T200A and E227A substitutions into the crystal structure of the new pandemic human H1 protein revealed the loss of potential hydrogen bond formation with Q191, which is part of the 190-loop of the receptor binding site, and with the penultimate galactose, respectively. Thus, a residue not belonging to the receptor binding site may affect the interaction of HA with its receptor. Interestingly, while alanine at position 200 is found in most new pandemic human viruses, the residue at position 227 in these viruses is invariably a glutamic acid.

Introduction

All influenza A virus (IAV) pandemics known so far originated from avian or swine IAV strains that managed to cross the species barrier to humans and acquire the capacity of human to human transmission. The most recent example of this was the new H1N1 swine-origin IAV that emerged in 2009 and rapidly spread around the world (Dawood et al., 2009; Ilyushina et al., 2010). The specificity of the interaction of hemagglutinin (HA) with sialic acid (SIA), the cellular receptor, largely explains the host range of IAVs (Taubenberger and Kash, 2010). Thus, viruses that infect humans bind preferentially to SIA linked to the penultimate galactose in a α2-6 configuration, while avian viruses prefer binding to SIA with α2-3 linkages (Connor et al., 1994). However, the adaptations in HA required for swine IAVs to become infectious for humans and to establish themselves in the human population are much less characterized.

The HA receptor binding site (RBS) is formed by three structural elements at the tip of the HA molecule, an α helix composed by residues 190-198 (the 190-helix) and two loop structures formed by residues 133-138 (the 130-loop) and 220-229 (the 220-loop). Four conserved residues, comprising Y98, W153 and H183 and Y195, form the base of the RBS (Skehel and Wiley, 2000). The amino acid residues in the RBS that are critical for the recognition of either avian or human receptors have been well characterized (Connor et al., 1994; Matrosovich et al., 2000; Naeve, Hinshaw, and Webster, 1984). For H1, glutamic acid and glycine residues at positions 190 and 225, respectively, result in binding to avian SIA receptors, while H1 proteins that carry aspartic acid residues at these positions interact with human SIA receptors (Glaser et al., 2005; Stevens et al., 2006a; Stevens et al., 2006b; Tumpey et al., 2007). For H2 and H3, mutations of glutamine and glycine residues at position 226 and 228 to leucine and serine, respectively, correlate with a shift from avian to human receptor specificity (Matrosovich et al., 2000). The same mutations also allow binding of H5 to human SIA receptors (Stevens et al., 2006b).

Besides birds, also pigs serve as a reservoir of new human IAVs. As pigs carry cell surface receptors for both avian and human IAVs, they may act as intermediate hosts or mixing vessels for the generation of IAV reassortants that can be transmitted to humans (Ito et al., 1998; Ma, Kahn, and Richt, 2008), as was recently demonstrated by the new H1N1 swine-origin IAV (Dawood et al., 2009). The swine-origin H1N1 virus is a reassortant with at least three parents. Six of the segments, including the one coding for HA, are closest in sequence to those of H1N2 'triple-reassortant' IAVs isolated from pigs in North America. The remaining two segments (for NA and M1) are from different Eurasian 'avian-like' viruses of pigs (Smith et al., 2009). Not much, however, is known about the adaptations required for swine IAVs to overcome barriers of cross-species transmission. While mutations in the IAV polymerase can enable such transmissions (Mehle and Doudna, 2009; Neumann and Kawaoka, 2006), efficient spread in the human population is very likely to also involve adaptive mutations in HA. These may involve changes in the binding preference of HA - from α2-3- to α2-6-sialyl glycans - as well as, for swine viruses already having an α2-6 preference, more subtle changes affecting the avidity and/or specificity with which the diverse spectrum of different α2-6-linked SIA receptors are recognized (Gambaryan et al., 2005; Nicholls et al., 2008).

In view of the important role of HA in host range tropism and pathogenesis, we decided to study and compare the receptor binding properties of the H1 proteins of closely related swine and new pandemic swine-origin IAVs. To this end we generated recombinant soluble trimeric H1 ectodomains of several IAVs and analyzed their sialic acid binding properties using fetuin solid phase and hemagglutination assays and glycan array analysis. The results show that closely related swine and new pandemic swine-origin H1 proteins differ dramatically in their ability to bind these receptors. While hardly any binding could be observed for the new pandemic swine-origin H1,

the swine H1 protein bound efficiently to several α2-6 SIA-containing glycans. The amino acid residues responsible for the difference in receptor binding were identified by analyzing chimeric H1 proteins as well as by performing systematic site directed mutagenesis of swine and new pandemic swine-origin H1 proteins. Molecular modeling of the responsible mutations (T200A and E227A) demonstrated the loss of two potential hydrogen bond interactions with Q191 in the 190-helix and with the penultimate galactose, respectively, thereby providing a rationale for the observed differences in H1-receptor binding.

Experimental Procedures

Genes, expression vectors, protein expression and purification. Codon optimized H1 encoding cDNAs (Genscript, USA) of A/Cal/04/09 (Accession; FJ966082; referred to as swine-origin H1), A/Swine/Ohio/01 (Accession; AF455675; referred to as swine H1), A/Kentucky/07 (Accession; CY028163; referred to as seasonal H1), A/Puerto Rico/8/34 (Accession; NP_040980.1; referred to as PR8 H1) and A/Duck/NZL/76 (Accession; ABB20429.1; referred to as duck H1) were cloned into the pCD5 expression as described previously (de Vries et al., 2010). The swine-origin IAV A/Cal/04/09 replicates efficiently in cell culture and has been shown to replicate in and transmit among guinea pigs with similar efficiency to that of a seasonal H3N2 influenza virus (Steel et al., 2010). Site directed mutagenesis of the H1-encoding sequences was performed with the Quickchange Site Directed Mutagenesis Kit (Stratagene CA). Chimeric HA expression plasmids were generated by conventional cloning using *SgrA*I, *Age*I, *Nhe*I and *Pac*I restriction enzymes (S2). The HA proteins were expressed in HEK293S GnT1(-) cells and purified from the cell culture supernatants as described previously .

HA-receptor binding assays. Binding of HA to fetuin was assessed using a fetuin solid phase binding assay similarly as described previously (Cornelissen et al., 2010; de Vries et al., 2010). Briefly, purified, soluble trimeric H1 was pre-complexed with horseradish peroxidase (HRP)-linked anti-Strep-tag mouse antibody and with HRP-linked anti-mouse IgG (4:2:1 molar ratio) prior to incubation of limiting dilutions on the fetuin-coated (1 µg/ml fetuin per well) 96-well Nunc MaxiSorp plates. HA-binding was subsequently detected using tetramethylbenzidine substrate (BioFX) in an ELISA reader (EL-808 [BioTEK]), reading the OD at 450 nm. Hemagglutination assays were performed with 0.5% chicken or human erythrocytes using HA pre-complexed as described previously (de Vries et al., 2010) at a starting concentration of 10µg/ml.

Glycan array analyses. Glycan array analysis of the HA proteins was performed by the Core H of the Consortium for Functional Glycomics similarly as described previously (Blixt et al., 2004; de Vries et al., 2010).

Modeling. 3D crystal structures of swine-origin H1 (PDB Accession number 3LZG (Xu et al., 2010)) and of the H1 protein from A/Swine/Iowa/30 (PDB 1RV0, in binary complex with an α2-6 linked SIA trisacharide (6LSTA) (Gamblin et al., 2004)) were downloaded from the PDB database. Substitutions T200A and E227A were modeled on the Cal/04/09 structure using Swissmodel (Schwede et al., 2003). Subsequent energy minimizations were not necessary as inspection of the modeled structure by GROMOS revealed no unfavorable energy interactions. Next, the Cα-backbone atoms of residues lining the receptor bindingsite of Swine/Iowa/30 were superpositioned with the corresponding atoms of the modeled Cal/04/09 (mutant) H1 protein. The RMS deviation of the superpositioned atoms was smaller than 1,02 Å allowing the SIA receptor to be copied from the Swine/Iowa/30 structure into the Cal/04/09 structures. Molecular interactions were further examined using the Swiss-pdb viewer (Guex and Peitsch, 1997).

Results

The H1 proteins of swine and new pandemic IAVs differ in their receptor binding properties

In a previous study (de Vries et al., 2010) we showed that recombinant soluble trimeric HA proteins produced in mammalian cells are excellent tools for performing HA-receptor binding studies. Here we exploited this approach to study and compare receptor binding properties of the H1 proteins of a new pandemic swine-origin H1N1 virus (swine-origin H1) and of a triple-reassortant swine H1N2 virus (swine H1). The HA gene segment of the swine-origin H1N1 virus is closest in sequence to those of the swine H1N2 'triple-reassortant' IAVs, with the particular swine H1 protein used in this study being the first hit in a protein blast search. The selected swine-origin and swine H1 proteins differ only at 26 amino acid residues in their ectodomains. As controls, soluble trimeric H1 proteins of a human seasonal H1N1 virus (seasonal H1), of IAV Duck/NZL/76/H1N3 (duck H1) and of IAV Puerto Rico/8/34/H1N1 (PR8 H1) were taken along. An alignment of these different H1 proteins is shown in supplementary Fig. S1. The purified proteins were first tested for their ability to bind fetuin (Fig 1). Fetuin is a blood glycoprotein with mono-, bi- and tri-antennary glycans containing α2-3 and α2-6 SIA in a 2:1 ratio (Baenziger and Fiete, 1979; Rogers et al., 1985; Spiro and Bhoyroo, 1974). Binding to fetuin by swine-origin H1 was hardly detectable. In comparison, swine and duck H1 exhibited strong binding to fetuin while seasonal and PR8 H1 displayed intermediate binding efficiency. The different binding properties of the swine and swine-origin H1 proteins were confirmed by hemagglutination assays using either chicken or human erythrocytes (supplementary Fig. S2).

Identification of the responsible residues

In order to identify the residues responsible for their different SIA-binding properties, a set of chimeric HA proteins was generated. In the first two, the HA1 domains were exchanged (Fig. 2A). Clearly, only the chimeric protein with the HA1 domain derived from the swine H1 (SO/S-NA) was able to efficiently bind to fetuin (Fig. 2B-C); the inverse chimeric protein (SO/S-AP) did not exhibit binding just like the swine-origin H1 protein. Additional chimeric proteins (Fig. 2D) were generated to narrow down the HA1 region responsible for the receptor binding difference. Chimeric proteins carrying the amino terminus of the swine or the swine-origin H1 protein (SO/S-NS and SO/S-SP proteins, respectively) were not affected in their (in)ability to bind fetuin (Fig. 2E-F). In contrast, exchanging the C-terminal HA1 region clearly affected the fetuin-binding capacity of the resulting H1 proteins (Fig. 2E-F). Thus, the chimeric protein, in which this region was derived from the swine H1 (SO/S-SA), demonstrated efficient fetuin binding, while the opposite was observed for the reciprocal chimera (SO/S-NS-AP) (Fig. 2E-F).

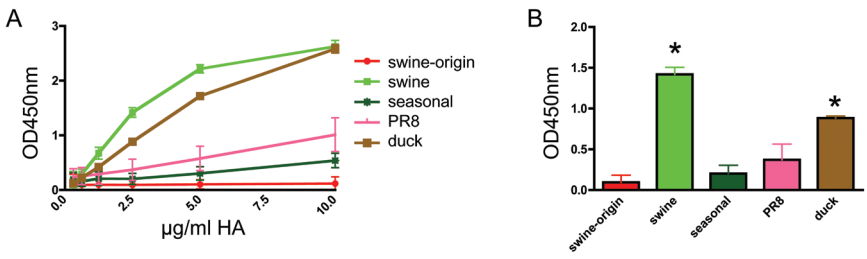

Fig. 1. Fetuin binding of recombinant soluble trimeric H1 proteins. (A) Limiting dilutions of recombinant soluble H1 trimers, complexed with HRP-conjugated antibodies, were applied in the fetuin binding assay. (B) Bar graph of the HA-fetuin binding at a HA concentration of 2.5 µg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine-origin H1 ($P<0.05$; Student's t-test).

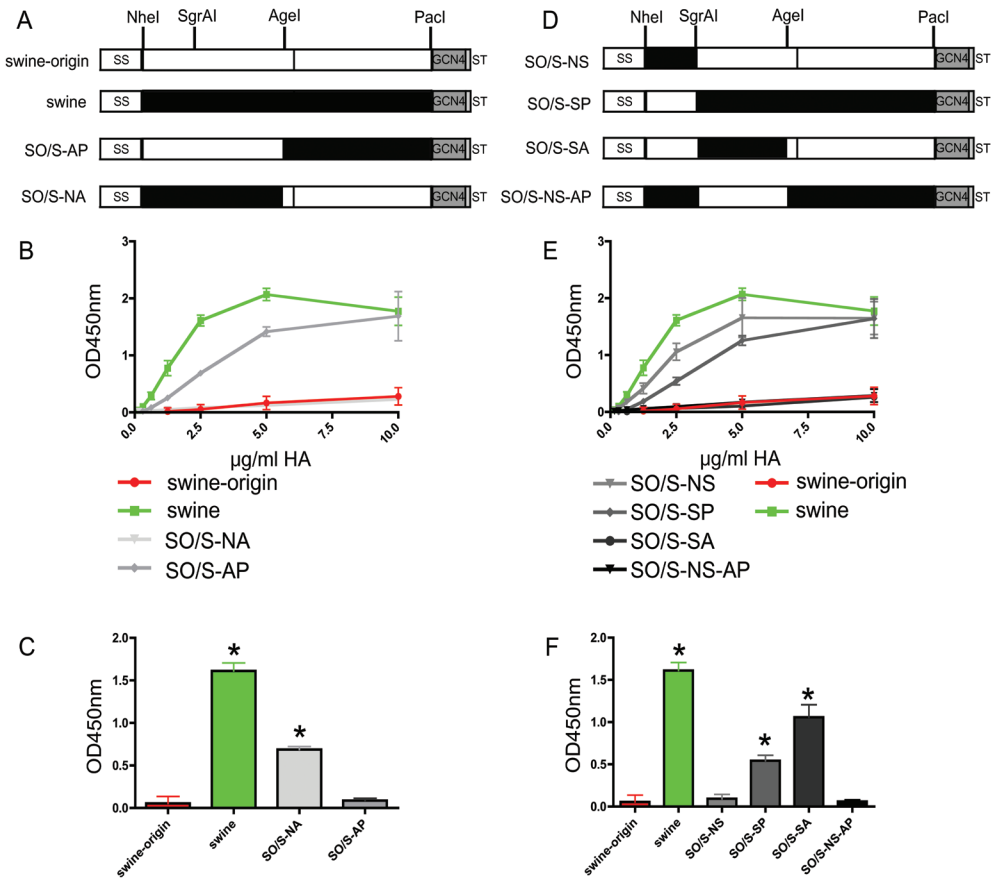

Fig. 2. Fetuin binding of chimeric H1 proteins. (A and D) Schematic representation of the soluble trimeric swine-origin and swine H1 proteins and chimers thereof. The HA ectodomains are preceded by a signal sequence (SS) and contain a GCN4 trimerization motif (GCN4) and the Strep-tagII (ST) at their carboxy terminus. The positions in the HA protein corresponding with the sequences recognized by the restriction enzymes (NheI, SgrAI, AgeI and PacI) used to generate the chimeric proteins are indicated. White and black boxes correspond with swine-origin and swine H1 protein sequences, respectively. (B and E) Limiting dilutions of recombinant soluble H1 trimers, complexed with a HRP-conjugated antibodies, were applied in the fetuin binding assay. (C and F) Bar graphs of the HA-fetuin binding at a HA concentration of 2.5 µg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine-origin H1 ($P<0.05$; Student's t-test).

The results show that the region between residues 143 and 332 of the H1 protein (H3 numbering) is responsible for the observed difference in the receptor binding between swine and swine-origin H1 proteins. Within this region, the two proteins differ at 14 positions (supplementary Fig. S1). To find out which amino acids are responsible for the binding difference we changed these residues one by one, in the background of the swine-origin H1 protein, to those of the swine H1 protein. All HA proteins containing a single amino acid mutation were expressed, purified and tested for fetuin binding. Only one of the mutated swine-origin H1 proteins, the one carrying the E227A mutation, showed a significant increase in fetuin binding (Fig 3A-B). The fetuin binding of this mutant protein was, however, less than that of the wild type swine H1 protein, indicating that additional residues must play a role.

In order to identify any additional residues important for the more efficient SIA-receptor binding of the swine H1 protein, the same mutations were made now in the background of the swine-origin E227A mutant protein. The subsequent fetuin binding assay showed that, with one exception, all

double mutant proteins displayed a similar intermediate SIA binding as the swine-origin E227A H1 protein, though some small differences were sometimes observed (Fig. 4A-B). The exception was the swine-origin T200A/E227A H1 protein, which bound to fetuin with similar efficiency as the swine H1 protein. Thus, while changing the T at position 200 in the swine-origin H1 protein did not have an apparent effect on fetuin binding (Fig. 3), in combination with the E227A mutation its consequences were significant (Fig. 4). Similar results were obtained when these HA proteins were tested in the hemagglutination assay (supplementary Fig. S2).

Next we analyzed whether the T200A mutation also had a synergistic effect on fetuin binding in combination with other mutations than the E227A substitution. Therefore, the same mutations were made again, now in the background of the swine-origin T200A H1 protein. However, none of the mutant HA proteins containing two amino acid substitutions displayed increased fetuin binding with the exception of the swine-origin T200A/E227A HA protein (Fig. 5A-B). These results demonstrate, that the positive effect of the T200A mutation on fetuin binding is only observed in combination with the E227A substitution.

To confirm the importance of the Ala residues found at position 200 and 227 in the swine protein for HA's strong fetuin binding, these amino acids were changed to the residues found in the swine-origin H1 protein, both separately and combined. The fetuin binding assay revealed that, while the effect of the A200T substitution was small, the A227E substitution had a much larger negative effect on SIA binding (Fig. 6). Again, the combination of both mutations had the most drastic effect, resulting in the same non-detectable fetuin binding as the swine-origin H1 protein. Thus, by introducing two substitutions in the swine-origin (T200A and E227A) or in the swine (A200T and A227E) H1 proteins, HA proteins are obtained with fetuin-binding properties that mirror each other. We conclude that the amino acid differences at these two positions are largely responsible for the different SIA-binding properties of the swine and swine-origin H1 proteins, at least as assessed by the fetuin-binding and hemagglutination assays.

Glycan array analysis

For a detailed study of the SIA binding properties of the swine-origin H1 proteins in comparison with the closely related swine and the seasonal H1 proteins, we subjected our soluble trimeric HA preparations to glycan array analysis in collaboration with the Consortium for Functional Glycomics. None of the proteins bound to α2-3 linked SIA-containing glycans (Fig. 7 and supplementary dataset

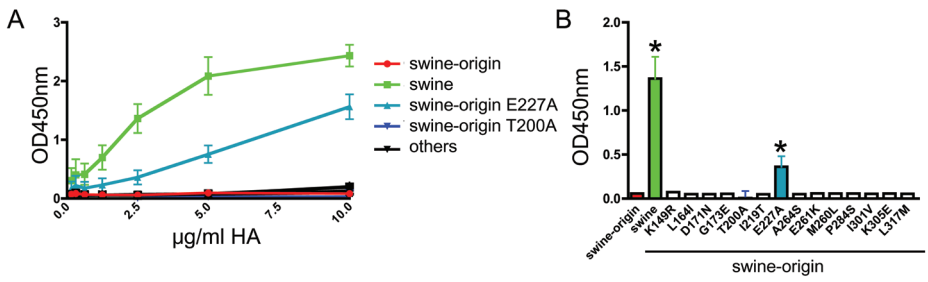

Fig. 3. Fetuin binding of swine-origin H1 proteins carrying single amino acid substitutions. (A) Limiting dilutions of swine-origin H1 proteins carrying single amino acid substitutions, complexed with a HRP-conjugated antibodies, were applied in the fetuin binding assay. Wild type swine-origin and swine H1 proteins were taken along as controls. (B) Bar graph of the HA-fetuin binding at a HA concentration of 2.5 μg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine-origin H1 ($P<0.05$; Student's t-test).

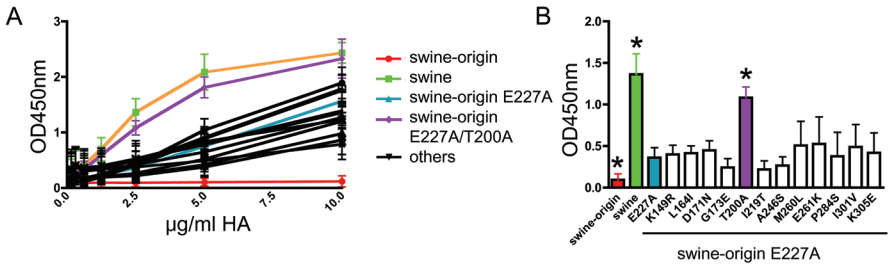

Fig. 4. Fetuin binding of swine-origin H1 proteins carrying amino acid substitutions in addition to E227A. (A) Limiting dilutions of swine-origin H1 proteins carrying amino acid substitutions in addition E227A, complexed with a HRP-conjugated antibodies, were applied in the fetuin binding assay. Wild type swine-origin and swine H1 proteins as well as the swine-origin E227A H1 protein were taken along as controls. (B) Bar graph of the HA-fetuin binding at a HA concentration of 2.5 μg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine-origin E227A H1 P<0.05; Student's t-test).

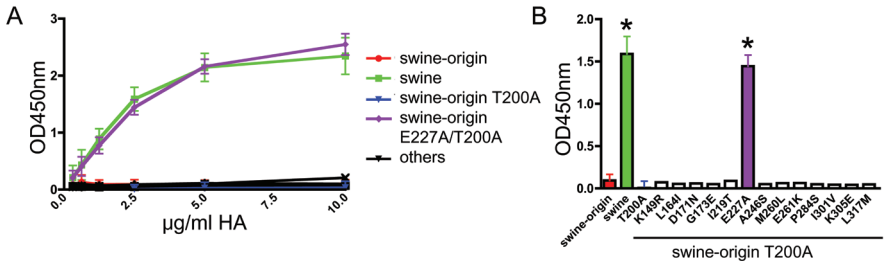

Fig. 5. Fetuin binding of swine-origin H1 proteins carrying amino acid substitutions in addition to T200A. (A) Limiting dilutions of swine-origin H1 proteins carrying amino acid substitutions in addition T200A, complexed with a HRP-conjugated antibodies, were applied in the fetuin binding assay. Wild type swine-origin and swine H1 proteins as well as the swine-origin T200A H1 protein were taken along as controls. (B) Bar graph of the HA-fetuin binding at a HA concentration of 2.5 μg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine-origin T200A H1 (P<0.05; Student's t-test).

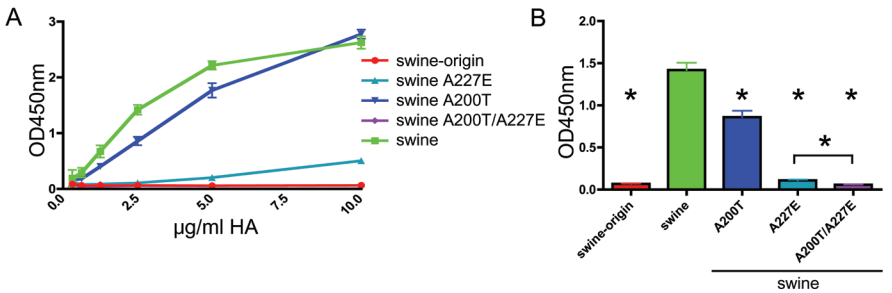

Fig. 6. Fetuin binding of swine H1 proteins carrying single or double amino acid substitutions. (A) Limiting dilutions of swine H1 proteins carrying single or double amino acid substitutions, complexed with a HRP-conjugated antibodies, were applied in the fetuin binding assay. Wild type swine-origin and swine H1 proteins were taken along as controls. (B) Bar graph of the HA-fetuin binding at a HA concentration of 2.5 μg/ml. Standard deviations are indicated. Asterisks indicate H1 binding significantly different from that of swine H1. The significant difference between the swine A227E and the swine A200T/A227E H1 proteins is also indicated (P<0.05; Student's t-test).

S3), as might be expected from the presence of residues D190 and D225 in all proteins (Glaser et al., 2005; Stevens et al., 2006a; Tumpey et al., 2007). Remarkably, the seasonal and the swine H1 proteins and the swine-origin T200A/E227A double-mutant H1 all bound most efficiently to the same set of 12 glycans, albeit with differences in their relative binding avidity. For example, only the swine H1 bound with high avidity to bi-antennary N-linked type glycans (#54-57; glycan numbering corresponds with that of the supplementary dataset S3, while their structures are shown in Fig. 7) containing two α2-6 linked SIAs. These N-linked type glycans have been demonstrated to occur in human upper respiratory epithelium (Chandrasekaran et al., 2008) and primary swine respiratory epithelial cells (Bateman et al., 2010). Note that all other bi-antennary glycans were not bound by any of the H1 proteins. Consistent with the fetuin-binding and hemagglutination assays, the swine-origin HA protein displayed hardly any binding to the glycan array.

Introduction of the T200A mutation into the swine-origin H1 protein resulted in modest binding to the same set of 12 glycan species, with the exception of the long, three lactosamine repeats-containing glycan #330, which was bound much more efficiently. However, when this glycan was fucosylated (#268), this increased binding was less apparent. Importantly, linear lactosamine repeat-containing glycans have been detected in swine and human respiratory epithelial cells (Bateman et al., 2010; Chandrasekaran et al., 2008). Introduction of the E227A mutation into the swine-origin HA protein

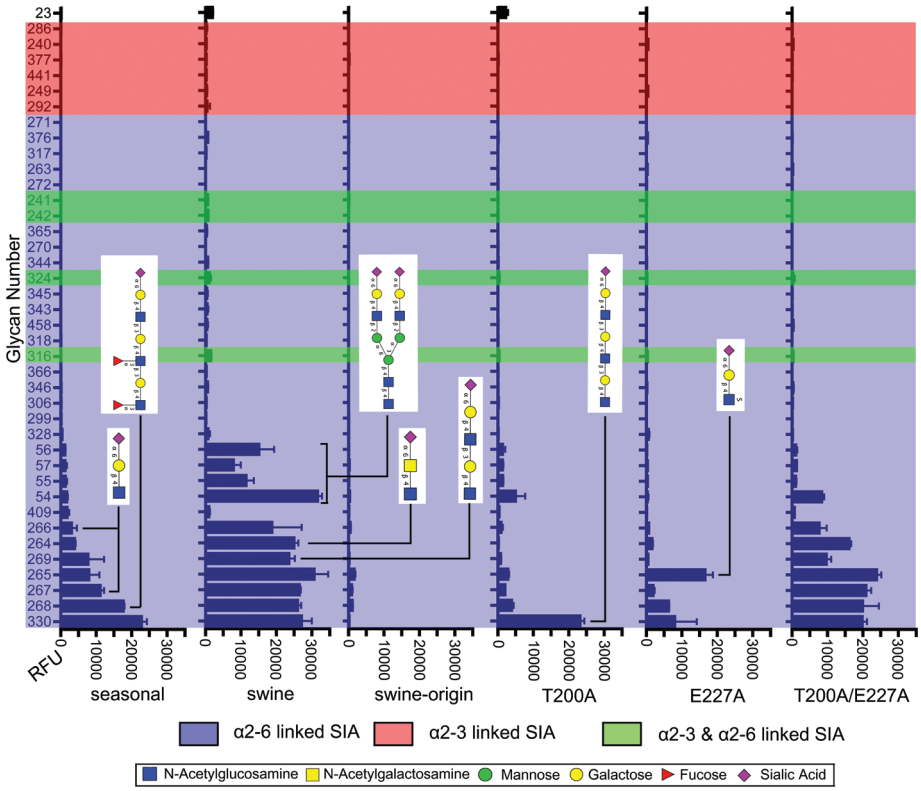

Fig. 7. Glycan array analysis of (mutant) H1 proteins. Seasonal, swine, swine-origin, swine-origin T200A (T200A), swine-origin E227A (E227A) and swine-origin T200A/E227A (T200A/E227A) H1 proteins were subjected to glycan array analysis. Glycan numbers are given on the y-axis, and are sorted according to the binding profile of the HA protein of the seasonal H1 protein. α2-6-linked, α2-3-linked and α2-3- and α2-6-linked sialyl glycans are indicated blue, red and green, respectively. Several glycan structures (generated with glycoworkbench (Bohne-Lang and von der Lieth, 2005)) are shown. The complete glycan array results as well as the identity of all glycans are shown in supplementary dataset S3.

also resulted in more efficient binding to the three lactosamine repeat-containing glycans #268 and 330, as well as to a sulphated single lactosamine repeat-containing glycan (#265). This increased binding was not apparent for similar non-sulphated glycans (#266-267). Combining the T200A and E227A mutations had a synergistic effect and resulted in a HA protein with a similar binding profile as the swine H1 protein, although the biantennary glycans (# 54-57) were less efficiently bound by the double mutant H1 protein.

Modeling of the T200A and E227A substitutions

We modeled the T200A and E227A substitutions into the crystal structure of the swine-origin H1 protein (Xu et al., 2010) and examined how changes in the molecular interactions could explain the altered binding with human SIA receptors. An α2-6 linked SIA trisaccharide (6LSTA) receptor was fitted into the modeled structure by superposition with the H1 protein from A/Swine/Iowa/30 in binary complex with 6LSTA (Gamblin et al., 2004). Fig. 8 shows the relevant part of the RBS of the swine-origin H1 and of the double mutant. The view of the RBS is such that the potential hydrogen bond interactions in the swine-origin H1 between T200 and Q191 and between E227 and penultimate galactose (the GlcNAc is not shown) are visible. Substitution of the residues at position 200 and 227 by alanine resulted in the loss of these potential hydrogen bonds. It is conceivable that the loss of a direct interaction of the receptor with position 227 in the RBS of the double mutant can affect the dynamic interactions between the receptor and the RBS, thereby resulting in altered, in this case increased, binding of particular glycans. Position 200, which is located on the surface, outside of the RBS, does not directly interact with the receptor. However, in the wild-type swine-origin H1 a potential hydrogen bond between T200 and Q191 occurs which is lost in the double mutant. Q191 is a highly conserved amino acid in the 190-helix, of which amino acids D190 and S193 have been shown to interact directly with the receptor. Any potential hydrogen bond with this helix may obviously influence its exact orientation and thereby have an indirect effect on receptor binding.

Amino acid distribution at position 200 and 227

We analyzed the amino acid distribution at positions 200 and 227 of all H1 proteins of IAVs of human, swine and avian origin (Table 1). The ancestral H1 from the 1918 pandemic strain contains A200 and A227. A200 is (almost) completely conserved in avian (100%) and human seasonal H1N1 (99.7%) viruses. Swine H1N1 and H1N2 viruses display a more diverse pattern. A200 is dominant in H1 from classical swine H1N1 (95%) and human-like H1 from H1N2 (96%) viruses. However, T200 is dominant in Eurasian swine H1N1 viruses (79%) and occurs frequently in triple reassortant H1N2 viruses (28%). Remarkably, T200 is only present in 1.4% of pandemic 2009 H1N1 viruses. The frequency of its occurrence remained at a low level since the start of the pandemic (data not shown).

The H1 proteins derived from the swine-origin new pandemic H1N1 virus invariably contain E227 (Table 1). The segment encoding this protein is derived from the H1N2 'triple-reassortant' influenza virus isolated from pigs in North America. The H1 proteins of these viruses contain either E (17%) or A (83%) at position 227 (Table 1). The triple-reassortant virus, in turn, obtained its HA-encoding segment from the classical swine H1N1 virus. Remarkably, most HA proteins of these latter viruses contain A at position 227 (91%), while E has never been detected.

Discussion

Transmission of IAVs from birds and swine to humans is posing a serious global health threat. While the molecular determinants governing the switch from avian to human receptor specificity have been elucidated for several HAs (Bateman et al., 2008; Connor et al., 1994; Matrosovich et al., 2000), much less is known about the HA requirements for pig to human transmission and subsequent

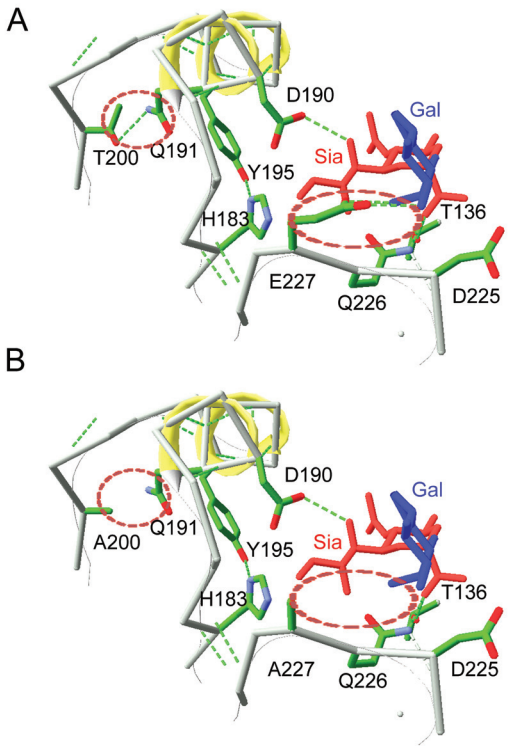

Fig. 8. Structural model of the swine-origin H1 RBS in complex with a α2-6-linked sialyl glycan. (A) Structural model of the swine-origin H1 RBS in complex with a α2-6-linked sialyl glycan. Key amino acids are indicated and shown in a stick representation (carbon, gray; oxygen, red; nitrogen, blue). The α2-6-linked SIA is shown in red and the penultimate galactose in blue. Potential hydrogen bond interactions are shown by the dotted lines. (B) Substitution of the residues at position 200 and 227, resulted in the loss of potential hydrogen bond interactions with Q191 and with the galactose. The red dotted circles highlight the presence or absence of the potential hydrogen bond interactions.

Table. 1. Frequency of residues at position 200 and 227 in H1

		HUMAN						SWINE				AVIAN
		H1N1						H1N1		H1N2		H1N1
			seasonal			swine-origin	classic swine	classic	Eurasian	triple reassortant	human-like	
Postion	AA[a]	1918-1957	1977	1978	1979-2010	2009-2010	1977-2010					
200	T	-	-	-	3	45	-	7	59	19	2	-
	A	41	6	14	831	3213	16	151	16	49	48	79
	S	-	-	-	-	20	-	1	-	-	-	-
227	E	-	-	5	832	3278	-	-	1	11	50	-
	A	30	6	8	-	-	16	146	73	55	-	79
	T	1	-	-	-	-	-	13	-	1	-	-
	S	1	-	-	-	-	-	-	-	1	-	-
	P	5	-	-	-	-	-	-	-	-	-	-
	H	4	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-	1	-	-	-
	K	-	-	1	2	-	-	-	-	-	-	-
Total[b]		41	6	14	834	3278	16	159	75	68	50	79

[a] Amino acids are indicated by single letters.
[b] Total number of unique full length sequences. Only for new pandemic H1N1 all full length sequences were counted.

efficient spread in the population. As a first step to address this point, we analyzed the differences in the receptor binding properties of the H1 proteins of closely related swine and new pandemic swine-origin IAVs. While the swine H1 protein efficiently bound to several α2-6-linked SIA-containing glycans, the swine-origin H1 protein displayed only very poor binding to these glycans. Although the two proteins differ at 26 amino acid residues, their different receptor binding properties were found to be largely determined by only two residues, located at positions 200 and 227. Substitution of both these residues was shown to have a synergistic effect. Importantly, reciprocal results were obtained when the residues at these positions were mutated in the context of the swine H1 protein. These findings provide new insights into H1-receptor interactions and show that residues 227 and 200, which are located within and outside of the RBS, respectively, can affect HA receptor binding properties.

In a previous study, in which glycan arrays were probed with whole viruses, dual receptor specificity was reported for new pandemic swine-origin H1N1 viruses (Childs et al., 2009). However, our recombinant trimeric swine-origin H1 preparations exclusively bound to α2-6- and not to α2-3-linked sialyl glycans, in agreement with other studies using recombinant new pandemic swine-origin H1 trimers (Maines et al., 2009; Yang, Carney, and Stevens, 2010). However, recombinant H1 proteins of several new pandemic swine-origin H1N1 viruses (Yang, Carney, and Stevens, 2010), which all contain A200, appeared to display more efficient binding to the glycan array than our recombinant H1 protein with the T200A substitution. These differences may be attributed to the H1 proteins differing at other residues, but also to the different expression systems used. While the HA proteins used by Yang and corkers were produced in insect cells (Yang, Carney, and Stevens, 2010), which contain paucimannose N-glycans, our H1 proteins were generated in HEK293S GnTI(-) cells, which produce proteins with high mannose N-glycans. We recently demonstrated that recombinant soluble HA proteins with paucimannose N-linked glycans display more promiscuous receptor binding when compared to proteins that carry high-mannose or desialylated complex N-glycans (de Vries et al., 2010).

In contrast to the swine H1 protein, the swine-origin H1 protein displayed only very modest receptor binding, for which the residues at position 200 and 227 were shown to be largely responsible. Yet we observed highly similar receptor specificities for these various H1 proteins. Close inspection of the glycan array data (Fig. 7 and dataset S3) revealed that selecting the 12 strongest binding glycans for each (mutant) H1 protein yielded almost completely overlapping sets. From these results we conclude that the amino acids at position 200 and 227 mainly contribute to the receptor binding affinity.

Our observations demonstrate that the identity of the residue at position 200, which is not part of RBS, can affect the interaction of HA with its receptor. Modeling of the T200A substitution into the structure of the swine-origin H1 protein (Xu et al., 2010), revealed the loss of a potential hydrogen bond with residue Q191 in the 190 loop, thereby probably affecting the interaction of H1 with SIA-containing glycans. Other studies already hinted at the putative involvement of residue 200 in H1-receptor binding. Characterization of zanamivir-selected drug-resistant H1N1 viruses not only revealed a massive deletion in the region encoding the NA active center, but also an A200T mutation in HA (Baz, Abed, and Boivin, 2007), in agreement with the notion that HA mutations resulting in weaker cell attachment often arise during *in vitro* selection with NA inhibitors (McKimm-Breschkin, 2000). Furthermore, passaging in naïve mice of high-avidity binding PR8 mutant viruses (H1N1), which had been selected in mice immunized with influenza vaccine, selected for additional HA substitutions, including an A200T substitution, that resulted in decreased cell binding (Hensley et al., 2009).

Substituting the residue at position 227, which is located within the RBS, had a much larger effect on receptor binding of H1 than substitution of the residue at position 200. Maines and coworkers (Maines et al., 2009) suggested that the presence of E227 in combination with I219 in the new pandemic H1 protein would disrupt optimal contacts with α2-6 sialylated glycans. In this study we demonstrate that the E227A substitution indeed results in increased receptor binding regardless, however, of the identity of the 219 residue. In agreement with our results, also for the H1 protein of PR8, substitution of the alanine at position 227, this time by a threonine residue, correlated with decreased cell binding (Hensley et al., 2009). According to our model (Fig. 8), the E227A substitution disrupts the potential hydrogen bond interaction with the penultimate galactose of the sialyl glycan. Loss of a hydrogen bond may affect the dynamic interactions between receptor and RBS and thereby change receptor binding affinity. However, the synergistic effect of substituting the residues at position 200 and 227 is not easily explained on the basis of a static model.

In contrast to the T200A substitution, which is found in most new pandemic swine-origin H1N1 viruses, the identity of residue 227 in the HA protein of all swine-origin H1N1 isolates is invariably a glutamic acid. The T200A substitution may provide a selective advantage to the swine-origin H1N1 virus, by its subtle effect on receptor binding (this study) and/or by modifying antigenicity (Hensley et al., 2009). However, it appears that the larger increase in receptor binding resulting from mutation of E227 is not compatible with spread of the swine-origin H1N1 virus in the human population. It will be of interest to determine the biological consequences of these mutations in H1 and to establish their relationship to the efficient propagation of H1N1 viruses in humans.

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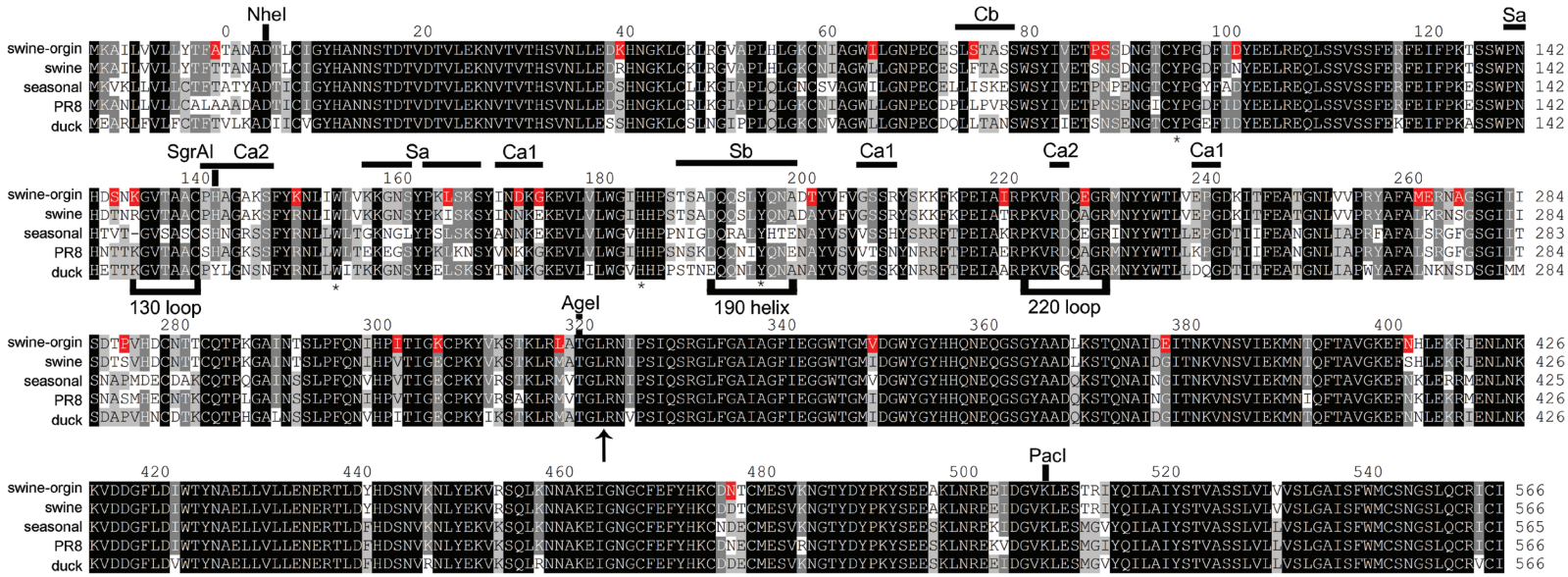
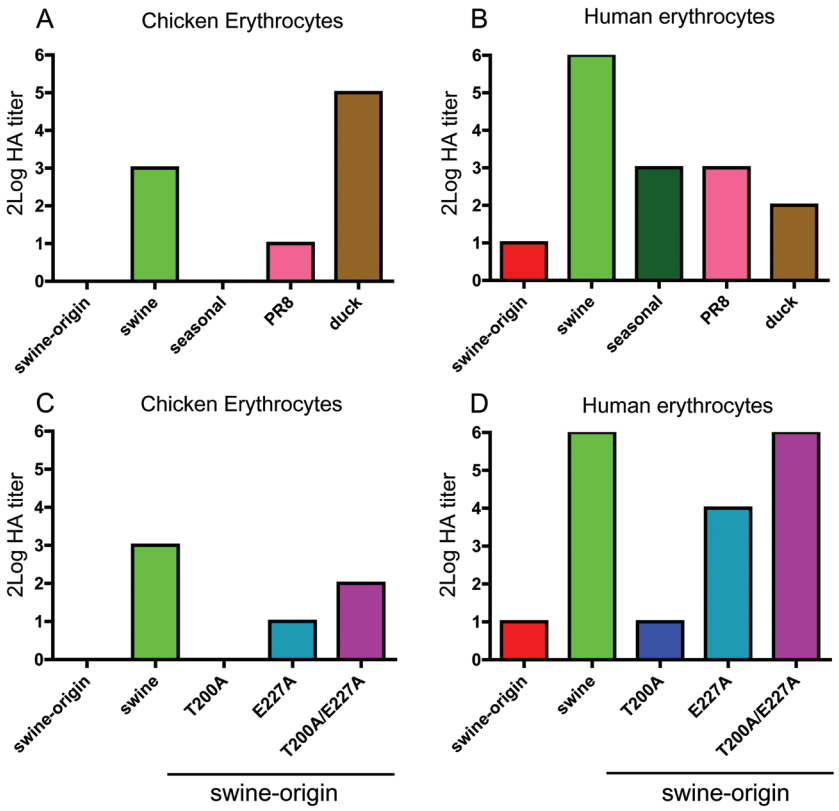


Supplementary Fig. S1. Alignment of the H1 proteins used in this study.
Alignment of the H1 proteins of A/Cal/04/09 (Accession# FJ966082; swine-origin H1), A/Swine/Ohio/01 (Accession# AF455675; swine H1), A/Kentucky/07 (Accession# CY028163; seasonal H1), PR8 (Accession# NP_040980.1; PR8 H1) and A/Duck/NZL/76 (Accession# ABB20429.1; duck H1). Differences between the swine-origin and swine H1 proteins are highlighted in red. The residues that form the 130-loop, 190-helix and 220-loop of the RBS are specified, while the four residues that form the base of the RBS (comprising Y94, W153 and H183 and Y195) are indicated by asterisks. The HA cleavage site is indicated by an arrowhead. NheI, SgrAI, AgeI and PacI indicate the positions in the HA protein corresponding with the sequences recognized by the restriction enzymes used to generate the chimeric proteins. Antigenic sites are indicated as described by Brownlee and Fodor (Philos. Trans. R. Soc. Lond. B. Biol. Sci. [2001] 356;1871-1876).



Supplementary Fig. S2. Hemagglutination assays.
Hemagglutination titers of the different pre-complexed H1 proteins are depicted. The titers were determined using either chicken (A and C) or human erythrocytes (B and D) making 2-fold dilutions starting with 10 μg/ml H1 protein.

Supplementary dataset S3. Raw glycan array data.
To be found online at: http://www.jbc.org/content/286/7/5868/suppl/DC1
The table shows the glycan numbers and structures in columns A and B, respectively. The average RFU value (average of 4) and the standard deviation are given for each HA protein analyzed. For each HA protein the 15 highest RFU values are shaded in grey. The data are sorted descending for the RFU values obtained with the seasonal H1 protein.

A Single Immunization with Soluble Recombinant Trimeric Hemagglutinin Protects Chickens against Highly Pathogenic Avian Influenza Virus H5N1.

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Abstract

Background: The highly pathogenic avian influenza (HPAI) virus H5N1 causes multi-organ disease and death in poultry, resulting in significant economic losses in the poultry industry. In addition, it poses a major public health threat as it can be transmitted directly from infected poultry to humans with very high (60%) mortality rate. Effective vaccination against HPAI H5N1 would protect commercial poultry and would thus provide an important control measure by reducing the likelihood of bird-to-bird and bird-to-human transmission.
Methodology/Principal findings: In the present study we evaluated the vaccine potential of recombinant soluble trimeric subtype 5 hemagglutinin ($sH5^3$) produced in mammalian cells. The secreted, purified $sH5^3$ was biologically active as demonstrated by its binding to ligands in a sialic acid-dependent manner. It was shown to protect chickens, in a dose-dependent manner, against a lethal challenge with H5N1 after a single vaccination. Protected animals did not shed challenge virus as determined by a quantitative RT-PCR on RNA isolated from trachea and cloaca swabs. Also in mice, vaccination with $sH5^3$ provided complete protection against challenge with HPAI H5N1.
Conclusions/Significance: Our results demonstrate that $sH5^3$ constitutes an attractive vaccine antigen for protection of chickens and mammals against HPAI H5N1. As these recombinant soluble hemagglutinin preparations can be produced with high yields and with relatively short lead time, they enable a rapid response to circulating and potentially pandemic influenza viruses.

Introduction

Influenza A viruses are enveloped, negative-strand RNA viruses with a segmented genome. They infect a large variety of animal species, although birds are considered to constitute the reservoir from which all influenza A viruses in other species originate (Munster and Fouchier, 2009; Webster et al., 1992). On the basis of the antigenic properties of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are classified into 16 HA (H1-16) and 9 NA (N1-9) subtypes. The highly pathogenic avian influenza (HPAI) virus H5N1 causes multi-organ disease and death in poultry, resulting in significant economic losses in the poultry industry. HPAI H5N1 also poses a major public health threat as it can be transmitted directly from infected poultry to humans with very high (60%) mortality rate. It is widely accepted that continued human exposure to influenza viruses circulating in wild and domestic avian species poses a permanent pandemic threat (Uyeki, 2008; Yen and Webster, 2009). Effective vaccination against HPAI H5N1 would protect commercial poultry and would thus provide an important control measure by reducing the likelihood of bird-to-bird as well as bird-to-human transmission. Therefore, the development of efficacious influenza vaccines is of high veterinary and public health importance.

Conventional vaccine preparations against HPAI H5N1 are produced by propagating virus in embryonated chicken eggs or mammalian cells. The vaccine viruses used are either reassortants or low pathogenic wild-type avian viruses. Either way, the viruses need to be selected for their ability to multiply in eggs or cultured cells, which may preclude the best genetic match to the circulating HPAI H5N1 strains. In addition, these vaccines have several other limitations as reviewed elsewhere (Ellebedy and Webby, 2009; Safdar and Cox, 2007). Hence, other vaccine strategies against HPAI H5N1 have been explored including the use of, among others, live attenuated influenza vaccines (Keitel and Atmar, 2009; Mueller et al., 2010), live vaccines based on heterologous viral vectors such as pox virus (Kreijtz et al., 2007), adenovirus (Gao et al., 2006), baculovirus (Wu et al., 2009) and Newcastle disease virus (Dinapoli et al., 2010), and DNA vaccination (Rao et al., 2008). While these different strategies often showed promising results, their applicability ultimately will depend on various important issues including safety, efficacy, production and costs (Ellebedy and Webby, 2009).

Protective immunity against influenza virus infection and disease is primarily conferred through HA via the induction of anti-HA antibodies. As protection from influenza virus infection correlates with anti-HA titers, nearly all vaccine approaches aim to induce high levels thereof. Therefore, the HA protein is the antigen of choice for the development of recombinant subunit vaccines to protect against HPAI H5N1. An influenza vaccine based on recombinant purified HA could offer the following advantages: I) The HA antigen can be produced using safe, quality-controlled and scalable conditions. II) There will be no need for virus cultivation, thus avoiding the necessity a) to obtain viruses that replicate efficiently in eggs or cell culture, b) to use biocontainment facilities and c) to inactivate the virus using procedures that may affect antigenicity and raise safety concerns. III) The recombinant HA protein can be highly purified thereby limiting adverse reactions caused e.g. by the presence of egg contaminants. IV) Immunization with recombinant HA will allow the serological differentiation of naturally infected from vaccinated animals/flocks (the so-called DIVA principle; (van Oirschot, 2001)). IV) Recombinant HA vaccines are manufactured with a relatively short lead time, allowing an accelerated response to emerging influenza strains.

Recombinant HA can be produced using different expression systems. When expressed in *E. coli* the resulting HA protein gave rise to the induction of hemagglutination inhibition (HI) titers upon immunization of mice (Biesova et al., 2009). However, as proper folding and trimerization of the HA protein requires multiple posttranslational modifications including glycosylation and disulfide bond formation, expression of the HA protein in higher eukaryotic systems is likely to result in

superior antigenicity. Thus, mammalian cell-derived HA trimers were found to induce much higher levels of neutralizing antibodies than similarly produced monomeric HA protein (Wei et al., 2008). The baculovirus expression system has been used to produce strain specific HA antigens in insect cells, which were shown to protect against a HPAI H5N1 challenge (Crawford et al., 1999). Such full-size HA proteins may, however, be limited in their efficacy because the membrane proteins may not retain their native membrane-bound structure upon purification (Bhattacharya et al.). Hence, the production of recombinant, soluble, stable HA trimers that are secreted from the cells seems like an attractive alternative approach. Such HA trimers, expressed either in insect or mammalian cells, were indeed shown to elicit neutralizing antibodies (Wei et al., 2008), and to partially protect mice against HPAI H5N1 challenge infection (Wang et al., 2009).

In view of their promising potential we have evaluated recombinant soluble HA trimers in chickens and mice for their ability to induce protective immunity against infection with HPAI H5N1. To this end, recombinant soluble H5 proteins provided with a GCN4 trimerization motif and a STREP-tag II, the latter for purification purposes, were expressed in mammalian cells. The recombinant soluble H5 trimers ($sH5^3$) were purified from the culture supernatants using a simple one-step purification protocol and characterized with respect to their oligomeric state and bioactivity. Subsequently, vaccination with the $sH5^3$ preparation was shown to provide complete protection against challenge with HPAI H5N1 both in mice and in chickens, in the latter already after a single immunization.

Materials and Methods

Genes and expression vectors

A cDNA clone corresponding to residues 18 to 523 (H3 numbering) of the HA from A/Viet Nam/1203/2004 (H5N1) (Genbank accession no. ABW90137.1) was synthesized using human-preferred codons by GenScript USA Inc. In this clone the predicted HA ectodomain protein lacks a multibasic cleavage site. The cDNA was cloned into the pCD5 expression vector for efficient expression in mammalian cells (Zeng et al., 2008). The pCD5 vector had been modified such that the HA-encoding cDNA was cloned in frame with DNA sequences coding for a signal sequence, an artificial GCN4 isoleucine zipper trimerization motif (KRMKQIEDKIEEIESKQKKIENEIARIKK) (Harbury et al., 1993) and the Strep-tag II (WSHPQFEK; IBA, Germany). The resulting vector encodes the soluble trimeric H5 protein designated as $sH5^3$.

Protein expression and purification

pCD5 expression vectors containing the HA ectodomain-encoding sequences were transfected into HEK293S GnTI(-)cells (Reeves, Hwa, and Khorana, 1999) using polyethyleneimine I (PEI) in a 1:5 ratio (µg DNA: µg PEI). At 6 h post transfection, the transfection mixture was replaced by 293 SFM II expression medium (Invitrogen), supplemented with sodium bicarbonate (3.7 g/liter), glucose (2.0 g/liter), Primatone RL-UF (3.0 g/liter), penicillin (100 units/ml), Streptomycin (100 µg/ml), glutaMAX (Gibco), and 1,5% dimethyl sulfoxide. Tissue culture supernatants were harvested 5–6 days post transfection. HA protein expression and secretion was confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). HA proteins were purified using Strep-tactin Sepharose beads according to the manufacturer's instructions (IBA, Germany). The concentration of purified protein was determined by using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according to the manufacturer's instructions.

Biological characterization of recombinant HA.

The oligomerization status of the $sH5^3$ proteins was determined by analyzing the elution profile using a Superdex200GL 10-300 column (GE Healthcare). Sialic acid-binding activity of $sH5^3$ was

assessed using a fetuin solid phase assay. 1µg/ml fetuin per well was used to coat 96-well Nunc MaxiSorp plates. When indicated in the figure legend, fetuin was treated with *Vibrio Cholera* derived neuraminidase (VCNA; Roche) followed by three washing steps. sH5[3] was pre-complexed with horseradish peroxidase (HRP)-linked anti-Strep-tag antibody (2:1 molar ratio) for 30 min at 0 ºC prior to incubation of limiting dilutions on the fetuin-coated plates (60 min, room temperature [RT]). HA-binding was subsequently detected using tetramethylbenzidine substrate (BioFX) in an ELISA reader (EL-808 [BioTEK]), reading the OD at 450nm.

Vaccination-challenge experiments in chickens

Animal studies were conducted at the Central Veterinary Institute (CVI), Lelystad, under BSL3 conditions and after approval by the Animal Ethic Committee. In the first chicken experiment, 6 weeks old SPF white Leghorn chickens (Lohmann, Cuxhaven, Germany) were used (10 chickens per group). One group of animals was immunized twice (on day 0 and 21) by intramuscular (i.m.) injection (0.5 ml) of 10 µg sH5[3] adjuvanted with Stimune as recommended by the manufacturer [Prionics, Lelystad]). As a challenge control, the other group received an equal volume of PBS in Stimune (mock vaccinated). Three weeks after the vaccination (day 42), the birds were challenged by inoculation with 10^5 median tissue culture infective dose ($TCID_{50}$) of A/Viet Nam/1194/04 virus (0.1 ml intranasally [i.n.] and 0.1 ml intratracheally [i.t.]) and monitored over a period of 14 days for signs of disease. Blood samples were collected at 3 weeks after each immunization (on day 21 and 42 post infection [p.i.]).

For the next experiment, 70 one-day-old layer hens (white Leghorn) were purchased from a local breeder. The chickens had been vaccinated against Newcastle disease virus and infectious bronchitis virus at the age of one day according to the farm's routine and were raised in CVI's animal facility. At the age of 6 weeks, the birds were transported to the BSL-3 facility and allocated to 7 experimental groups of 10 birds each. The animals of six groups were immunized once (on day 21) or twice (on day 0 and 21) by i.m. injection of 10, 2 or 0.4 µg sH5[3] antigen adjuvanted with Stimune. When immunized twice, the same doses were given on day 0 and 21. As a challenge control, one group was mock-vaccinated twice (on day 0 and 21) with PBS in Stimune. Four weeks after vaccination (day 49), blood samples were taken and the chickens were challenged as above and observed daily for clinical signs during 14 days. Trachea and cloaca swabs were taken from each chicken on day 2, 4 and 7 p.i.

Vaccination-challenge experiment in mice

Female, specified pathogen-free (SPF) 9-week-old BALB/c mice (Charles River Laboratories; 10 animals per group) were immunized once (on day 21) or twice (on day 0 and 21) by i.m. injection (0.2 ml) of 2 µg sH5[3] adjuvanted with Stimune. As a challenge control, one group of mice was mock vaccinated. Three weeks after the vaccination, mice were anaesthetized with ketamin/xylazin by intraperitoneal injection and inoculated intranasally with 50 µl of H5N1 A/Viet nam/1194/04 containing ~10 median lethal dose (LD_{50}; 3.7 ^{10}log $TCID_{50}$; provided by Dr. Alan Hay from the WHO Influenza Centre at the National Institute for Medical Research, London). The mice were weighed daily and examined for signs of illness during the next 14 days. Clinical signs were recorded using a scoring system (0 = no clinical signs; 1 =rough coat; 2 = rough coat, less reactive, passive during handling; 3 = rough coat, rolled up, laboured breathing, passive during handling; 4 = rough coat, rolled up, laboured breathing, unresponsive). Animals reaching a score of 4 were euthanized. Surviving animals were bled and sacrificed on day 14 p.i.

Virus detection assay

Trachea and cloaca swabs were stored in cold tryptose phosphate broth supplemented with antibiotics. The medium was clarified by low-speed centrifugation and the supernatant was harvested, aliquoted

and stored at -70°C. Upon thawing, trachea and cloaca swabs sampled from the same bird on the same day were pooled and the viral RNA was extracted from 200 µl using a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). Subsequently, cDNA was synthesized using reverse primer 5'-CACTGGGCACGGTGAGC- 3' and part of the M1 gene was amplified by running 45 cycles of Light Cycler PCR using primer 5'-CTTCTAACCGAGGTCGAAACGTA-3' as the reverse primer in the presence of the TaqMan fluorescent probe 5'-6FAM-TCAGGCCCCCTCAAAGCCGA-X-ph. Negative and positive control samples were tested in parallel. The lower limit of detection was determined to be approximately 500 $TCID_{50}$. Some samples gave inconclusive results, meaning that they gave only a very weak signal (fluorescence < 0.07) after >31 cycles.

Hemagglutination inhibition (HI) assay

Heat-inactivated immune sera from chicken blood samples were tested for hemagglutination inhibition (HI) activity with 1% chicken red blood cells and 4 hemagglutinating units (HAU) of H5N1 (A/Viet Nam/1194/04 NIBRG-14). In addition, the chicken sera were tested for HI activity using 8 HAU (67 ng) of recombinant soluble trimeric HA protein. To this end the recombinant proteins were precomplexed with the anti-Strep-tag antibody as described above, mixed with limiting dilutions of the chicken sera and incubated with 0.5 % chicken red blood cells. Red button formation was scored as evidence of hemagglutination. Antibody titers were expressed as the reciprocal of the highest serum dilution showing HI. Immune sera prepared from mouse blood samples were treated with VCNA, heat-inactivated at 56 °C for 30 min and tested by HI assay using 8 HAU of $sH5^3$ as described above.

ELISA

Total antibody titers against $sH5^3$ were determined by using a $sH5^3$-specific ELISA. To this end, 96-well Nunc MaxiSorp plates coated with 0.5 µg $sH5^3$ per well were incubated with limiting dilutions of chicken or mouse sera. After extensive washing, the plates were incubated with goat-anti-chicken or goat-anti-mouse antibodies conjugated with HRP. Peroxidase activity was visualized using tetramethylbenzidine substrate (BioFX) and an ELISA reader (EL-808 [BioTEK]), reading the OD at 450nm. The OD values of the 250-fold diluted samples, which were in the logarithmic phase of the curve, were plotted.

Results

Expression, purification, and characterization of $sH5^3$.

In order to express soluble trimeric subtype 5 HA ($sH5^3$) in mammalian cells, the H5 ectodomain-coding sequence was first cloned into an appropriate expression vector. In the pCD5 vector used, the H5-sequence was preceded by a signal peptide-encoding sequence, to allow efficient secretion of the recombinant protein, and followed by sequences coding for the GCN4 isoleucine-zipper trimerizaton motif (Harbury et al., 1993) and the Strep-tag II, the latter for purification purposes (Fig. 1A). Expression of the H5 ectodomain was achieved by transient transfection of the expression plasmid into HEK cells. Expression and secretion of the H5 protein was verified by subjecting cell culture supernatant to gel electrophoresis followed by western blotting using an antibody directed against Strep-tag II (Fig. 1B). The results show that the recombinant H5 protein could be readily detected in the cell culture supernatant after transfection of the cells with the expression plasmid, but not after mock transfection. The secreted H5 protein was purified in a single step protocol by using Strep-tactin sepharose beads. Protein yields varied between 0.4-1 mg of recombinant protein per 100 ml cell culture medium. After purification of the H5 protein from the cell culture supernatant, the oligomeric state of the H5 protein was analyzed by gel filtration column chromatography (Fig. 1C). The bulk of the H5 protein eluted with the velocity of an oligomer, while only a minor fraction was

found as aggregates in the void volume. The trimeric nature of the H5 oligomer was confirmed using blue-native gel electrophoresis followed by western blotting (Fig. 1D). When the H5 preparation was heat-denatured for increasing time periods prior to electrophoresis, the initially trimeric HA species dissociated into dimers and monomers. The biological activity of the purified sH5^3 was studied using a solid phase-binding assay with the sialylated blood glycoprotein fetuin. Binding of sH5^3 was measured by means of the HRP conjugated to the anti-Strep-tag II antibody as detailed in the Material and Methods section. The H5 preparation exhibited a concentration dependent binding to the fetuin. This binding was sialic acid-dependent, as no binding was observed when the fetuin had been treated with neuraminidase (VCNA; Fig. 1E). In conclusion, biologically active sH5^3 was efficiently produced using a mammalian expression system and readily purified.

Efficacy of sH5^3 as a vaccine against lethal HPAI H5N1 infection in chickens

To examine the immunogenicity of sH5^3 and its potential as a vaccine a first experiment in chickens was performed, in which 10 chickens were vaccinated twice (on day 0 and 21) i.m. with 10 µg of Stimune-adjuvanted sH5^3 and challenged 3 weeks later by i.n./i.t. inoculation of a lethal dose of A/

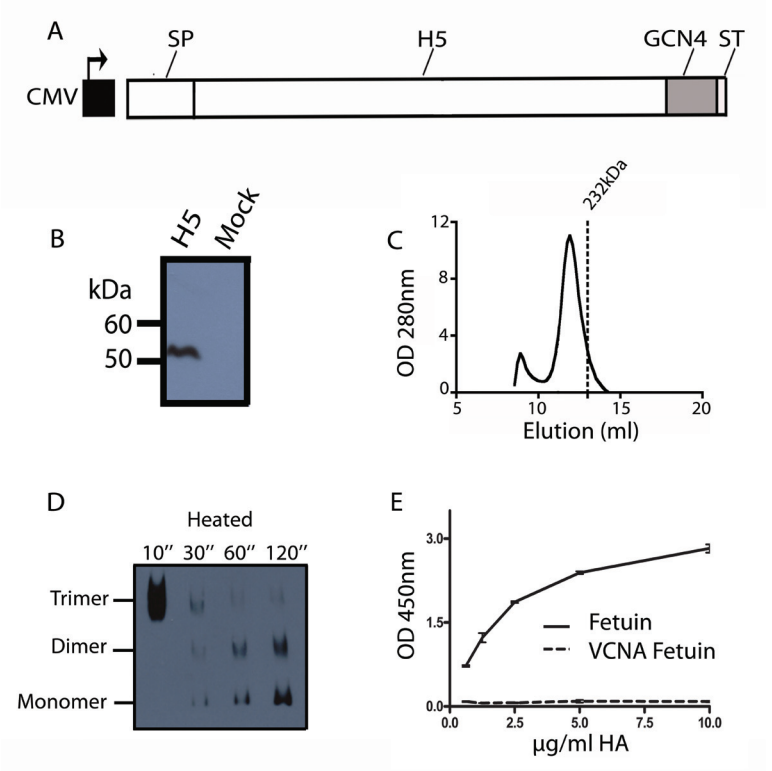

Figure 1. Expression, purification and biological activity of recombinant, soluble trimeric H5 protein. (A) Schematic representation of the H5 expression cassettes used. The H5 ectodomain encoding sequence (H5) was cloned in frame with DNA sequences coding for a signal peptide (SP), the GCN4 isoleucine zipper trimerization motif (GCN4) and the Strep-tag II (ST) under the control of a CMV promoter. (B) H5 expression and secretion into the culture media was analyzed by SDS-PAGE followed by western blotting. The recombinant protein was detected using a mouse anti-Strep-tag antibody. (C) Analysis of purified recombinant H5 proteins by gel filtration. Shown is the elution profile of a H5 protein preparation using a Superdex200GL 10-300 column. The elution of a 232kDa catalase control is indicated by the line. (D) Blue native-PAGE analysis of the recombinant H5 protein. The position in the gel of the momomeric, dimeric and trimeric ectodomain species observed after heating of the HA sample prior to electrophoresis is indicated. (E) Recombinant soluble H5 trimers were complexed with a HRP-conjugated mouse antibody directed against the Strep-tag prior to their application in a fetuin binding assay. HA binding was also assessed after treatment of fetuin with VCNA (fetuin + VCNA).

Viet Nam/1194/04 virus. Another 10 birds were mock-vaccinated to serve as challenge controls. As shown in Fig. 2, the boost vaccination with 10 μg sH5[3] conferred complete protection. None of the vaccinated chickens died or showed symptoms indicative of influenza-related disease, whereas all mock-vaccinated chickens succumbed within 2 days. Serological analysis showed that none of the mock-vaccinated animals contained antibodies against sH5[3] as determined by a sH5[3]-specific ELISA (Fig. 2B). In contrast, all immunized animals demonstrated appreciable levels of HA antibodies already after a single immunization and these levels increased further after the boost. These total antibody levels against sH5[3] correlated nicely with the HI titers against H5N1 (Fig. 2C). All mock-vaccinated chickens had a HI titer below the detection limit. HI antibody titers observed after the sH5[3] boost reached with a maximum of 1024 and a minimum of 64, which was apparently still sufficient to protect the animal against the lethal challenge. Interestingly, 50% of the birds developed HI antibody titers equal to 64 or higher already three weeks after the first vaccination. In addition, HI titers were also determined by using sH5[3] rather than H5N1 virus as the hemagglutinating agent, which gave essentially similar results (compare Fig. 2D and 2C), demonstrating the reliability of the assay. In summary, the results show that chickens vaccinated twice with sH5[3] are protected against a lethal challenge with H5N1. The HI titers observed suggested that one vaccination might already be sufficient to confer protection against HPAI H5N1.

Vaccination efficacy in chickens: effect of antigen dose and vaccination booster

These results prompted us to determine the minimal sH5[3] dose required to confer protection and to examine whether a single dose could already be sufficient. In the second vaccination-challenge experiment, chickens were thus vaccinated with 10, 2 or 0.4 μg of sH5[3] either once or twice and challenged four weeks later by infection with a lethal dose of A/Viet Nam/1194/04 as described above. The results are shown in Fig. 3. Again, all mock-vaccinated birds succumbed to the infection within 2 days. Vaccinating twice with a dose of 0.4 μg of sH5[3] was sufficient to protect 90% of the chickens against mortality, while all chickens survived when a dose of 2 or 10 μg was similarly administered (Fig. 3A). Interestingly, also single vaccination with sH5[3] could induce sufficient immunity to protect chickens against lethal infection (Fig. 3B); when a dose of 2 μg was given only one chicken died (90% protection), whereas a dose of 10 μg was protective to all birds. Even after a single dose of 0.4 μg, 60% of the chickens were protected against death.

Serological analysis showed that protection against the lethal H5N1 challenge correlated well with the observed antibody levels against sH5[3] as determined by ELISA (Fig. 3C-D) and by HI assay (Fig. 3E-F). Both assays revealed a dose-dependent antibody response, which was substantially enhanced after the booster immunization. Relatively high HA antibody levels were observed after two immunizations, even with the lower dose, except in two animals, one of which did not survive the challenge (Fig. 3C). Also after a single immunization, significant antibody levels were measured, except again in the low dose group. Here, 5 animals had hardly measurable ELISA titers. Consistently, 4 of these animals succumbed to the challenge. Also the one animal that died after a single immunization with 2 μg of sH5[3] did not have detectable sH5[3] antibodies. Essentially the same results as with the ELISA were obtained with the HI assay using sH5[3] as the hemagglutinating agent (Fig. 3E-F). Thus, the animals that succumbed to the lethal challenge after a single immunization also exhibited the lowest HI titers.

Shedding of challenge virus from vaccinated chickens

We analyzed whether vaccination with sH5[3] decreased or prevented chickens from shedding challenge virus. For practical reasons, virus shedding was analyzed by a quantitative RT PCR rather than by measuring infectious virus titers. To this end, trachea and cloaca swabs were taken from

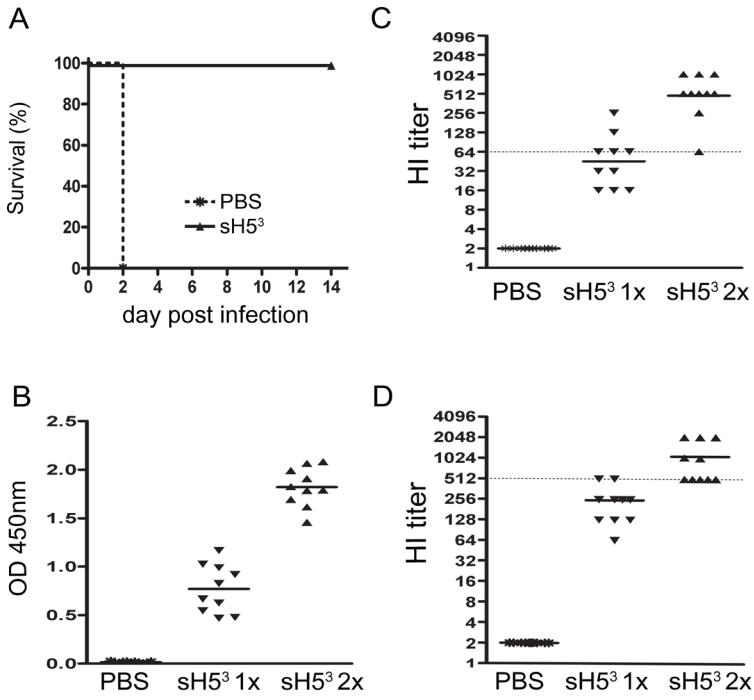

Figure 2. sH5³ vaccination in chickens. Ten chickens were immunized two times with 10 ug sH5³ (on day 0 and 21). As a challenge control, chickens were mock-treated (PBS). Three weeks after the vaccination, all birds were challenged with 10^5 $TCID_{50}$ of HPAI H5N1 A/Viet Nam/1194/04 (A) Kaplan-Meier survival curves, indicating percentage mortality on each day for each group (B-C-D) Blood samples were collected 3 weeks after the first immunization (sH5³ 1x) and 3 weeks after the second vaccination (sH5³ 2x). The sH5³ antibody levels as determined by ELISA (B), the HI titers against H5N1 (NIBRG-14) (C) and the HI titers against sH5³ (D) in serum for each bird. Bars represent geometric means per group. The dotted lines indicate the lowest antibody levels correlating with protection in this experiment.

the chickens of the vaccine dose titration experiment of Fig. 3 at 2, 4 and 7 days after the challenge inoculation. The tracheal and the cloacal swab sampled from each chicken at each particular day were pooled and the presence of viral RNA in the pooled swabs was analyzed using a quantitative RT PCR assay detecting the M1 gene. The results are shown in Table 1. Of the chickens that received a booster vaccination, only 2 birds, both of which had received the lowest amount of antigen, tested positive. Notably, these were the two animals that developed the lowest sH5³-specific antibody titers (Fig. 3C). One of these animals did not survive the challenge. Virus shedding could not be detected in any of the other birds, although 3 swabs gave inconclusive results. Of the chickens vaccinated only once, all animals that died tested positive. None of the birds vaccinated with 10 µg sH5³ tested positive. Of the chickens vaccinated with a lower dose and surviving, two tested positive, but only at day 2 p.i. In conclusion, the vaccinated birds that could control the lethal HPAI H5N1 challenge infection exhibited minimum or no virus shedding.

Efficacy of sH5³ vaccine against a lethal HPAI H5N1 infection in mice.

Finally we examined whether sH5³ would also confer protection in mice. Therefore, 2 groups of 10 mice were vaccinated either once (on day 21) or twice (on day 0 and 21) with 2 µg of sH5³ adjuvanted with Stimune and challenged three weeks later by intranasal inoculation with ~ 10 LD_{50} of H5N1 A/Viet Nam/1194/04. The percentage of mice surviving the infection, median clinical scores and body weights per group observed after the challenge inoculation are shown in Fig. 4. All mock-vaccinated mice succumbed to infection or had to be euthanized by day 9 p.i.. These mice showed severe clinical signs, including respiratory distress and significant weight loss, which continued

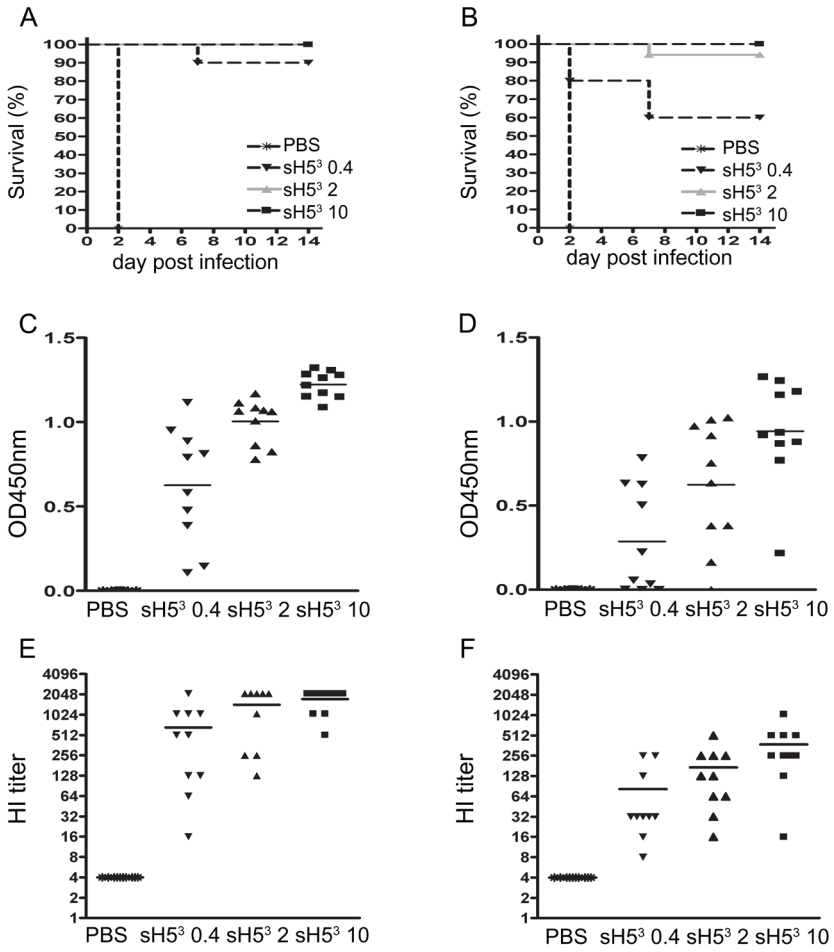

Figure 3. sH5³ dose titration after a single or boost vaccination in chickens. Seven groups of 10 chickens were immunized i.m. with 10, 2 or 0.4 µg sH5³ either once or twice with 3 weeks interval. As a challenge control, one group was mock-treated (PBS). Four weeks after the vaccination, all chickens were challenged with 10^5 $TCID_{50}$ of HPAI H5N1 A/Viet Nam/1194/04. Kaplan-Meier survival curves, indicating percentage mortality on each day for each group that was (mock-)vaccinated twice (A) or once (B). (C-D) The sH5³ antibody levels at the day of challenge as determined by ELISA for each chicken that was (mock-)vaccinated twice (C) or once (D). (E-F) Serum HI titers in the same sera, measured against sH5³ for the chickens that were (mock-)vaccinated twice (E) or once (F). Bars represent the geometric means for the test groups.

until the animals died. A booster vaccination with sH5³ provided 100% protection against the lethal dose of A/Viet Nam/1194/04; none of the mice showed significant signs of disease and their body weights remained constant. A single vaccination with sH5³ did not protect mice against disease and concurrent weight loss; yet 40% of the mice survived. These mice started to recover from day 9 p.i. onwards as demonstrated by their regain of body weight. (Fig. 4C). Fig. 4D shows the pre-challenge anti-sH5³ antibody levels in individual serum samples,as determined by ELISA. Such antibodies could be detected in most vaccinated animals. However, after a single immunization, these levels remained very low compared to those in animals that received a booster vaccination. These results were essentially confirmed by determining the HI titers against sH5³ in the same serum samples. With the exception of one animal, the serum of which demonstrated high levels of auto-agglutination, all mice displayed high HI titers when vaccinated twice and low HI titers when vaccinated once. Thus, mice vaccinated twice with sH5³ were protected against a lethal challenge with HPAI H5N1, while

Table 1. Virus detection in trachea and cloaca swabs collected from vaccinated chickens after H5N1 infection

	Virus detection in swab samples collected from vaccination groups on indicated day p.i.																		
	1x vaccination									2x vaccination									
	10			2			0.4			10			2			0.4			0
Chicken #	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2
1	-	-	-	+	+	±†	-	-	-	-	-	-	-	-	-	-	-	-	+†
2	-	-	-	-	-	-	+	+	+†	-	-	±	-	-	-	-	+	+†	+†
3	-	-	-	-	-	-	+	-	-	-	-	-	±	-	-	-	-	-	+†
4	-	-	-	+	-	-	+	x†	x†	-	-	-	-	-	-	-	-	-	+†
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+†
6	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+†
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+†
8	±	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	+†
9	-	-	-	-	±	-	+	+	+†	-	-	-	-	-	-	-	-	-	+†
10	-	-	-	-	-	-	+	x†	x†	-	-	-	-	±	-	-	-	-	+†

+ = positive; ± = weak signal (CP >31.0 and fluorescence <0.07); - = negative; x = not available

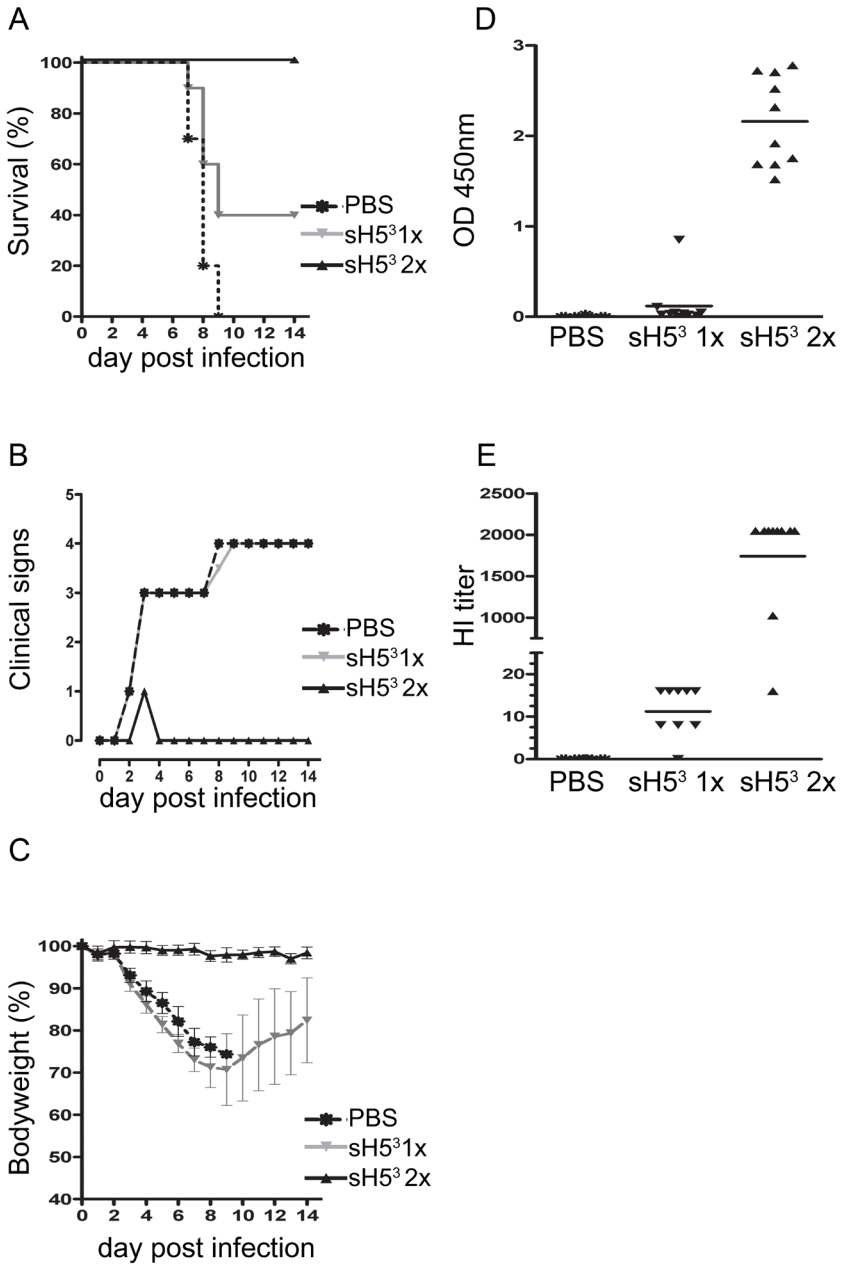

Figure 4. sH5³ vaccination of mice. Groups of 10 BALB/c mice were immunized i.m. with 2 µg sH5³ either once (day 21) or twice with a 3-week interval (day 0 and day 21). As a challenge control, one group of mice was mock-treated (PBS) twice (day 0 and day 21). Three weeks after the vaccination, mice were infected with ~ 10 LD_{50} of A/Viet Nam/1194/04 and monitored daily for clinical signs and body weight during the next 14 days. (A) Kaplan-Meier survival curve indicating percentage mortality on each day for each group. (B) Median clinical scores per group. (C) Mean body weights per group expressed as percentage of starting body weight, plotted as a function of time. Error bars represent the standard deviation. (D-E) Blood samples were collected at the day of challenge. sH5³ antibody levels as determined by ELISA (D). HI titers against sH5³ (E). Bars represent geometric means.

a single vaccination provided partial protection. The differences in protection correlated with the observed serum HI titers.

Discussion

Despite all the - understandable - attention drawn in the past year by media and scientific community to the new pandemic influenza A virus H1N1, a large influenza threat continues to be posed by HPAI H5N1. In 2009, HPAI H5N1 was found in wild birds in Germany, China, Mongolia and the Russion Federation, several outbreaks of the virus in poultry were reported in Viet Nam, Hong Kong, Nepal, India, Bangladesh, Egypt, Lao DPR, and Cambodia, while H5N1 remained endemic in large areas of Indonesia. That same year also dozens of confirmed human cases were reported (WHO timeline of major events; http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html). Even though the virus has so far remained restricted in its ability to infect humans and initiate efficient human-to-human transmission, its persistence and spread among wild birds and poultry holds a continual risk of the emergence of a pandemic strain. This threat can be reduced by vaccination of poultry against H5N1 as this would limit the propagation of the virus and minimize the risk of bird-to-human transmission. In addition, in case a human pandemic H5N1strain would emerge, there would be an immediate need for effective and reliable vaccines matching the pandemic strain. In the present study we evaluated the vaccine potential of a recombinant soluble H5 protein ($sH5^3$) to protect chickens and mice against a lethal infection with HPAI H5N1. The recombinant HA vaccine, which has a short development cycle, proved to be protective after a single immunization in its natural host, while a booster vaccination was needed to confer complete protection in a mammalian model. In addition, the $sH5^3$ induced immunity prevented viral shedding from chickens. These promising results warrant further research into the development of recombinant soluble HA as a fast, safe and effective alternative vaccine not only against H5N1, but against other influenza A viruses as well.

The recombinant HA expression cassette was constructed such that the HA protein was produced and secreted by cells in high yields as a bioactive trimer. The importance of the oligomeric state of the HA protein for the induction of neutralizing antibodies has recently been demonstrated (Wei et al., 2008). High-molecular-weight oligomers and trimers, but not monomers, were found to efficiently induce neutralizing antibodies in mice. This difference was attributed to the preferential induction of antibodies against epitopes present in the monomeric, but not in the trimeric form (Wei et al., 2008). While the soluble recombinant HA trimers were purified using metal affinity chromatography followed by ion-exchange chromatography in previous studies (Wang et al., 2009; Wei et al., 2008), we used a protocol based on the Strep-tag system (Terpe, 2003). Proteins with a Strep-tag exhibit high affinity towards Strep-tactin, an engineered form of streptavidin. By exploiting this highly specific interaction, Strep-tagged proteins can be isolated in one step. Furthermore, because the Strep-tag elutes under gentle, physiological conditions it is especially suited for the generation of native proteins (Zwicker et al., 1999), a characteristic that in the case of HA may contribute to the ability of the recombinant protein to induce neutralizing antibodies.

The efficacy of the $sH5^3$ vaccine was first studied in chickens. Adjuvanted with Stimune, a water-in-oil adjuvant also known as Specol, the $sH5^3$ protein formulation induced an immunity that was completely protective against a lethal H5N1 challenge after administration of two doses ($\geq$ 2 μg $sH5^3$/dose). Importantly, our vaccine preparation also protected chickens after a single immunization. While 100% of the chickens were protected after vaccination with 10 μg $sH5^3$, 90% were protected when using 2 μg. Similar HA doses were previously needed to protect chickens with a vaccine preparation consisting of full length HA proteins, which had been purified from insect cell cultures infected with recombinant baculovirus expressing the H5 gene, emulsified in a water-in-oil adjuvant (Crawford et al., 1999). These results are consistent with the observation that mammalian cell-produced HA trimers elicited similar levels of neutralizing antibodies as trimeric HA produced

in insect cells (Wei et al., 2008). The efficacy of our $sH5^3$ vaccine preparation was furthermore demonstrated by the absence of viral RNA in the protected birds. This would imply that a vaccinated flock can pose a barrier against further spread of circulating virus.

Most conventional influenza vaccines require two vaccination rounds to produce antibody titers sufficiently high to confer full protection to chickens. In this regard, vaccination with sHA^3 provides potential advantages over other vaccine approaches. However, the production costs per dose of sHA^3 compared to egg-cultured inactivated whole-virus vaccines might be higher, even though recombinant protein expression in mammalian cell culture systems has been shown to be highly scalable and productive, with expression levels up to the order of grams of protein per liter (Durocher and Butler, 2009; Tchoudakova et al., 2009). sHA^3 vaccination could however be economically feasible in epidemic situations when millions of chickens have to be vaccinated individually, provided that a single preventive vaccination would suffice. Moreover, vaccinated flocks housed in endangered regions rapidly achieve a state of protective immunity. This may be a particularly valuable feature in the event of a pandemic, when the virus transmission cycle needs to be interrupted as soon as possible and the risk of exposure of farmers, veterinarians and people in monitoring teams to potentially zoonotic HPAI should be limited to the upmost extent.

The efficacy of the $sH5^3$ vaccine was also studied in mice. The vaccine preparation was completely protective against a lethal H5N1 challenge after 2 doses. Immunization with a single dose resulted in 40% survival. These differences in protection levels correlated with the observed anti-$sH5^3$ titers in the animals' sera. As the dose (2 μg) received by the mice is at least comparable, relative to their body weights, to the dose that conferred complete protection in chickens after a single immunization (10 μg), these results appear to suggest that the $sH5^3$ is more effective in conferring a protective immune response in chickens than in mice. The reason for this apparent discrepancy is unclear and warrants further investigations. In the only other study so far that used soluble HA trimers as a vaccine preparation in mice, much less protection against challenge with H5N1 was observed after two immunizations (Wang et al., 2009). Although the H5 trimer produced in this latter study differed from the one that we used, with respect to the trimerization motif (t4 foldon vs GCN4 trimerization motif) and the purification tag (His tag vs Strep tag II), the difference is more likely to be explained by the different adjuvant used (Alum vs Stimune). Alum is known to induce low antibody titers when used with subunit vaccines (Gupta, 1998), while Stimune has been reported to generate long-lasting, functional antibody responses (Beck et al., 2003; Herfst et al., 2007; Leenaars et al., 1994). Stimune is however not licensed for human use.

In conclusion, we have shown that the $sH5^3$ protein produced in mammalian cells elicited protective immune responses in mice and chickens when adjuvanted with Stimune. Chickens protected against the lethal H5N1 challenge also did not shed the virus at day 7 post infection. As these recombinant HA vaccines can be manufactured with high yields and a relatively short lead time, they offer an attractive alternative vaccination strategy, which will allow a rapid response to circulating and potentially pandemic influenza viruses.

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Author contributions
Conceived and designed the experiments: LC RdV PR CdH. Performed the experiments: LC RdV EdBL AR. Analyzed the data: LC RdV CdH. Wrote the paper: LC PR CdH.

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Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus

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Abstract
In 2009 a new influenza A/H1N1 virus strain ("pandemic (H1N1) 2009", H1N1v) emerged that rapidly spread around the world. The virus is suspected to have originated in swine through reassortment and to have subsequently crossed the species-barrier towards humans. Several cases of reintroduction into pigs have since been reported, which could possibly create a reservoir for human exposure or ultimately become endemic in the pig population with similar clinical disease problems as current swine influenza strains. A soluble trimer of hemagglutinin (HA), derived from the H1N1v, was used as a vaccine in pigs to investigate the extent to which this vaccine would be able to protect pigs against infection with the H1N1v influenza strain, especially with respect to reducing virus replication and excretion. In a group of unvaccinated control pigs, no clinical symptoms were observed, but (histo)pathological changes consistent with an influenza infection were found on days 1 and 3 after inoculation. Live virus was isolated from the upper and lower respiratory tract, with titres up to 10^6 $TCID_{50}$ per gram of tissue. Furthermore, live virus was detected in brain samples. Control pigs were shedding live virus for up to 6 days after infection, with titres of up to 10^5 $TCID_{50}$ per nasal or oropharyngeal swab. The soluble H1N1v HA trimer diminished virus replication and excretion after a double vaccination and subsequent challenge. Live virus could not be detected in any of the samples taken from the vaccinated pigs. Vaccines based on soluble HA trimers provide an attractive alternative to the current inactivated vaccines.

Introduction

In April 2009 a new influenza A/H1N1 virus strain was detected in two children in Southern California, both suffering from respiratory disease (2009). Full sequence analysis showed that this new influenza strain, currently named "pandemic (H1N1) 2009" (H1N1v), is likely a reassortant between North American and Eurasian swine influenza strains (Garten et al., 2009; Smith et al., 2009). Unlike most other introductions of swine influenza strains in the human population, this strain was successful in human-to-human transmission. The virus spread quickly to other countries and continents and finally, on the 11th of June 2009, the WHO declared this outbreak to be a pandemic, the first one since 1968 (Hong Kong flu).

On 28 April 2009, the Canadian Food Inspection Agency became involved in the first field infection of swine with this H1N1v (Weingartl et al., 2010). Introduction of the virus through an infected human was suspected, but could not be proven. On the 25th of June, a second swine herd, in Argentina, was reported to the World Organization for Animal Health (OIE) as being infected (Pereda et al., 2010). Also in this case, introduction through infected humans was suspected, but could not be confirmed. In both cases the clinical symptoms in the pigs were rather mild and recovery of the pigs was uneventful. Many more such cases in swine herds have since been detected, in countries all over the world.

The susceptibility of pigs to this particular virus strain has been confirmed in several experimental studies (Brookes et al., 2010; Lange et al., 2009; Vincent et al., 2010). Clinical symptoms in pigs were shown to be similar to those caused by endemic swine influenza strains. It was also shown that virus transmission to susceptible pigs, at least those naïve for antibodies against any swine influenza viruses, readily occurs. Whether the H1N1v is able to outcompete endemic H1N1 and/or H1N2 strains, or whether it would be able to co-exist with these endemic strains in swine, is as yet unknown. In such cases pigs may become a reservoir from which repeated introductions into the human population could occur.

It can be expected that the more widespread the infection becomes in humans, the more often the virus will be introduced in swine. Infected pigs may therefore become a source of infection for humans, even if the virus would not succeed in becoming endemic in the pig population. Humans in contact with high concentrations of infected pigs may be exposed to much higher amounts of virus than when exposed to infected humans. This could result in much more severe clinical symptoms, even in a higher mortality. Possible contact persons are not just the farmers and their family, but also include veterinarians, pig consultants, traders, transporters, visitors of pig markets and slaughterhouse personnel.

A way to decrease the risk for people involved may be vaccination of pigs, with the primary aim of reducing virus excretion and therefore exposure of humans to the virus. Conventional vaccines consist of whole viruses propagated in either embryonated chicken eggs or cell cultures, which are subsequently inactivated and adjuvanted. In case new such vaccines, based on new influenza subtypes, are needed, the development, registration and subsequent production takes a relatively long time, taking care of safety, efficacy and production issues. As an alternative a recombinant purified hemagglutinin (HA) could be used as a vaccine. One such recombinant, a secretable, soluble trimer of the HA ectodomain from the H1N1v influenza strain, was constructed and formulated as a vaccine to be tested in swine. The aim of this study was to determine to what extent this vaccine is able to protect against infection with the H1N1v influenza strain, especially with respect to reducing virus replication and excretion. It was shown that the HA trimer was almost complete able to prevent virus replication and excretion after a double vaccination.

Materials & methods

Study Desgin

The study was carried out with 18 pigs, divided in two groups of 9. In one group the pigs were vaccinated twice, with a four week interval. At the age of 10 weeks they were vaccinated for the first time. The other group was an unvaccinated control group. Three weeks after the second vaccination the animals in both groups were challenged, resp. inoculated with the H1N1v virus. At days 1 and 3 post inoculation (p.i.) 3 pigs from each group were euthanized. The remaining 3 pigs in each group were euthanized at day 21 p.i., the end of the experiment,

The design of the experiment was evaluated and approved by the Ethical Committee for Animal Experiments of the Animal Sciences Group.

Animals

Nine-week-old piglets were purchased from a high health breeding herd in which no seroconversions against any influenza subtype had been observed for more than 2 years. Before purchasing the pigs, all were tested individually with an NP-ELISA (IDEXX) and in hemagglutination inhibition assays against H1N1, H1N2 and H3N2 influenza virus strains that are endemic in the swine population.

Vaccine and vaccination

Based on H3 numbering, a cDNA clone corresponding to residues 16 to 524 of the HA from A/California/04/2009(H1N1) (Genbank accession no. ABW90137.1) was synthesized using human-preferred codons by GenScript USA Inc. The HA ectodomain encoding cDNA was cloned into the pCD5 expression vector for efficient expression in mammalian cells (Zeng et al., 2008). The pCD5-Cal/04/09 vector had been modified such that the HA-encoding cDNA was cloned in frame with DNA sequences coding for a signal sequence, a GCN4 isoleucine zipper trimerization motif (KRMKQIEDKIEEIESKQKKIENEIARIKK) (Harbury et al., 1993) and the Strep-tagII (WSHPQFEK; IBA, Germany). The HA ectodomain was expressed in HEK293T as previously described (de Vries et al., 2010). HA protein expression and secretion was confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). Secreted HA proteins were purified using Strep-tactin sepharose beads according to the manufacturer's instructions (IBA, Germany). The concentration of purified protein was determined by using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according the manufacturer's instructions. Oligomeric status of the HA protein was determined by analyzing the elution profile using a Superdex200GL 10-300 column and by blue-native gel-electrophoresis. The vaccine was formulated with Specol (Boersma et al., 1992; Bokhout, Van Gaalen, and Van Der Heijden, 1981) as an adjuvant, at 25 µg HA per dose of 2ml. Pigs were vaccinated intramuscularly.

Virus and inoculation

Influenza virus A/Netherlands/602/2009 (H1N1)v was isolated from the first confirmed case in the Netherlands (Munster et al., 2009). The patient was a 3-year old boy, developing a fever and symptoms of respiratory disease after returning from Mexico with his family. A nasal swab was taken before the patient was treated with oseltamivir. Virus was initially grown on embryonated eggs, and subsequently passaged on Madin-Darby canine kidney (MDCK) cells before it was used to inoculate the pigs. This virus differs by 8 amino acids from the A/California/4/2009 (H1N1)v strain. Because it is, however, closer to the consensus sequence, it is considered representative of the circulating H1N1v influenza strains. Pigs were inoculated with a dose of $10^{7.5}$ $TCID_{50}$, suspended in 2 ml PBS, of which 1 ml was nebulised within each nostril.

Observations and sampling
Clinical symptoms and body temperature were recorded daily from day 3 before inoculation until the end of the experiment. At days 1, 2, and 3 p.i. clinical symptoms and body-temperature were recorded twice per day with a 12 hour interval.

Serum samples were collected during both times of vaccination, at the time of inoculation, and 7, 10, 14 and 21 days p.i.

Oropharyngeal and nasal swabs were collected daily from all animals still alive from day 0 to 11 p.i., and on days 14, 17 and 21 p.i. For oropharyngeal swabs multi-layered gauze dressings in a pair of tweezers were used to scrape the palatine tonsils at the dorsal pharyngeal wall, behind the soft palate. Nasal swabs were collected using sterile rayon swabs (Medical Wire & Equipment, Corsham, United Kingdom). The swabs were suspended in 4 ml (oropharyngeal swabs) or 2 ml (nasal swabs) medium (Eagle minimum essential medium (EMEM) (Gibco, Invitrogen, Breda, The Netherlands) with 5% fetal bovine serum (FBS), and 10% antibiotics). After centrifugation (1800 g for 15 min) the samples were stored at -70°C until analysis.

At days 1 and 3 p.i. 3 pigs from each group were euthanized and a gross pathological examination was performed. Thirteen different tissue samples were collected from each of these pigs for histological and/or virological examinations: nasal mucosa from the turbinates, tonsils, trachea, tracheobronchial lymph nodes (TBLN), six pieces of lung, brainstem, cerebrum and cerebellum. The lung pieces originated from the right apical lobe (lung 1), the right cardiac lobe (lung 2), the right diaphragmatic lobe (lung 3), the left diaphragmatic lobe (lung 4), the left cardiac lobe (lung 5), and the left apical lobe (lung 6).

For (immuno)histology, tissue samples were fixed in 10% neutral buffered formalin for a maximum of 48 hours, embedded in paraffin and tissue slides were stained with hematoxylin and eosin. For immunohistological evaluation tissue slides were mounted on silicon coated glass slides, deparaffinised and exposed to 1% H_2O_2 to block endogenous peroxidase. After washing, the slides were treated with protease type XXIV (0.1mg/ml, diluted in PBS, Sigma®, order nr. P8038) for 10 minutes. Samples were incubated with 10% normal goat serum and thereafter incubated with a murine monoclonal antibody, directed against the Influenza A virus nucleoprotein (HB65 MCA) for 45 minutes. After rinsing, slides were incubated with a HRP labelled polymer conjugated to an anti-murine IgG antibody (DAKO Envision™+ System) and to visualize the immunohistochemical signal followed by treatment with diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin eosin.

For virological examination, 0.1 g from each tissue sample was added to 0.6 ml of medium (same as used for the swabs), and homogenized using the MagNa Lyser (Roche Applied Science) for 30 sec at 3500 g. After centrifugation (9500 g for 5 min), 0.4 ml of the supernatant was added to a further 1.2 ml of medium and stored at −70 °C until analysis.

At day 21 p.i. the remaining pigs where euthanized. Lungs were collected for a broncho-alveolar lavage, using 50 ml of cold (4°C) phosphate-buffered saline (PBS). The broncho-alveolar lavage fluid (BALF) obtained was centrifuged (9500 g for 5 min) and stored at −70 °C until analysis.

PCR
Nasal swabs, oropharyngeal swabs, tissue homogenates and BALF were all tested with a quantitative real time RT-PCR (qRT-PCR). A one-tube qRT-PCR was performed to detect the matrix gene of the influenza virus. The Qiagen one-step RT-PCR kit was used with a 25 µl reaction mixture

containing 1 µl of kit-supplied enzyme mixture, 1 µl dNTP mix, 4 U of RNase inhibitor (Promega, Madison, WI), 0.5 µM of each primer M-Fw (5′-CTTCTAACCGAGGTCGAAACGTA-3′), M-Rev (5′-CACTGGGCACGGTGAGC-3′), and 0.3 µM of probe M (5'-6FAM-TCAGGCCCCCTCAAAGCCGA-X-ph). The qRT-PCR was performed with the MX4000 (Stratagene®, Texas). The RT-PCR program consisted of 30 min at 50 °C and 15 min at 95 °C. A three-step cycling protocol was used as follows: 95 °C for 5 s, 58 °C for 15 s, and of 72 °C for 20 s for 45 cycles. In each PCR run a standard curve was included with a known virus concentration. Results of the PCR are expressed as $TCID_{50}$-equivalents per swab or per gram of tissue. $TCID_{50}$-equivalents are a relative measure and not necessarily represent live virus.

Virus isolation

Nasal swabs, oropharyngeal swabs, tissue homogenates and BALF were all tested in a virus isolation with end-titration on MDCK-I-BD5 cells (Meijer et al., 2004). Samples were initially diluted with the same amount of GMEM/EMEM medium containing 1% bovine serum albumin and antibiotics (twofold dilution). This initial dilution was serially diluted tenfold in the same medium. The diluted samples (100 µl/well) were mixed with 150 µl of 2×10^5 MDCK-I-BD5 cells/ml and incubated for 48 hours at 37°C and 5% CO_2. The monolayers were subsequently washed with PBS, frozen at −20 °C and fixed with 4% cold (4°C) paraformaldehyde for 10 min. After washing, viral NP-protein containing cells were stained using HRPO-conjugated monoclonal antibody HB65 and 3-amino-9-ethyl-carbozole (AEC; Sigma–Aldrich, The Netherlands) as a substrate for HRPO. Samples were tested in eightfold and titres were calculated according to the method of Spearman-Kärber (Finney, 1978). Virus titres are expressed as $TCID_{50}$ per swab or per gram of tissue.

Hemagglutination inhibition test

The hemagglutination inhibition (HI) test was carried out as described before (OIE, 2008). Before testing, samples were inactivated for 30 minutes at 56°C. Subsequently they were pre-treated with receptor destroying enzyme (RDE) and chicken red blood cells to remove non-specific agglutinins and hemagglutination inhibitors. Starting at an initial dilution of 1:10, sample were tested in two-fold dilution series. Samples were incubated for 60 minutes after adding antigen and another 45 minutes after adding chicken red blood cells and subsequently read. The antigens used in the test were the A/Netherlands/602/2009 (H1N1)v and, for swine influenza, the A/Swine/Best/96 (H1N1) (Loeffen et al., 1999) and A/Swine/Gent/7625/99 (H1N2) (Van Reeth, Brown, and Pensaert, 2000). All were standardised at 4 hemagglutinating units per 25 µl.

Virus neutralisation test

The virus neutralisation tests were performed on MDCK-I-BD5 cells (Meijer et al., 2004). Sera were heat inactivated for 30 min at 56 °C before testing. Twofold serial dilutions of the sera were made in GMEM/EMEM medium containing 1% bovine serum albumin and antibiotics in 96-well plates. The diluted sera (50 µl/well) were mixed with 100 $TCID_{50}$ of the influenza viruses (50 µl) and incubated at 37 °C and 5% CO_2 for 1 h. Thereafter 150 µl of 2×10^5 MDCK-I-BD5 cells/ml were added to each well. The plates were incubated at 37 °C and 5% CO_2 for 48 h. The monolayers were washed with PBS, frozen at −20 °C and fixed with 4% cold (4 °C) paraformaldehyde for 10 min. After washing, viral NP-protein containing cells were stained using HRPO-conjugated monoclonal antibody HB65 and 3-amino-9-ethyl-carbozole (AEC; Sigma–Aldrich, The Netherlands) as a substrate for HRPO. A complete lack of staining was scored as positive neutralisation. VN-antibody titres were expressed as the reciprocal of the highest serum dilution giving positive neutralisation.

Results

Clinincal symptoms

No clinical symptoms were observed in any of the inoculated animals, neither in the control group, nor in the vaccinated group. Body temperatures of all animals remained within normal range during the whole animal experiment. One of the pigs from the vaccinated group died between the first and second vaccination of unrelated causes (Mulberry Heart Disease) and could not be replaced. In this group therefore only 2 pigs were left after day 3 p.i. until the end of the experiment at day 21 p.i.

Pathological and histological findings

At day 1 p.i. some reduced retraction of the lungs was observed in one of the control pigs, and some moderate hyperaemia of the nasal mucosa in one of the vaccinated pigs. Histology of the lungs revealed a slight to mild focal interstitial pneumonia in all control pigs, accompanied with a mild catarrhal bronchiolitis in one of them. A slight focal interstitial pneumonia was present in one of the vaccinated pigs. Immunohistochemistry showed the presence of virus in lungs and nasal mucosa of all control pigs, and in some individual cases also in the trachea, tonsil and tracheobronchial lymph node. Vaccinated pigs were all negative in the immunohistochemistry.

Gross pathology revealed at 3 days p.i. a mild to moderate focal or multifocal pneumonia in all control pigs. In two of the vaccinated pigs a mild reduced retraction of the lungs was observed, with some moderate hyperaemia of the trachea in one of these cases, and some moderate hyperaemia of the nasal mucosa in the other. Histology revealed a mild to moderate interstitial pneumonia in all three control pigs, with a moderate catarrhal bronchitis/bronchiolitis with focal epithelial necrosis and intra luminal cell debris in two of these pigs. Two of the three vaccinated pigs showed some slight interstitial pneumonia. Immunohistochemistry of the lungs was again positive in all three control pigs, with 2 of them also positive in the nasal mucosa and trachea. Vaccinated pigs were all negative in the immunohistochemistry.

Antigen detection

From all control pigs, live virus could already be isolated at day 1 p.i. from nasal and oropharyngeal swabs, at titres ranging from $10^{2.4}$ to $10^{6.4}$ $TCID_{50}$ per swab. Comparable virus titres were observed until day 4 p.i., declining thereafter. No live virus could be isolated from day 6 p.i. (nasal swabs) or day 7 p.i. (oropharyngeal swabs) onward, respectively. Virus titres seemed overall slightly higher in oropharyngeal swabs than in nasal swabs. From none of the vaccinated pigs live virus could be isolated from nasal or oropharyngeal swabs at any time (Fig 1A and 1B).

Viral genome titres peaked on the same days as live virus, but could be detected somewhat longer, until day 10 p.i. in oropharyngeal swabs and day 9 p.i. in nasal swabs from control animals (although one further single weak positive nasal swab was found on day 14 p.i.). From the vaccinated pigs, only on day 1 p.i. genome was detected from multiple animals, but at low amounts (Fig 1C and 1D).

On day 1 p.i. live virus could be isolated from the control animals from the upper and lower respiratory tract, with the highest titres in the nasal mucosa and trachea. Low amounts of live virus were also detected in the cerebrum and cerebellum. No live virus was isolated from TBLN (Fig 2A). On day 3 p.i. live virus was only detected from the upper and lower respiratory tract, but no longer from parts of the central nervous system and still not from the TBLN (Fig 2B). From the vaccinated animals no live virus could be isolated from any of the tissue samples at either time point. (Fig 2A and 2B)

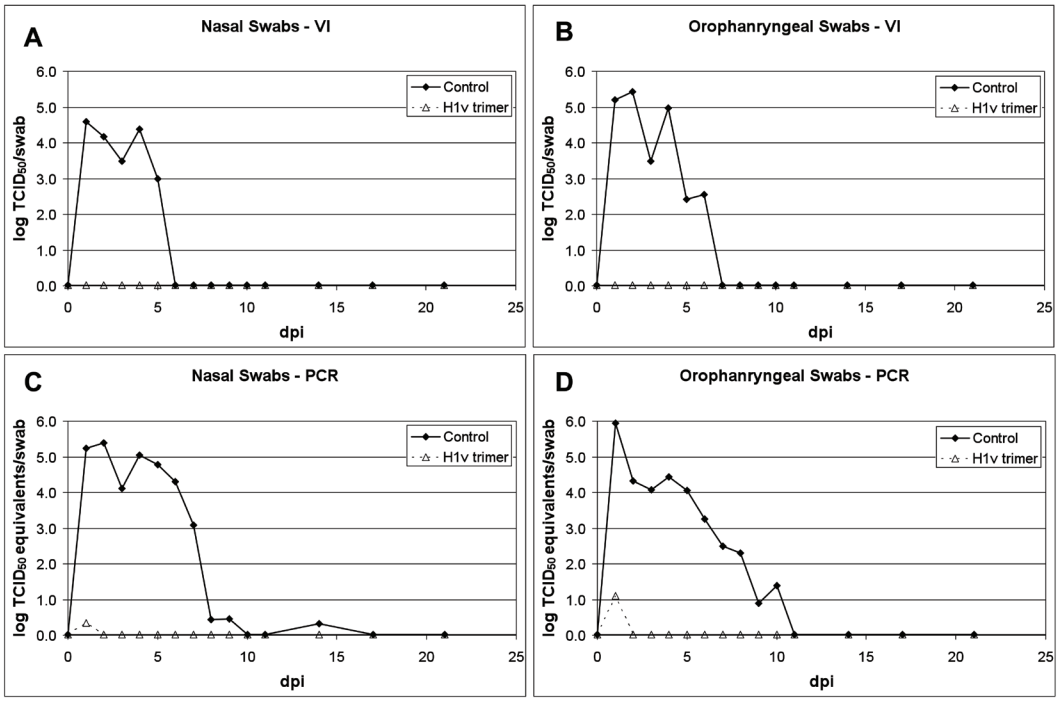

Figure 1: Virus Isolation (VI; in log $TCID_{50}$) and PCR (in log $TCID_{50}$ equivalents) titres in nasal and oropharyngeal swabs.

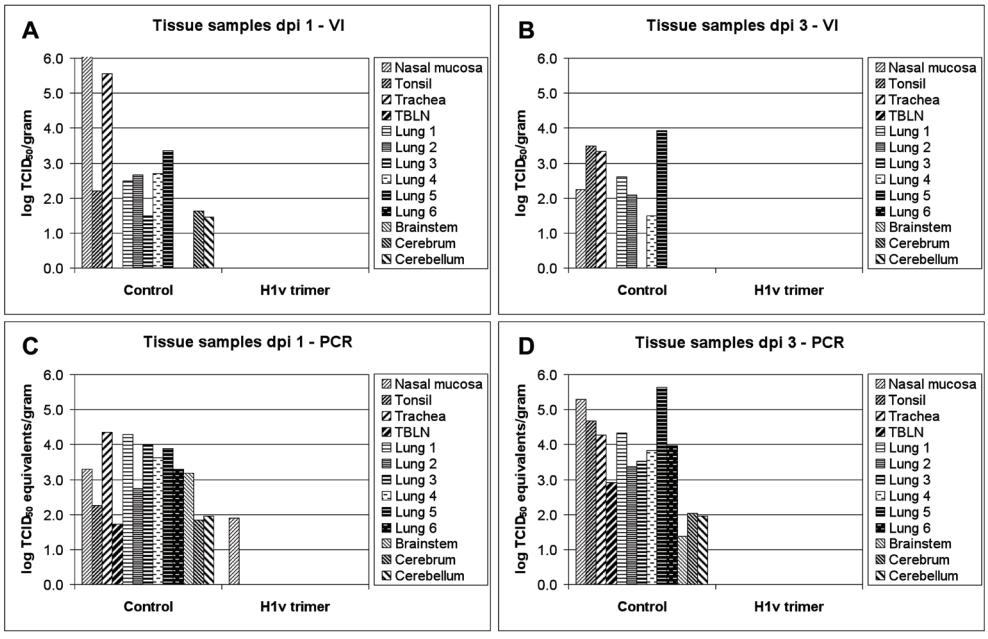

Figure 2: Virus Isolation (VI; in log $TCID_{50}$) and PCR (in log $TCID_{50}$ equivalents) titres in 13 different pieces of tissue.

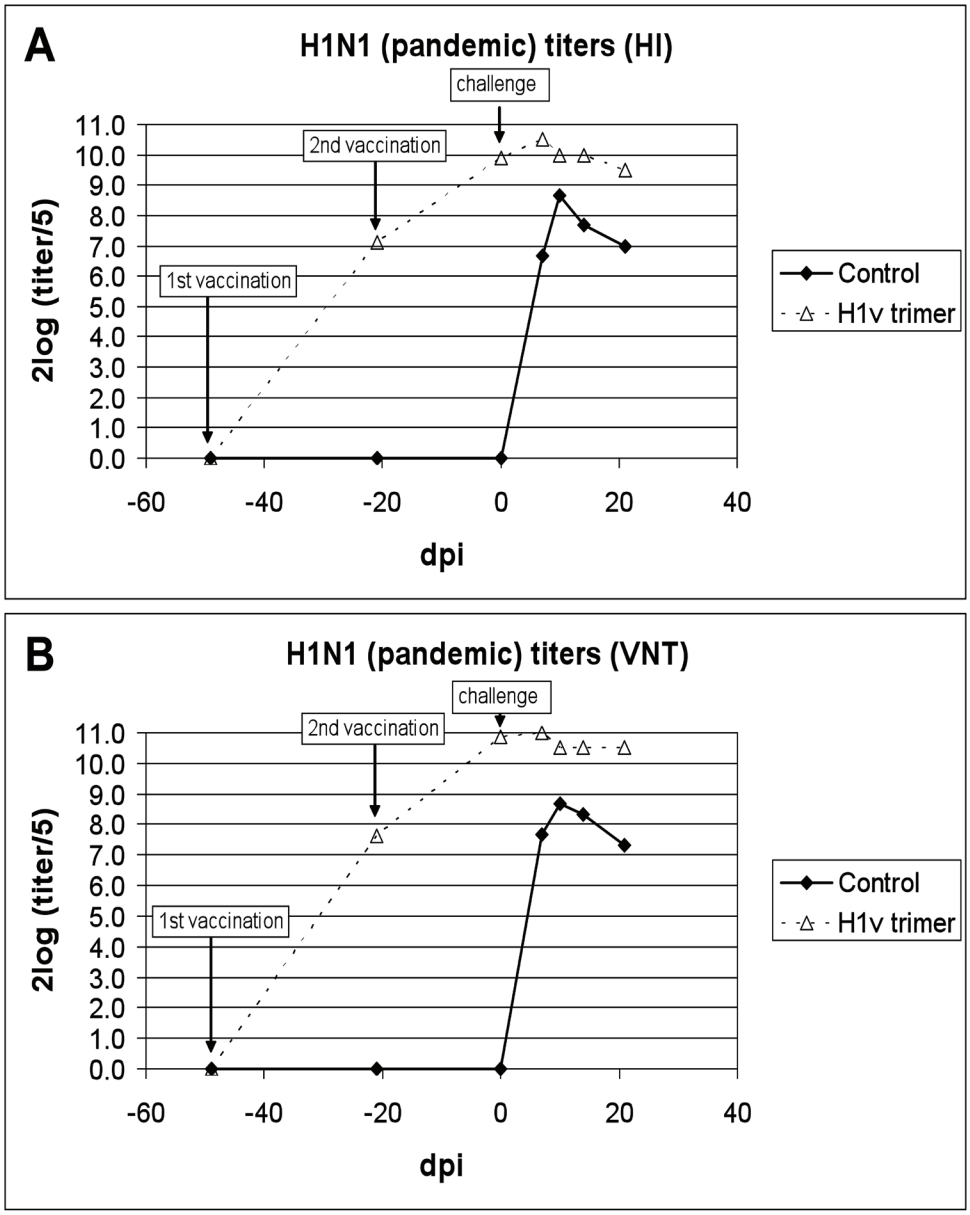

Figure 3: Development of hemagglutination inhibition (HI) and virus neutralisation (VNT) titres against H1N1v after vaccination and subsequent challenge.

On day 1 and day 3 p.i. virus genome could be detected by PCR from all tissue samples from the control pigs, including from the TBLN and central nervous system. In only one of the vaccinated animals, viral genome was detected in nasal mucosa at day 1 p.i. (Fig 2C and 2D).

BALF from pigs euthanized at day 21 p.i. was negative in the PCR.

Serology
Already after the first vaccination, at the time of the second vaccination, high antibody titres against the homologous H1N1v strain were seen, both in the HI-test (Fig 3A) and in a VNT (Fig 3B). The second vaccination resulted in a further rise of these antibody titres to levels >10,000.

After inoculation with the challenge virus, the non-vaccinated animals responded with titres up to 2560, peaking at 10 days p.i. and then decreasing again. In the vaccinated animals almost no changes were seen in the levels of the titres after the challenge (Fig 3A and 3B).

Cross-reactivity, both after vaccination and after inoculation/challenge, was seen in HI-tests and VNT when a swine influenza strain of subtype H1N1 was used in the test, but not when an H1N2 strain of swine origin was used. Results for the HI-tests are shown in Fig 4. VNT results are not shown as they were almost identical to the HI-results.

Discussion

The soluble H1N1v HA trimer was almost completely able to prevent virus replication and excretion after a double vaccination and subsequent homologues challenge. Live virus could not be detected in any of the samples taken from the vaccinated pigs. Viral genome was only detected at day 1 p.i. in nasal and oropharyngeal swabs and at day 1 p.i. in the nasal mucosa from one of the euthanized pigs. The amount of genome detected from the swabs was very low, but genome could be detected in multiple animals. This viral genome may very well represent residual challenge virus. However, some very limited virus replication in the upper respiratory tract in the vaccinated groups can not be excluded, as high levels of virus replication were observed already seen at day 1 p.i. in the control group.

A recombinant purified HA has several advantages compared to whole inactivated vaccines. Most importantly, the development of an HA-based vaccine has a relatively short lead time, allowing for a fast response to a potential emergency situation. It can be produced using safe and scalable conditions, without the need of growing live viruses and the disadvantages related to that. HA vaccines also allow for the use as marker vaccines, although this will depend also on other circulating influenza strains in the target population. Marker vaccines make it possible to serologically detect and monitor infections in a vaccinated population, allowing for the collection of invaluable epidemiological data.

The advantage of recombinant HA trimers over recombinant HA monomers is that the former induce higher levels of neutralising antibodies (Wei et al., 2008). In part this is likely due to the fact that trimers mimic the natural membrane-bound structure, including the relevant epitopes to induce neutralising antibodies against. Trimeric HA preparations therefore seem more promising vaccine candidates than previously used HA monomers.

Vaccination of pigs reduces the exposure of humans to the influenza virus almost completely. In case pigs are deemed a potential source of infection for humans, vaccination of herds at risk, or even the entire pig population, therefore seems a realistic option. The vaccine could however also be used for humans themselves. Similar results with an HA trimer based on H5N1 in poultry and mice (Cornelissen et al., 2010), but also ferrets (Bosch et al., 2010), suggest that the use of these recombinant HA trimers is promising in general.

In this experiment we used a rather high dose of HA as proof of principle for the soluble trimer. Further studies would need to determine the efficacy of the vaccine at lower doses. The lower the dose, the easier it would be to produce sufficient quantities of vaccine in a short time, which is one of

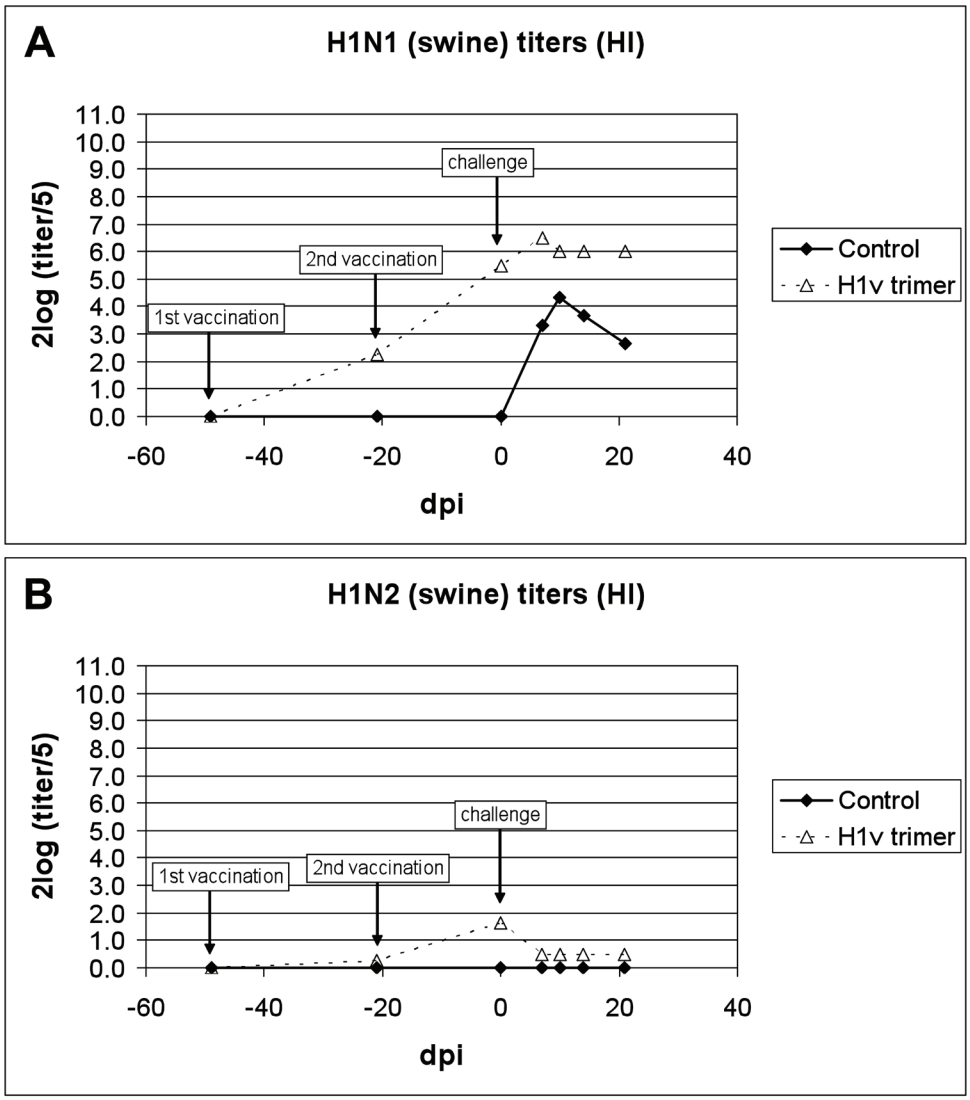

Figure 4: Cross-reaction in the hemagglutination inhibition (HI) test when using H1N1 and H1N2 subtypes of swine origin after vaccination and challenge.

the most crucial issues during a pandemic or other emergency situation. Furthermore, it would make the vaccine more cost-affordable, which is especially relevant for continuous use of the vaccine in pig herds, for instance for use of this kind of vaccines against swine influenza strains that are endemic.

Contrary to previous inoculation studies with the H1N1v influenza virus (Brookes et al., 2010; Lange et al., 2009; Vincent et al., 2010), no clinical symptoms were seen in the inoculated control animals. Nevertheless, virus titres from nasal and oropharyngeal swabs were higher than published before (Brookes et al., 2010), and also relatively high virus titres were found in all parts of the lungs, providing sufficient evidence that the inoculation itself was successful. Furthermore, pathological

changes, both macroscopic and microscopic, were abundantly present in the unvaccinated controls, while only some minor changes were seen in some of the vaccinated pigs. In our study the pigs were much older than in the other published studies. Whether this explains the lack of clinical symptoms, remains to be seen. In a previous study with swine influenza virus in naïve pigs, clinical symptoms seemed to be even more severe in older pigs (Loeffen et al., 2003).

Antibodies against the H1N1v influenza virus are readily detected by an HI assay in which a current European swine strain of the H1N1 subtype is used. This cross-reactivity may result in difficulties to correctly identify infections in swine with H1N1v influenza strains by serology. Infections with swine H1N1 influenza strains are very common in many European countries, with seroprevalences in sows up to 80%, and herd prevalences up to more than 95% (Van Reeth et al., 2008). On the other hand, due to this high prevalence of H1N1 antibodies, it may be more difficult for the H1N1v influenza virus to become endemic in the swine population. Currently no reports can be found that suggest a wide spread of H1N1v influenza virus in swine populations where other H1N1 strains are endemic. It remains to be seen how the epidemiology of H1N1v will develop, whether it will be able to co-circulate with current H1N1 strains or whether one strains will eventually predominating the other. Furthermore, recombination with current swine strains in Europe could occur, as happened before with the European swine H3N2 (Castrucci et al., 1993) and H1N2 strain (Brown et al., 1998). This could increase the potential of the H1N1v influenza strain to become endemic in the swine population.

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Recombinant soluble, multimeric HA and NA exhibit distinctive types of protection against pandemic swine-origin 2009 A(H1N1) influenza virus infection in ferrets

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Abstract
The emergence and subsequent swift and global spread of the swine-origin influenza virus A(H1N1) in 2009 once again emphasizes the strong need for effective vaccines that can be developed rapidly and applied safely. With this aim we produced soluble, multimeric forms of the 2009 A(H1N1) HA (sHA_3) and NA (sNA_4) surface glycoproteins using a virus-free mammalian expression system and evaluated their efficacy as vaccines in ferrets. Immunization twice with 3.75-µg doses of these antigens elicited strong antibody responses, which were adjuvant-dependent. Interestingly, co-administration of both antigens strongly enhanced the HA-specific, not the NA-specific responses. Distinct patterns of protection were observed upon challenge inoculation with the homologous H1N1 virus. Whereas vaccination with sHA_3 dramatically reduced virus replication - e.g. by lowering pulmonary titers by about 5 $\log_{10}$ units -, immunization with sNA_4 markedly decreased the clinical effects of infection such as body weight loss and lung pathology. Clearly, optimal protection was achieved by the combination of the two antigens. Our observations demonstrate the great vaccine potential of multimeric HA and NA ectodomains, as these can be easily, rapidly, flexibly and safely produced in high quantities. In particular our study underscores the underrated importance of NA in influenza vaccination, which we found to profoundly and specifically contribute to protection by HA. Its inclusion in a vaccine is likely to reduce the HA dose required and to broaden the protective immunity.

Introduction

The recent emergence of the pandemic swine-origin 2009 A(H1N1) influenza virus strongly emphasizes the potential of influenza viruses to cause morbidity and mortality in the human population on a global scale. Worldwide over 200 countries and overseas territories or communities have reported laboratory-confirmed cases of the pandemic virus including more than 16,000 deaths. Vaccination is the primary method to prevent or lower the burden of influenza disease. However, as illustrated again by the 2009 pandemic, a rapid response during the early phase of an outbreak is hampered by the time-consuming vaccine strain preparation and vaccine manufacturing process currently used. This, combined with the notorious capacity of influenza viruses to escape from existing immunity by antigenic drift and shift, stresses the need for novel, safe and preferably broadly effective vaccines that can be produced rapidly and in flexible response to newly emerging antigenic variants.

The currently licensed influenza virus vaccines are composed of the viral envelope glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). Antibodies elicited by these two large glycoproteins have distinct properties in immunity against influenza virus. Antibodies to HA generally neutralize viral infectivity by interference with virus binding to sialic acid receptors on the target cells or, subsequently, by preventing the fusion of the viral and cellular membrane through which the viral genome gains access to the target cell. Antibodies to NA disable release of progeny virus from infected cells by inhibiting the NA-associated receptor destroying enzymatic activity. The HA-mediated humoral immunity has been characterized most extensively and shown to prevent virus infection. The contribution of NA antibodies to preventing disease has been studied less well. They appeared to produce a kind of permissive immunity (Johansson, Bucher, and Kilbourne, 1989) characterized by a decrease in infectious virus release from apical surfaces of infected epithelia (Chen et al., 1999; Deroo, Jou, and Fiers, 1996; Johansson, Bucher, and Kilbourne, 1989; Johansson, Grajower, and Kilbourne, 1993; Sandbulte et al., 2007; Schulman, Khakpour, and Kilbourne, 1968; Sylte and Suarez, 2009), reducing the probability of virus shedding and spread into the environment.

Immunization with the combination of HA and NA provides enhanced protection against influenza (Chen et al., 1999; Johansson, 1999; Johansson and Kilbourne, 1993). Although HA and NA are equivalently immunogenic (Johansson, Bucher, and Kilbourne, 1989), the humoral immune response towards conventional inactivated vaccines or virus infection is naturally skewed towards HA since HA and NA occur on the viral surface at an approximately 4:1 ratio (Webster, Laver, and Kilbourne, 1968). In addition, in intact virions the HA immunologically outcompetes NA in B and T cell priming as shown in mice (Johansson, Moran, and Kilbourne, 1987). This antigenic competition is not seen in vaccinated animals when HA and NA are administered separately (Johansson and Kilbourne, 1993; Powers, Kilbourne, and Johansson, 1996). The currently licensed pandemic vaccines as well as the seasonal trivalent vaccines are generally prepared from whole viruses and are hence biased to contain more HA than NA antigen. Adapting the HA-NA ratio in vaccine formulations in favor of NA may provide a more balanced humoral immune response resulting in higher NA antibody levels and increased protection against disease (Johansson, Pokorny, and Tiso, 2002; Sylte and Suarez, 2009).

Recombinantly produced HA and NA antigens allow the development of vaccines in which the relative amounts of both antigens can be easily controlled. Eukaryotic expression systems, both mammalian and insect, are the preferred platforms for production of such glycoproteins in view of their better preservation of the proteins' natural antigenic structure. We have addressed the efficacy of recombinantly produced HA and NA subunits of the 2009 A(H1N1) influenza virus as vaccines against homotypic influenza virus in a ferret model, with particular emphasis on the contribution of the NA antigen. Thus we expressed soluble, multimeric forms of the HA and NA antigens of the

pandemic H1N1 virus in a mammalian expression system, purified the glycoproteins by single-step affinity chromatography and subsequently immunized ferrets either with one or with both antigens and with or without ISCOM Matrix M as an adjuvant. The animals responded serologically to both antigens, but only when administered with the adjuvant. Interestingly, inclusion of NA in the vaccine enhanced the levels of HA antibodies and of virus neutralizing activity. Significant protection, as judged particularly from the dramatically ($5\log_{10}$ units) reduced viral lung titers, was observed upon homologous challenge in the animals immunized with HA-containing vaccines in combination with ISCOM Matrix M. Interestingly, vaccine formulations containing NA clearly reduced the clinical effects of infection.

Materials and Methods

Influenza A challenge virus

Influenza virus A/Netherlands/602/2009 was isolated from the first case of a laboratory confirmed 2009 A(H1N1) infection in The Netherlands by inoculation of 11-day old embryonated chicken eggs (Munster et al., 2009). Virus stocks of influenza virus A/Netherlands/602/2009(H1N1) were prepared by infecting confluent Madin-Darby Canine Kidney (MDCK) cells. After cytopathologic changes were complete, culture supernatants were cleared by low speed centrifugation and stored at −70°C. Infectious virus titers were determined in MDCK cells as described previously (Rimmelzwaan et al., 1998). All experiments with these viruses were performed under Bio Safety Level (BSL)-3 conditions.

Preparation of HA and NA antigens

Human codon optimized sequences encoding the soluble hemagglutinin ectodomain (sHA; a.a.17-522) and the neuraminidase head domain (sNA; a.a.75-469) of influenza virus A/California/04/2009(H1N1) were synthesized (GenScript) and cloned into a derivative of expression plasmid pS1-Ig (Li et al., 2003) for expression in HEK293T cells. The HA gene was preceded by a sequence encoding an N-terminal CD5 signal peptide and followed by sequences encoding a C-terminal artificial GCN4 trimerization domain (GCN4-pII) (Harbury et al., 1993) and a *Strep*-tag for affinity purification (IBA GmbH) as described recently (Cornelissen et al.; de Vries et al.). The NA gene was preceded by sequences successively coding for an N-terminal CD5 signal peptide, a double *Strep*-tag (One-STrEP; IBA GmbH) and an artificial GCN4 tetramerization domain (GCN4-pLI) (Harbury et al., 1993).

Protein expression and purification

HEK293T cells were transfected with the sHA and sNA expression plasmids using polyethyleneimine (PEI) in a 1:5 ratio (μg DNA : μg PEI). After a 6 h incubation period the transfection medium was replaced by 293 SFM II expression medium (Invitrogen) supplemented with sodium bicarbonate (3.7 g/liter), glucose (2.0 g/liter), Primatone RL-UF (3.0 g/liter), penicillin (100 units/ml), streptomycin (100 μg/ml), glutaMAX (Gibco), and 1.5% DMSO. Tissue culture supernatants were harvested 5–6 days post transfection and sHA and sNA proteins were purified from the culture medium using *Strep*-Tactin affinity chromatography (IBA GmbH). sHA and sNA protein expression and purification was confirmed by western blotting using a *Strep*-Tactin-HRP conjugate (IBA GmbH; data not shown) and SDS-PAGE analysis. Oligomerization of the proteins was determined by gel filtration chromatography and by blue-native-PAGE analysis. Quantification of protein amounts was done using BSA as a reference.

Ferrets

Healthy young adult outbred female ferrets (*Mustela putorius furo*; between 6 and 12 months old) were purchased from a commercial breeder. The animals were checked for the absence of antibodies

against circulating seasonal A/H1N1 and A/H3N2 influenza viruses and against the swine-origin influenza A/NL/602/09 virus by hemagglutination inhibition assay. An independent animal ethics committee approved the experimental protocol before the start of the experiment.

Immunizations and infections

Thirty-six seronegative ferrets were divided into six groups of 6 ferrets each and vaccinated twice with the following formulations: 3.75 µg sHA_3 + 3.75 µg sNA_4 in phosphate buffered saline (PBS) (group 1); 3.75 µg sHA_3 in ISCOM Matrix M (IMM, Isconova, Uppsala, Sweden) (group 2); 3.75 µg sNA_4 in IMM (group 3); 3.75 µg sHA_3 + 3.75 µg sNA_4 in IMM (group 4); PBS (group 5); IMM (group 6). Vaccinations were performed with an interval of 20 days under anesthesia with ketamine in the quadriceps muscles of the hindleg in a total volume of 1ml. Ferrets were housed in groups and received food and water *ad libitum*. At 32 days after the last vaccination, the animals were anesthetized with ketamine/medetomidine (reversed with atipamezole), weighed and subsequently challenged intratracheally with $1x10^6$ $TCID_{50}$ of influenza A/NL/602/09(H1N1) in a volume of 3 ml PBS (Del Giudice et al., 2009; van den Brand et al.). Ferrets were subsequently monitored three times daily for the development of clinical signs. Before infection and two and four days after infection, nose and throat swabs of each ferret were collected while ferrets were anesthetized with ketamine. Four days after inoculation, animals were weighed and subsequently euthanized by exsanguination while under anesthesia with ketamine and medetomidine. Necropsies were performed according to standard procedures. One ferret of group 1 died between the first and second vaccination due to reasons unrelated to the experiment.

Serology

Serum samples were collected before vaccination, at the day of second vaccination (day 20) and at the day of challenge (day 52). Sera were stored at –20°C until use. Sera were tested for the presence of anti-HA antibodies using a hemagglutination inhibition assay (HI-assay) with 1% turkey erythrocytes and for the presence of virus neutralizing antibodies using a micro virus neutralization assay (VN-assay) as described previously (Frank et al., 1980; Palmer D, 1975). Sera were tested for the presence of antibodies reactive with influenza A/NL/602/09(H1N1). For this purpose, reverse genetics viruses were produced. The titers obtained with these viruses were comparable to those against the wild-type strains (data not shown). Positive control serum specific for influenza A/NL/602/09(H1N1) was obtained from a ferret infected with this virus (Munster et al., 2009). Other H1N1 influenza viruses used in the HI-assay were A/Netherlands/386/86 (NL/86), A/Netherlands/25/80 (NL/80), A/New Jersey/8/76 (NJ/76), A/Swine/shope/1/56 (Sw/56), A/Italy/1443/76 (It/76), A/Iowa/15/30 (Io/30), A/Puerto Rico/8/34 (Pr/34) and A/Brisbane/59/07 (IVR-148 vaccine strain; IVR/148). Serum samples of ferrets infected with these viruses were used as a positive control in this assay (de Jong et al., 2001).

Sera were also tested for the presence of neuraminidase inhibiting (NI) antibodies using a previously described fetuin-based assay (Lambre et al., 1991). Briefly, 96-well Nunc MaxiSorp plates were coated overnight at 4˚C with 100 µl of 5 µg/ml fetuin. Sixty-µl volumes of serially diluted serum samples were incubated for 30 minutes at 37°C with an equal volume of sNA_4 containing culture supernatant (prediluted in PBS-Ca/Mg [0.901mM/0.493mM] to give a half-maximum OD_{450} of 1.5) after which 100 µl of the mixture was added to the fetuin-coated wells. After one hour incubation at 37°C, the plates were washed and neuraminidase activity was subsequently measured by adding peroxidase-labelled peanut agglutinin (2.5 µg/ml; Sigma), incubating for 1 h at room temperature, washing the plates and adding 100 µl of peroxidase substrate (TMB) to each well. After 5 minutes, the reaction was stopped by the addition of 100 µl of 0.3 M phosphoric acid and OD values were measured at 450 nm using an ELISA reader (EL-808 [BioTEK]). To test the sera for cross-reactive NI antibodies, sNA_4 expression constructs similar to the ones described above

for A/California/04/2009(H1N1) were also made for the head domains of A/Kentucky/UR06-0258/2007(H1N1) (a.a.75-470) and A/turkey/Turkey/1/2005(H5N1) influenza virus (a.a.55-449). Sera specific for influenza A/NL/602/09(H1N1) and A/turkey/Turkey/1/2005(H5N1) obtained from a ferret infected with these viruses were used as a positive control.

Virus replication in the upper and lower respiratory tract

Samples of all lobes of the right lung and of the accessory lobe were collected of the infected ferrets, snap frozen on dry ice with ethanol and stored at –70°C until further processing. Lung samples were weighed and subsequently homogenized with a FastPrep-24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, The Netherlands) and centrifuged briefly. Nose and throat swabs were stored directly at -70°C in the same medium as used to homogenize the lung samples. Quadruplicate 10-fold serial dilutions of throat, nose and lung samples were used to infect MDCK cells as described previously (Rimmelzwaan et al., 1998). HA activity of the culture supernatants collected 5 days post inoculation was used as indicator of infection. The titers were calculated according to the Spearman-Karber method and expressed as log $TCID_{50}$ per gram for lung tissue or per ml for swabs (Karber, 1931).

Histopathology

Four days post inoculation (dpi) with influenza A/NL/602/09 virus, ferrets were euthanized and lungs were observed macroscopically and weighed before samples from the right lungs were collected to determine the virus titers. Subsequently left lung lobes were inflated with 10% neutral buffered formalin. After fixation and embedding in paraffin, lungs were sectioned at 4 µm and tissue sections were examined by staining with hematoxylin and eosin (HE).

Statistical Analysis

Significance among animal groups was analyzed by one-way ANOVA and Tukey test subsequent to ANOVA. Differences were considered significant at $P<0.05$.

Results

Production of sHA_3 and sNA_4 antigens

Constructs were designed to express the trimeric HA ectodomain (a.a.17-522) and the tetrameric NA head domain (a.a.75-469) of the 2009 A(H1N1) influenza virus as pictured in Fig.1A. The

Fig.1 Design and expression of soluble, multimeric HA (sHA) and NA (sNA) proteins of 2009 A(H1N1) influenza virus. A) Schematic representation of the recombinantly expressed sHA and sNA protein constructs. sHA: the HA ectodomain (a.a.17-522) is expressed with an N-terminal CD5 signal peptide and a C-terminal trimerization (GCN4-pII) GCN4 domain and *Strep*-Tag (ST), respectively. sNA: the NA head domain (a.a.75-469) is expressed with an N-terminal CD5 signal peptide, a OneSTrEP (OS) peptide and a tetramerization (GCN4-pLI) GCN4 domain. (B) Coomassie blue stained reducing SDS-PAGE of affinity-purified sHA and sNA proteins.

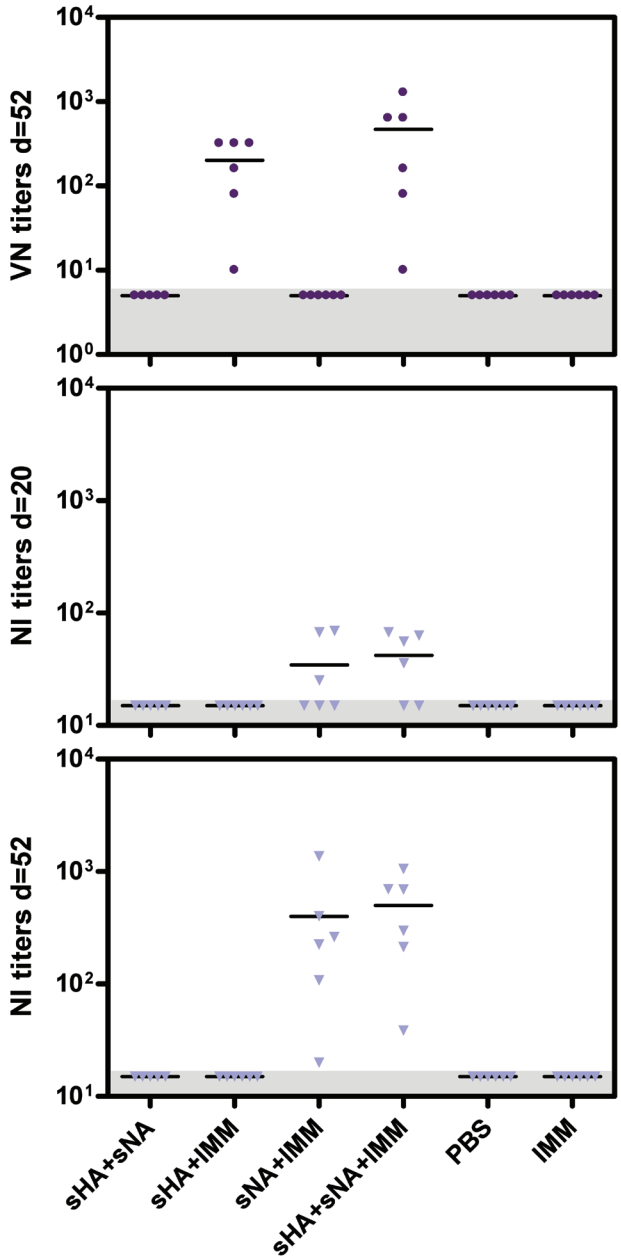

Fig.2 Antibody response to vaccination with multimeric 2009 A(H1N1) influenza virus HA and NA antigens. Ferrets were immunized on day 0 and day 20 with: 3.75 µg sHA_3+3.75 µg sNA_4 (sHA+sNA); 3.75 µg sHA_3 in adjuvant (ISCOM Matrix M [IMM]; sHA+IMM); 3.75 µg sNA_4 in adjuvant (sNA+IMM); 3.75 µg sHA_3+3.75 µg sNA_4 in adjuvant (sHA+sNA+IMM), PBS or IMM, as indicated. The antibody response to the 2009 A(H1N1) influenza virus was evaluated by hemagglutination inhibition (HI; upper panel), virus neutralization (VN; second panel from the top) and neuraminidase inhibition (NI) assays (lower panels). Each dot represents the result of one ferret. Horizontal lines represent means. The horizontal grey bar indicates the detection limit of the assay.

sHA_3 and sNA_4 proteins were produced by expression in HEK293T cells and purified from the culture medium by affinity chromatography yielding glycoproteins of the expected size (Fig.1B). Gel filtration analysis indicated the trimeric and tetrameric oligomeric nature of the HA and NA subunits, respectively (data not shown). The multimeric complexes were also biologically active, further confirming their native state, as judged by their sialic acid binding (sHA_3; **chapter 3**) and neuraminidase activity (sNA_4; below).

Antibody responses induced by immunization with sHA_3 and sNA_4

The glycoproteins were tested for their ability to induce protective immunity against homologous virus challenge. Ferrets were immunized at days 0 and 20 with sHA_3+sNA_4 without adjuvant (sHA+sNA), with sHA_3+sNA_4 adjuvanted with ISCOM Matrix M (IMM; sHA+sNA+IMM), or with similarly adjuvanted sHA_3 (sHA+IMM) or sNA_4 (sNA+IMM). Sera were collected at the day of the second immunization and pre-challenge (days 20 and 52) and antibody responses were measured by HI and VN assays against the homologous virus and by NI assay (Fig.2). No responses with any of these assays were observed in control animals vaccinated with PBS or with adjuvant only, but also not in the animals immunized with the non-adjuvanted mixture of sHA_3 and sNA_4. In contrast, the immunizations with adjuvanted sHA_3 (sHA+IMM) induced high HI titers, with a geometric mean titer of 91 at day of challenge. Interestingly, the additional inclusion of sNA_4 (sHA+sNA+IMM) significantly increased the average HI titers to 468 ($p<0.05$; one-way ANOVA and Tukey test). Also the NI titrations revealed the adjuvant-dependent induction of NA antibodies, which were low after one (Fig.2) but strongly boosted after two immunizations (Fig.2). However, in this case no clear augmentation of these titers was observed due to the co-administration of sHA_3. Consistent with the observed HI titers, high VN titers were found both in the sHA+IMM and in the sHA+sNA+IMM vaccinated animals (Fig.2). Also here the co-administration of the sNA_4 antigen with the sHA+sNA+IMM vaccine had resulted in an increase in the mean VN titer, average values ranging from 1:202 to 1:468 in the sHA+IMM and sHA+sNA+IMM groups, respectively.

Protection against clinical signs after infection with 2009 A(H1N1) influenza virus

Vaccinated ferrets were challenged with 10^6 $TCID_{50}$ 2009 A(H1N1) 5 weeks after the second vaccination. From day two after inoculation onwards, clinical signs were observed in inoculated ferrets which included breathing difficulties, lethargy, decreased appetite and weight loss. In general, only mild clinical signs were observed in ferrets of groups 2, 3 and 4, while more severe symptoms were observed in ferrets of groups 1, 5 and 6. Loss of body weight became obvious in the PBS- and IMM-vaccinated control groups as well as in the non-adjuvanted sHA+sNA vaccine group (Fig.3). Interestingly, the animals immunized with sHA+IMM showed nearly similar weight losses, while body weights were not significantly affected after vaccination with both the sNA_4 containing formulations (groups sNA+IMM and sHA+sNA+IMM). More or less consistently, the lung weights of the ferrets determined *post mortem* showed a corresponding tendency of adjuvanted sNA_4-vaccinated animals having the least disease-related increase due to lung consolidation (Fig.3).

Gross pathologic and histopathologic findings in the lungs of ferrets

Four days after inoculation with influenza virus 2009 A(H1N1) virus, the lungs of the ferrets were examined macroscopically and weighed before samples were taken for assessing virus replication and histopathological changes. Dark red and firm consolidated areas were observed macroscopically in lungs of inoculated ferrets. The percentage of affected lung tissue was estimated and varied between groups. Mean percentages of affected areas in the lungs of about 50% were observed in ferrets of groups 1, 5 and 6, while the extent of consolidation was less pronounced in ferrets of groups 2, 3 and 4, which showed less than 25% of the lung area being affected (Fig.3). Also the relative lung weight was lower in these groups compared to that in ferrets of groups 1, 5 and 6 (Fig.3).

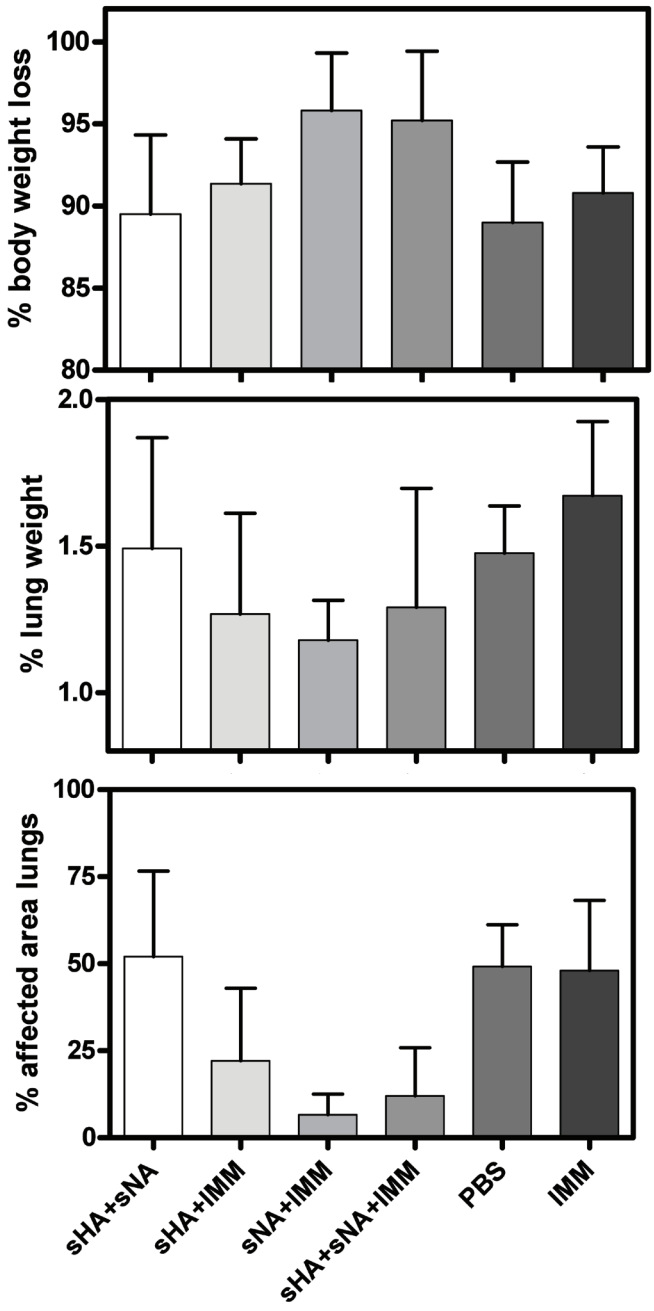

Fig.3 Clinical effects after challenge inoculation with 2009 A(H1N1) influenza virus. Ferrets immunized as described in the legend to Fig.2 were inoculated intratracheally on day 52 with 10^6 $TCID_{50}$ of virus. Body weight losses are expressed as percentage of body weight before infection (upper panel). Lung weights are expressed as percentage of body weight, as an indicator of lung consolidation (middle panel). Lungs were observed macroscopically and scored for lung area percentage displaying consolidated areas (bottom panel). Mean values are displayed; error bars indicate standard deviations. The horizontal grey bar indicates the detection limit of the assay.

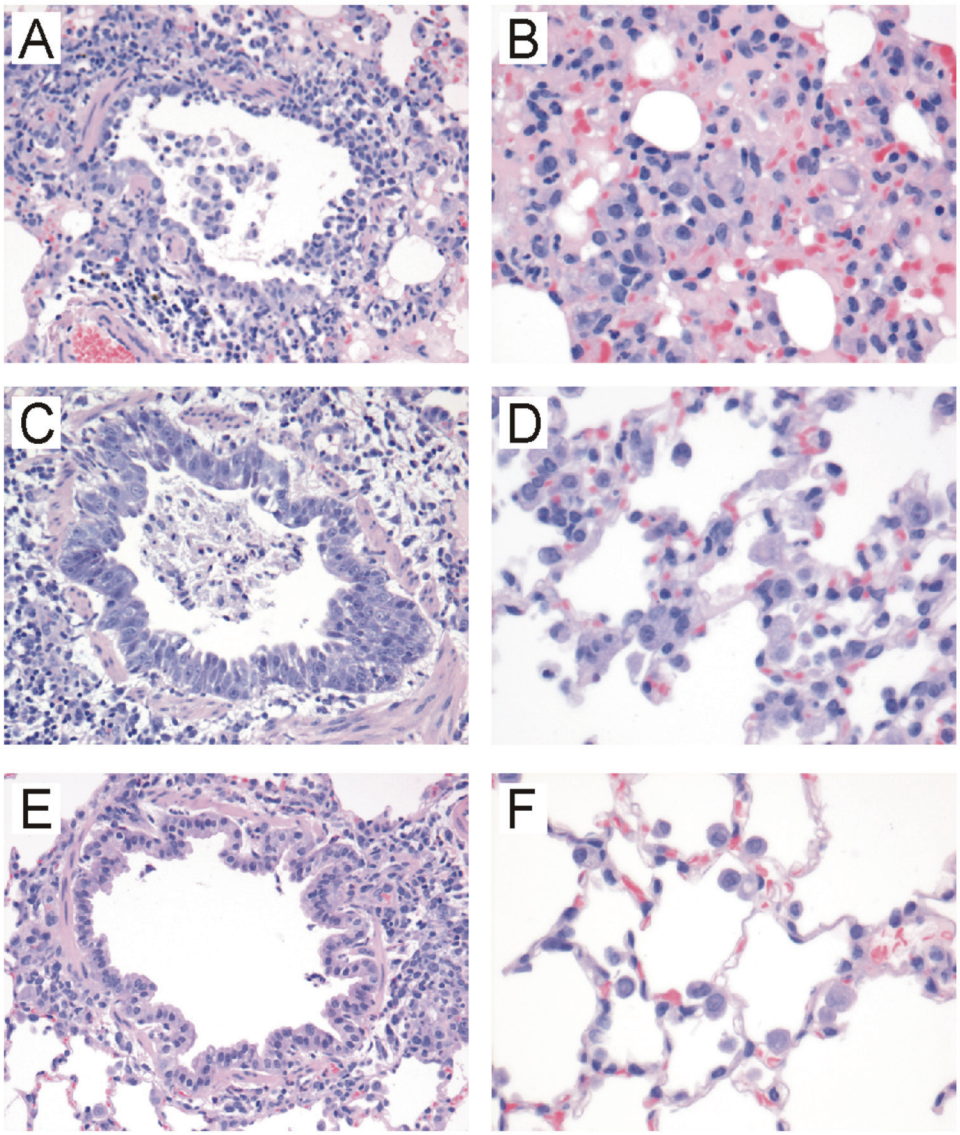

Fig.4 Examples of histopathologic findings in lungs of ferrets after inoculation. A) Inflammatory infiltrates and loss of epithelial cells in the bronchiolar walls and cellular debris in the bronchiolar lumen observed in the lungs of unprotected ferrets mock-vaccinated with PBS or adjuvant only (IMM) or vaccinated with the non-adjuvanted sHA_3 + sNA_4. B) Proteinaceous fluid (edema) and infiltrate of inflammatory cells in the alveoli of lungs of ferrets mock-vaccinated with PBS or adjuvant only (IMM) or vaccinated with the non-adjuvanted sHA_3 + sNA_4. C) Peribronchiolar infiltrate and cellular debris in bronchiole of ferret vaccinated with sHA+IMM. D) Inflammatory infiltrate in the alveolar septa and hypertrophy and hyperplasia of type II pneumocytes in lungs of ferrets vaccinated with sHA+IMM. E) Peribronchiolar infiltrate observed in lungs of ferrets of the sNA+IMM and sHA+sNA+IMM groups. F) Absence of inflammatory cells and hyperplasia of type II pneumocytes in alveoli of lungs of ferrets of the sNA+IMM and sHA+sNA+IMM groups. H&E staining; magnification 20x (bronchioli) and 40x (alveoli).

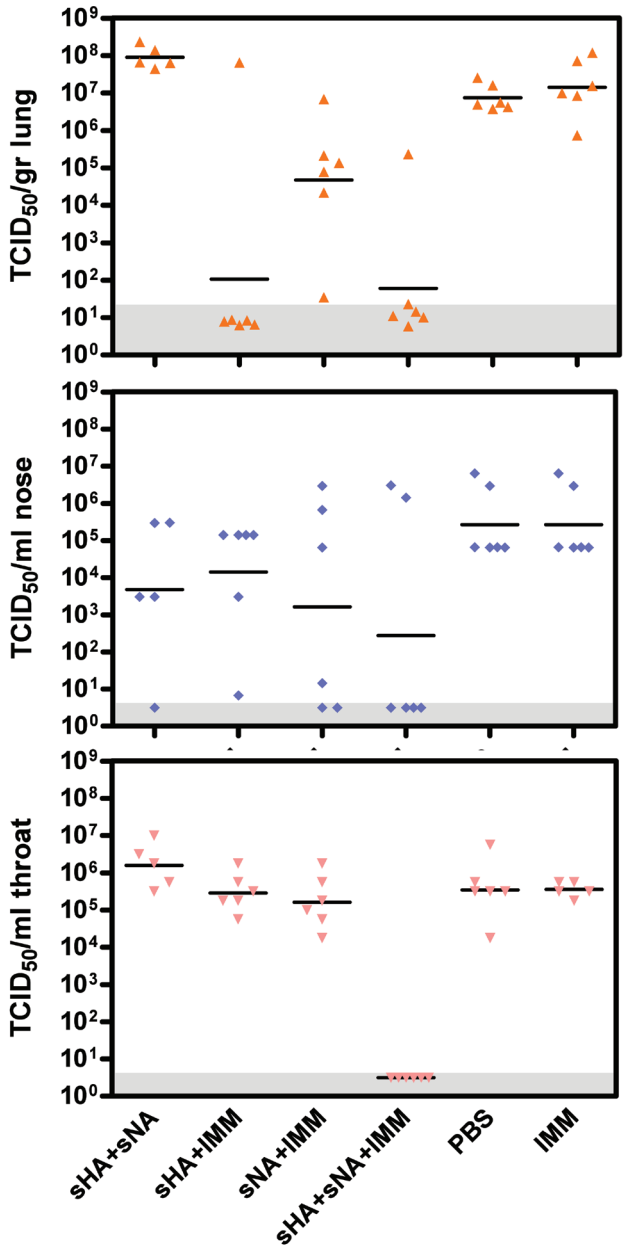

Fig.5 Viral titers in lungs, nose and throat of challenge-inoculated animals. Virus replication in the ferrets immunized and challenged as described in the legend to Fig.3 was analyzed 4 days after inoculation. Virus titers were determined in lung homogenates (upper panel), nose swaps (middle panel) and throat swaps (bottom panel). Titers were assayed by means of end-point titration in MDCK cells. Each dot represents the result of one ferret. Horizontal lines represent means. The horizontal grey bar indicates the detection limit of the assay.

Histopathological changes observed at day 4 post inoculation in the lungs of ferrets mock-vaccinated with PBS or adjuvant only (IMM) or vaccinated with the non-adjuvanted sHA_3 + sNA_4 were characteristic for a moderate to severe necrotizing broncho-interstitial pneumonia. Multifocally, many neutrophils and macrophages and variable numbers of erythrocytes, edema fluid and fibrin were present in the alveoli of the lungs of these ferrets. In addition, inflammatory infiltrates were present in the alveolar septa, in the bronchioles, in the bronchi and in the walls of bronchi and bronchioli. A dramatic reduction in histopathological changes was observed in the adjuvanted sNA_4-vaccinated animals (sNA+IMM and sHA+sNA+IMM groups), while ferrets immunized with sHA+IMM were partially protected from developing pathology (Fig.4).

Protection against virus replication in the upper and lower respiratory tract

To measure the effect of vaccination on the virus replication in the respiratory tract, virus titers were determined in lungs, throat and nose 4 days after inoculation. As shown in Fig.5 the challenge virus replicated efficiently in the lungs of the control ferrets (PBS and IMM groups) and in the animals immunized with the non-adjuvanted mixture of sHA_3 and sNA_4 (sHA+sNA group), with mean viral titers of approximately 10^{7}-10^{8} $TCID_{50}$/gram tissue. These viral loads were reduced by about 5 log_{10} units in the animals immunized with the sHA_3 protein in adjuvant (sHA+IMM group) and in animals co-immunized with sHA_3 and sNA_4 in adjuvant (sHA+sNA+IMM group). Mean viral loads were reduced by 2-3 log_{10} units in animals immunized with adjuvanted sNA_4 antigen (sNA+IMM group).

High viral loads in the nose were observed in the control animals (PBS and IMM groups; Fig.5) at day 4 after the challenge. Though not statistically significant due to the large variations in titers within groups, these viral loads appeared somewhat lower in the animals immunized with adjuvanted sHA_3 or adjuvanted sNA_4 or with the non-adjuvanted sHA_3 + sNA_4 combination. The highest reduction of nose viral titers was found in animals immunized with the adjuvanted combination of sHA_3 and sNA_4 antigens. Viral titers in the throat were generally high and not significantly affected by vaccination except in the animals vaccinated with the adjuvanted combination of sHA_3 and sNA_4. These ferrets did not have detectable titers in the throat.

Cross-reacting antibody responses induced by immunization with sHA_3 and sNA_4

To investigate whether the antibodies induced by the sHA_3 and sNA_4 antigens could cross-react with other H1N1 influenza viruses we performed additional HI and NI assays with the post vaccination sera. As expected, the highest HI titers were measured against the homologous virus while various extents of cross-reactivity were observed with a range of other H1-strains (Fig.6A). Thus, no cross-reactivity was detected for A/Swine/shope/1/56, A/Italy/1443/76, A/Iowa/15/30, A/PR/8/34 and IVR/148, whereas significant cross-reactivity was measured against A/NL/25/80 and A/NewJersey/8/76 and, particularly, against A/NL/386/86, more or less consistent with the sequence similarities of their antigenic domains (see table 1). This was the case with the sera from both the sHA_3+IMM and the sHA_3+sNA_4+IMM vaccinated animals. Consistent with the earlier observed differences in HI activity against the homologous virus (Fig.2), the levels of cross-reactivity were markedly higher with the sera from ferrets immunized with sHA_3+sNA_4+IMM than with those from sHA_3+IMM immunized animals, confirming again the enhancing effect of the sNA_4 antigen. HI titers against each strain were detected in control sera of ferrets infected with the homologous influenza A/H1N1 virus (data not shown).

To investigate the cross-reactivity of the NA antibodies we produced sNA_4 glycoprotein complexes of two other N1 influenza viruses, the human H1N1 strain A/Kentucky/UR06-0258/2007 and the avian H5N1 strain A/turkey/Turkey/1/2005. When tested in our NI assay there was a strong neuraminidase inhibiting activity with the pooled sera of the sNA_4+IMM and sHA_3+sNA_4+IMM

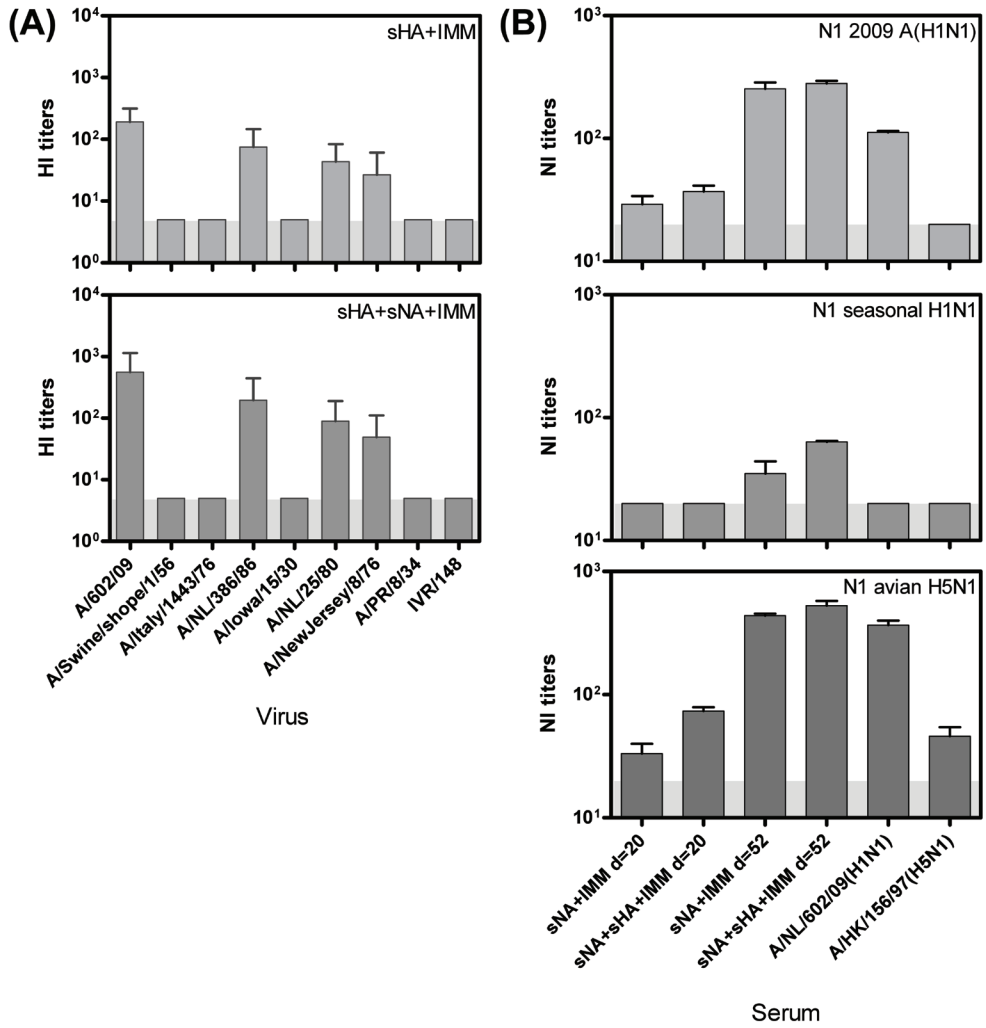

Fig.6 Induction of cross-neutralizing antibodies by vaccination with multimeric 2009 A(H1N1) influenza virus sHA_3 and sNA_4 antigens. (A) Sera of ferrets immunized twice with sHA_3 or sHA_3+sNA_4, both in adjuvant, as described in the legend to Fig.2, were tested in an HI assay for activity towards different influenza viruses including A/Swine/shope/1/56, A/Italy/1443/76, A/NL/386/86, A/Iowa/15/30, A/NL/25/80, A/NewJersey/8/76, A/PR/8/34 and IVR/148 influenza H1N1. Mean titers are displayed; error bars indicate standard deviation. (B) Sera of ferrets immunized once or twice with sNA_4 or sHA_3+sNA_4, both in adjuvant, were pooled and tested in a NI assay for activity against the sNA_4 of A/Kentucky/UR06-0258/2007(H1N1) and A/turkey/Turkey/1/2005(H5N1) influenza virus. The NA of A/California/04/2009(H1N1) was taken along as a positive control. Positive control sera specific for A/NL/602/09(H1N1) or A/turkey/Turkey/1/2005(H5N1) influenza virus were obtained from a ferret infected with these viruses. Average titers of two replicates are displayed; error bars indicate standard deviations. The horizontal grey bar indicates the detection limit of the assay.

immunized animals against the avian H5N1 virus sNA_4 protein while some inhibition of the seasonal H1N1 virus sNA_4 protein was observed (Fig.6B). Of note, a control serum derived from a H5N1 virus infected chicken tested negative against both human H1N1 virus sNA_4 proteins.

Discussion

For the induction of protective immune responses against influenza viruses the externally exposed parts of the virion glycoproteins are considered to be the key targets. As these antigens naturally

occur in virions as multimeric structures we have prepared these ectodomain complexes of the 2009 A(H1N1) influenza virus in the form of soluble HA trimers (sHA_3) and soluble NA tetramers (sNA_4). When these antigens were administered to ferrets in combination with the adjuvant ISCOM Matrix M either alone or in combination, protective immunity against a homologous challenge was induced. While the effect of immunization with sHA_3 reduced pulmonary virus titers significantly, immune responses to sNA_4 reduced the severity of disease. Although the differences per parameter were not significant, the sNA_4 vaccinated animals displayed an overall lower morbidity with respect to weight loss, lung consolidation, lung lesions and lung histopathology, compared to sHA_3 vaccinated animals.. The strongest reduction in virus titers in the upper and lower respiratory tract was seen in animals vaccinated with the adjuvanted sHA_3+sNA_4 mixture indicating that the more optimal protection against influenza is conferred by immunity elicited by the combination of these antigens. This is the first study showing the distinctive effects of immunization with soluble forms of HA and NA antigens and, particularly, the added value of combining these recombinant soluble antigens for eliciting protective immunity against influenza viruses, in particular towards the pandemic 2009 A(H1N1) influenza virus.

Correlates of protection against infection have so far not been extensively investigated for the pandemic 2009 A(H1N1) influenza virus. In our study the strong reduction in mean pulmonary virus titers after challenge infection correlated with the presence of HI and VN titers whereas the marked reduction in lung pathogenicity was associated with the presence of NI titers, indicating that optimal protection against influenza is achieved by immunity against both HA and NA antigens. The level of serum antibodies inhibiting hemagglutination as determined by an HI assay is generally accepted as a measure of influenza immunity. Antibodies neutralizing NA activity are also likely to play a direct role in decreasing virus replication by preventing virus release from infected cells (Kilbourne et al., 1968; Sylte and Suarez, 2009).

An interesting finding of our study was the differential effect of combining the HA and NA antigens on the antibody levels induced against each of these antigens. As compared with the NI titer observed after immunization with (adjuvanted) sNA_4 alone, the additional inclusion of sHA_3 in the vaccine

Table 1: Sequence homology of the antigenic regions within HA of different H1N1 strains.

H1N1 strains:	GenBank Protein Accession number:	% sequence homology HA antigenic domains (Soundararajan et al., 2009) relative to A/California/04/09
A/California/04/09	ACQ76318.1	100
A/Netherlands/602/09	ACQ45338.1	98.6
A/Swine/shope/1/56	*	n.a.
A/Italy/1443/76	*	n.a.
A/Netherlands/386/86	AAK51350.1	66.2
A/Iowa/15/30	AAD25303.1	76.1
A/Netherlands/25/80	AAK51352.1	67.6
A/NewJersey/8/76	AAA43210.1	78.9
A/PuertoRico/8/34	ACV89502.1	64.8
A/Brisbane/59/07 (IVR-148 vaccine strain)	ADI99532.1	56.3

* sequence not available; n.a. not applicable

was without much effect, confirming the lack of antigenic competition between the two antigens when co-administered other than in the context of a virion. In contrast, strongly increased VN titers and statistically higher HI titers were observed upon vaccination with the (adjuvanted) antigen combination as compared to vaccination with sHA_3 only. These enhanced antibody levels correlated with the increased reduction in virus titers in the upper respiratory tract while the enhanced reduction in pathogenic effects correlated with NA-specific immune responses. The mechanism by which the NA antigen affects the immune reaction to the HA antigen is quite intriguing and currently not understood, and hence warrants further investigation.

Typical for influenza A viruses, antigenic variants of HA and NA within a certain virus subtype able to escape from existing immunity are gradually selected in the human population. This process of antigenic drift calls for the almost annual adjustment of the seasonal vaccine composition in response to newly arising variants. Also in view of the threat of future influenza pandemics, caused for instance by an avian H5N1 virus, future vaccine development should be directed to inducing broadly protective immunity. Studies in mice have suggested that inclusion of NA in the vaccine increases the protection level against drifted strains within the same subtype (Brett and Johansson, 2005). This type of cross-protective immunity may be engendered - at least in part - by humoral immunity against NA since NA antibodies against seasonal human H1N1 have been shown in mice to have intrasubtypic protectivity against H5N1 (Sandbulte et al., 2007). Our *in vitro* NI assays also clearly demonstrated the 2009 A(H1N1) NA-specific humoral immunity induced in ferrets to exhibit cross-neutralizing activity against sNA_4 of a human seasonal H1N1 and - more potently - against sNA_4 of an avian H5N1 strain. In the approximately 50 amino acids that constitute the putative antigenic regions (Soundararajan et al., 2009), the NA sequence of pandemic A/California/04/2009(H1N1) has 64% identity compared to that of avian A/turkey/Turkey/1/2005(H5N1) NA but only 36% compared to that of a human A/Kentucky/UR06-0258/2007(H1N1), which is consistent with the observed cross-reactivity. The 2009 A(H1N1) HA-specific cross-neutralizing activity, as we measured using the standard HI assay, was restricted to the swine H1N1 virus strains strains A/NL/386/86, A/NL/25/80 and A/NewJersey/8/76 while no cross-reactivity was observed against the vaccine strain (IVR/148), which is quite consistent with the sequence homology of the relevant antigenic regions within HA. Essentially the same results were obtained with ferret sera obtained from ferrets infected with the influenza A/NL/602/09 virus (data not shown). NA has been found to have a lower rate of mutations, perhaps as a result of the lower immuno-selective pressure on this antigen during natural infection (Abed et al., 2002; Kilbourne, Johansson, and Grajower, 1990). It has been proposed that influenza vaccines including the more slowly evolving NA may hence be less vulnerable to antigenic changes in newly emerging viruses and thereby provide longer-lasting immunity (Johansson and Brett, 2007). Prevalent NA-specific cross-reactive humoral immunity elicited by seasonal H1N1 infection or vaccination may be a tentative explanation for the moderate severity and mortality rates of 2009 A(H1N1) influenza virus in humans and may provide a dampening effect on severity of a possible future H5N1 pandemic.

Interestingly, only in ferrets vaccinated with one or both antigens in combination with the adjuvant ISCOM Matrix M, antibody responses were detected and ferrets were protected against challenge infection. These observations further highlight the importance of the use of a proper adjuvant in influenza vaccine formulations. The adjuvant used in the current study, ISCOM Matrix M, is a formulation based on proprietary state-of-the-art Immune-Stimulating-Complex technology. Compared to earlier generations of ISCOMs adjuvants, ISCOM Matrix M lacks toxicity and is considered safe while retaining its strong adjuvant activity. Also in other preclinical studies with influenza A/H5N1 virus vaccines it has been demonstrated that this adjuvant potentiates specific antibody responses (Madhun et al., 2009).

The recombinant subunit approach that we applied in our studies provides several advantages for the production of influenza virus vaccines compared to more traditional virus-dependent methods and may hence be extremely valuable in combating seasonal epidemics and future pandemics. The HA-to-NA ratio of the vaccine can easily be adjusted due to the independent expression of HA and NA. The reactogenicity to the vaccine may be decreased since the recombinant proteins are highly purified and free of egg-derived proteins. The response time for vaccine preparation can be reduced since the genes encoding the antigens can be rapidly cloned into the expression vectors, bypassing the necessity for reverse genetics or adaptation of viruses to growth in culture systems. The glycoproteins' antigenicity reflects that of the natural strains since direct gene cloning excludes the risk of immunogenicity changes caused by mutations acquired during virus propagation in culture systems. Furthermore, the recombinant subunit approach obviates the deleterious effects on antigenicity often associated with classical virus inactivation. Lastly, the approach avoids the need for high biocontainment facilities necessary to grow virus-dependent vaccines. The advances in production quality and quantity of recombinant pharmaceuticals in mammalian cell lines make these expression systems increasingly feasible for clinical application (Durocher and Butler, 2009). Recombinant full-length HA (Cox, 2008; Huber and McCullers, 2008; Lakey et al., 1996; Powers et al., 1995; Treanor et al., 2001) and NA (Kilbourne et al., 2004) and soluble forms of HA (Cornelissen et al.; Wang et al., 2009) - but not of NA - have been previously described to function as effective vaccine antigens against influenza virus. A major advantage of expressing soluble, multimeric forms of the HA and NA antigens rather than their full-length counterparts is their highly efficient, single-step affinity purification from the culture supernatant, hence not requiring the solubilization of membranes using detergents.

This study particularly underscores the underrated importance of NA in protective vaccination against influenza. Since the current inactivated influenza virus vaccines are standardized only for the amount of HA, the NA content is variable as is, consequently, the frequency and level of seroconversion to NA, which is often rather poor (Kendal, Bozeman, and Ennis, 1980; Kilbourne, 1987). Our results are in line with previous results (Chen et al., 1999; Johansson, 1999; Johansson, Matthews, and Kilbourne, 1998; Johansson, Pokorny, and Tiso, 2002; Sandbulte et al., 2007) supporting the notion that future vaccines including sufficient immunogenic amounts of neuraminidase in influenza vaccines may provide a better and possibly more broadly effective immune response towards influenza viruses. In addition, inclusion of NA in the vaccine may also reduce the dose of HA antigen required for the induction of protective immunity. Further studies into these aspects are certainly warranted.

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Glycan-dependent immunogenicity of recombinant soluble trimeric hemagglutinin

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Abstract
Recombinant soluble trimeric influenza A virus (IAV) hemagglutinin (sHA_3) is an effective vaccine against a (lethal) challenge with IAV. Here we investigated to what extent the glycosylation status of the sHA_3 glycoprotein would affect its immunogenicity. Thus, different glycosylation forms of subtype H5 trimeric HA protein ($sH5_3$) were produced by expression in insect cells and different mammalian cells, some in the presence of inhibitors of N-glycan modifying enzymes; unglycosylated $sH5_3$ was prepared by enzymatic removal of the oligosaccharides. More specifically the following $sH5_3$ preparations were evaluated: (1) HA proteins carrying complex glycans produced in HEK293T cells, (2) HA proteins carrying 9 terminal mannoses expressed in HEK293T cells treated with kifunensine, (3) HA proteins containing 5 terminal mannoses derived from HEK293S GnT1(-) cells, (4) insect cell-produced HA proteins carrying paucimannose N-glycans, and (5) HEK293S GnTI(-) cell-produced HA proteins treated with endoglycosidase H, thus carrying side chains composed of only a single *N*-acetylglucosamine each. The different glycosylation states were confirmed by comparative electrophoretic analysis of the HA proteins and by mass spectrometric analysis of released glycans. The immunogenicity of the HA preparations, adjuvanted with Stimune, was studied in chickens and mice. The results consistently demonstrate that HA proteins carrying terminal mannose moieties induce significantly lower hemagglutination inhibition antibody titers as compared to both fully glycosylated antigens and HA proteins carrying single *N*-acetylglucosamine side chains. We conclude that the glycosylation state of recombinant antigens is a factor of significant importance when developing glycoprotein-based vaccines such as recombinant HA proteins.

Introduction

Influenza A viruses (IAVs) are important human and animal pathogens. Because of the worldwide spread of IAV infection in animal and man, eradication of IAV is not possible. Vaccination provides the most effective way to control IAVs and to protect against IAV infection and disease. Despite the efficacy of the approved influenza vaccines, there is room for new developments as has been reviewed elsewhere (Ellebedy and Webby, 2009; Safdar and Cox, 2007).

As protection against influenza virus infection and disease correlates with serum anti-hemagglutinin (HA) antibody levels the use of recombinant HA proteins may provide an attractive vaccination approach. Recombinant HA antigens can be produced using safe, quality controlled and scalable conditions without the need for virus cultivation. Furthermore, adverse reactions may be limited as the HA preparations can be highly purified and do not contain egg contaminants. In addition, recombinant HA proteins can be manufactured with short lead-time, allowing an accelerated response to emerging IAV strains.

Expression of recombinant HA proteins in higher eukaryotic expression systems is likely to result in superior antigens when compared to other expression systems as folding and trimerization of the HA protein is known to depend on multiple co- and post-translational modifications including glycosylation and disulfide bond formation. In agreement herewith, mammalian cell-derived HA trimers were shown to induce higher levels of neutralizing antibodies than their momomeric counterparts (Weldon et al., 2010). We and others have previously demonstrated the efficacy of recombinant HA protein preparations from insect or mammalian cells to protect against IAV infection and disease (Bosch et al., 2010; Cornelissen et al., 2010; Loeffen et al., 2011; Wang et al., 2009; Wei et al., 2008).

N-linked glycosylation is essential for proper folding, oligomerization and transport of the HA protein (Hanson et al., 2009). In addition, glycosylation may affect HA-receptor binding and fusion (Ohuchi et al., 1997; Ohuchi, Ohuchi, and Matsumoto, 1999). Loss of N-glycan sites neighboring the receptor-binding site or enzymatic truncation of the N-glycan structures were both shown to result in increased affinity of HA for receptors (Ohuchi et al., 1997). We and others recently showed that HA proteins that only differ in their glycosylation status, as a result of different production or processing conditions, possess different receptor fine specificities (de Vries et al., 2010; Wang et al., 2009). Furthermore, HA-linked oligosaccharides may also function in masking antigenic regions, while on the other hand HA glycans may also serve as a target for recognition of IAV by the innate immune system (Reading et al., 2007; Skehel et al., 1984). Antibodies raised against HA protein bearing only a single *N*-acetylglucosamine (GlcNAc) at each glycosylation site were reported to show better binding affinity and neutralization activity against IAV compared to antibodies raised against fully glycosylated HA (Wang et al., 2009).

In view of the apparent importance of HA glycosylation in eliciting protective antibodies, we decided to compare the immune response induced by recombinant soluble trimeric HA proteins of H5 subtype ($sH5_3$), which differ in their glycosylation states. The results show that proteins carrying terminal mannoses induce lower antibody titers than HA proteins carrying fully glycosylated N-linked side chains or single GlcNAc residues. HA proteins carrying the latter two modifications did not appear to differ in their antibody response.

Materials and Methods

Genes and expression vectors

cDNA clones corresponding to residues 18 to 523 (H3 numbering) of HA from A/Viet Nam/1203/2004 (H5N1) (Genbank accession no. ABW90137.1) and A/Mallard/Denmark/64650/03 (H5N7) (Genbank

accession no. AAT07996.1) were synthesized using human-preferred codons by GenScript USA Inc. H5 derived from the H5N7 virus (designated as $sH5_3^{N7}$) lacks a glycosylation site on the head and has 94.7% sequence identity to H5 derived H5N1 (designated $sH5_3^{N1}$). Cloning the H5 sequences into the appropriate expression vector was done as described previously (Cornelissen et al., 2010). Briefly, cDNA was cloned into the pCD5 expression vector for efficient expression in mammalian cells or into the pMT-Bip vector (Invitrogen) for expression in Schneider S2 cells. The HA-encoding cDNA was cloned in frame with DNA sequences coding for a signal sequence, an artificial GCN4 isoleucine zipper trimerization motif (RMKQIEDKIEEIESKQKKIENEIARIKK) and the Strep-tag II (WSHPQFEK; IBA, Germany).

Protein expression and purification

pCD5 expression vectors containing the HA ectodomain-encoding sequences were transfected into HEK293T and HEK293S GnTI(-) cells (Reeves et al., 2002) as described previously (Cornelissen et al., 2010). HA proteins secreted by the cells were purified using Strep-Tactin sepharose beads according to the manufacturer's instructions (IBA, Germany). When indicated, HA trimers bound to Strep-Tactin beads were treated with Endoglycosidase H (EndoH) for 3 hrs at 37°C (2μU/ml), followed by three washing steps prior to elution of the protein from the beads. *Drosophila* Schneider S2 cells were co-transfected with pMT-Bip vector encoding the HA ectodomain and pCoBlast using Cellfectin in a 19:1 ratio, and stable cell lines were selected according to manufacturer's protocols (Invitrogen) as described previously (de Vries et al., 2010). HA proteins were purified as described above. HA protein expression and secretion was confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). The concentration of purified protein was determined by using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according to the manufacturer's instructions.

Characterization of recombinant HA.

Oligomerization of the $sH5^3$ proteins was confirmed by Blue Native PAGE as described previously (de Vries et al., 2010). For DC-SIGN-binding experiments, 5 μg/ml HA was coated on 96-well Nunc MaxiSorp plates for 16 hrs at 4 ºC in PBS. The plates were blocked with with a solution of 3% BSA and 0.1% Tween20 in PBS for 2 hrs at room temperature (RT) followed by three washes with 0.05% Tween20 in PBS. Subsequently, serially diluted DC-SIGN-Fc (R&D systems) was added for 2 hrs at RT followed by three washes with 0.05% Tween20 in PBS. HRP-labeled goat-anti-human IgG was added for 1 hr at RT at a 1:1000 dilution, followed by three washes with 0.05% Tween20 in PBS. Peroxidase activity was visualized using tetramethylbenzidine substrate (BioFX) and an ELISA reader (EL-808 [BioTEK]), reading the OD at 450nm.

Glycan release, purification, labeling and mass spectrometry

The $sH5_3$ proteins were treated with the endoglycosidase PNGase F (NEB). The N-glycan mixture released by the PNGase F-treatment was applied to a C18 RP-cartridge (500mg; JT Baker, Phillipsburg, NJ). The flow through and wash fractions (2ml 10% acetonitril (AcN) and 4ml water) of these cartridges were subsequently applied to carbon cartridges (150mg Carbograph; Grace, Deerfield, IL). After a wash with 6ml water, glycans were eluted with 3ml 25% AcN and 3ml 50% AcN containing 0,1%TFA. The purified N-glycans were subsequently labeled with the fluorophore 2-aminobenzoic acid (2-AA), as described elsewhere (Ruhaak et al., 2010). Labeled glycans in 75% AcN were loaded on Biogel P10 (BIO-RAD Hercules, CA) conditioned with 80% AcN. After a wash with 80% AcN glycans were eluted with water, and analyzed with an Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in the negative-ion reflectron mode. DHB (Bruker Daltonics, Bremen, Germany) was used as matrix.

Immunization experiments
Animal studies were conducted at the Central Veterinary Institute (CVI), Lelystad, and at the Central Laboratory Animal Research Facility (CLARF) Utrecht after approval by the appropriate Animal Ethics Committees. Thirty, one-day-old layer hens (white Leghorn) were purchased from a local breeder. The chickens had been vaccinated against Newcastle disease virus and infectious bronchitis virus at the age of one day according to the farm's routine and were raised in the CVI's animal facility. At the age of 6 weeks, the birds were allocated to 3 experimental groups of 10 birds each. The animals were immunized twice (on day 0 and 21) by intramuscular (i.m.) injection of 2 μg $sH5^3$ antigen adjuvanted with Stimune (Prionic). As a control, one group was mock-vaccinated twice (on day 0 and 21) with PBS in Stimune. Blood was taken before the second vaccination and three weeks after the second vaccination. For the mouse experiments, female, 9-week-old C57B/6 mice obtained from Charles River Laboratories were immunized twice (on day 0 and 21) by i.m. injection (0.05 ml) of 4 μg $sH5^3$ antigen adjuvanted with Stimune. Six animals per group were used. As a control, in each experiment one group of mice was mock vaccinated twice. Blood was taken before and three weeks after the second vaccination.

Hemagglutination inhibition (HI) assay
Vibrio Cholera Neuraminidase (VCNA) treated and heat-inactivated immune sera from mice and chicken blood samples were tested for hemagglutination inhibition (HI) activity with 8 (125 ng) hemagglutinating units (HAU) of homologous antibody-complexed $sH5^3$ produced in HEK293T or HEK293S GnTI(-) cells as described previously (Cornelissen et al., 2010). To this end the recombinant proteins were precomplexed with the anti-Strep-tag and anti-mouse antibodies as described previously (Cornelissen et al., 2010), mixed with limiting dilutions of the mice sera and incubated with 0.5 % chicken red blood cells in PBS containing 1% BSA. Red button formation was scored as evidence of hemagglutination. Antibody titers were expressed as the reciprocal of the highest serum dilution showing HI.

Statistical Analysis
Significance among animal groups was analyzed by Student's t-test or by one-way analysis of variance (ANOVA) and Tukey test after ANOVA. Differences were considered significant at $P<0.05$.

Results

HA-glycan-dependent induction of antibodies against $sH5_3^{N1}$
Previously, we showed that recombinant soluble trimeric HA proteins can protect chickens, mice, pigs, and ferrets against a (lethal) challenge with the homologous IAV. The recombinant proteins used to vaccinate the animals were either produced in HEK293T cells (Bosch et al., 2010; Loeffen et al., 2011) or in HEK293S GnTI(-) cells which are deficient in N-acetylglucosaminyltransferase I (GnTI) activity, resulting in HA proteins containing 5 terminal mannoses (Cornelissen et al., 2010; Reeves et al., 2002). During one of these vaccination-challenge experiments, in which chickens were vaccinated with 2μg $sH5_3^{N1}$ produced in HEK293S GnTI(-) cells (Cornelissen et al., 2010), a parallel experiment was performed, in which other chickens were vaccinated twice with the same amount of recombinant protein produced in HEK293T cells. These recombinant proteins only differ in their glycosylation status. While HEK293T cell-produced HA protein contains complex glycans, the HA proteins made in the HEK293S GnTI(-) cells carry high mannose oligosaccharides. While none of the chickens that were vaccinated twice with 2μg $sH5_3^{N1}$ died or showed symptoms indicative of influenza-related disease after challenge by intranasal / intratracheal inoculation of a lethal dose of A/Vietnam/1194/04 virus ((Cornelissen et al., 2010)], and data not shown), differences were observed in HI antibody titers, depending on the cells from which the recombinant proteins were derived (Fig. 1A). Although all immunized animals developed appreciable HA antibody titers both

after the first vaccination and after the boost, HI titers were significantly higher after vaccination with HEK293T cell-derived $sH5_3^{N1}$ than after vaccination with HEK293S GnTI(-) cell-derived HA protein. The mock-immunized chickens had a HI titer below the detection limit.

To confirm and extend these observations, we switched to another animal model. Thus, mice were immunized twice with 4 μg of purified $sH5_3^{N1}$ produced in HEK293T or in HEK293S GnTI(-) cells. In addition, another group was taken along, in which mice were immunized with $sH5_3^{N1}$ produced in HEK293T cells in the presence of kifunensine (KIF). Both the HEK293S GnTI(-) cell-produced HA protein and the HA protein produced in HEK293T cells in the presence of KIF carry high mannose glycans, containing either 5 or 9 mannoses, respectively. Three weeks after the second immunization, blood samples were taken and HA antibody titers in the serum were determined. The results are shown in Figure 1B. Again, the HA proteins expressed in HEK293T cells in the absence of KIF, thus containing complex glycans, induced the highest HI antibody titer. The mice immunized with the protein preparations containing the high mannose glycans displayed comparable, but significantly lower HI antibody titers. No appreciable HI antibody titers were detected after mock-immunization.

To confirm their different glycosylation states, the protein preparations were subjected to gel electrophoresis (Fig. 1C). The $sH5_3^{N1}$ proteins carrying the high mannose glycans migrated somewhat faster in the gel than the HEK293T cell-produced HA, with the HEK293S GnTI(-) cell-produced HA protein, which carries the smallest N-glycan side chains, demonstrating the highest electrophoretic mobility. As expected, only the proteins with high mannose oligosaccharides were sensitive to EndoH, in agreement with the known specificity of this endoglycosidase (Maley et al., 1989). When the HA proteins derived from the different expression systems were treated with PNGase F an enzyme that removes all N-linked glycans, they migrated with similar mobility in the gel consistent with the HA proteins having identical protein backbones and only differing in their N-linked glycosylation.

HA-glycan-dependent induction of antibodies against $sH5_3^{N7}$

Next, we investigated whether the detrimental effect of terminal mannose moieties on the immune response was antigen-specific, or could also be observed by using a different HA protein. Therefore, soluble trimeric H5 from a different H5 virus, H5N7 ($sH5_3^{N7}$) was prepared. This H5 protein carries one oligosaccharide side chain less than $sH5_3^{N1}$. In addition, we expanded our set of protein preparations by including HEK293S GnTI(-) cell-produced HA protein treated with EndoH, which results in an HA protein that carries single GlcNAc residues rather than oligosaccharide side chains, and HA protein produced in S2 insect cells, which carries paucimannose side chains with 3 mannose moieties (Kim et al., 2005). The glycoproteins were analyzed by gel electrophoresis as shown in Figure 2. The electrophoretic mobilities of the glycoproteins differed according to the length of their glycan side chains. Thus, while HEK293T cell-produced HA displayed the lowest electrophoretic mobility, according to $sH5_3^{n7}$ carrying large complex glycans, the highest mobility was observed for the EndoH-treated protein, which carries only single GlcNAc residues. The terminal mannose-containing HA proteins migrated according to their theoretical number of mannose moieties (9, 5, and 3 mannoses for HEK293T cell-produced HA in the presence of KIF, HEK293S GnTI(-) cell-produced HA and S2 cell-produced HA, respectively (Kim et al., 2005)). Of these HA glycoproteins, the H5 protein produced in insect cells was resistant to treatment with EndoH, as EndoH removes only *N*-linked sugars containing more than three terminal mannose moieties (Maley et al., 1989). When the HA proteins were deglycosylated using PNGase F, they all comigrated in the gel consistent with that HA proteins having identical polypeptide backbones irrespective of the cell type from which they originated

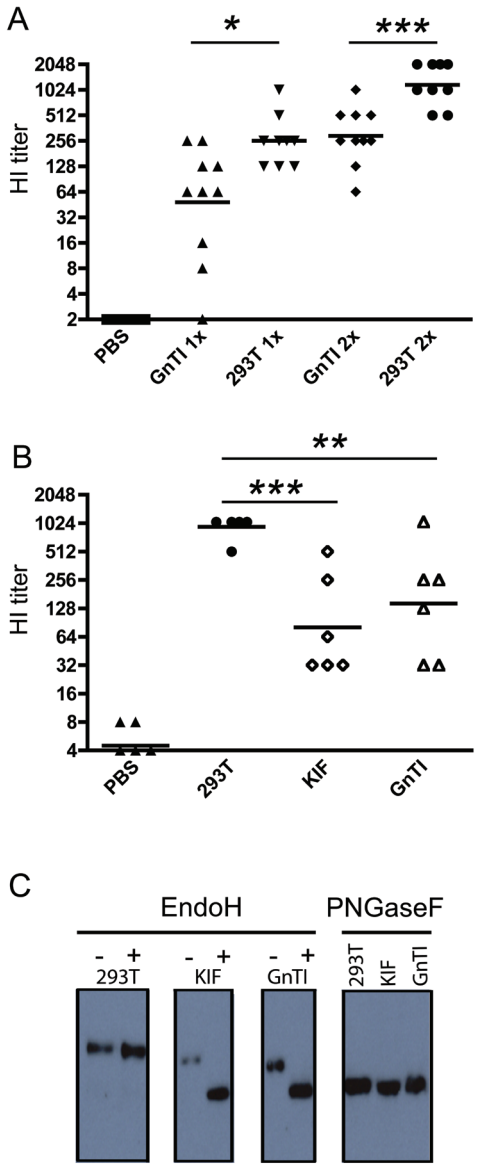

Figure 1. HA-glycan-dependent induction of antibodies against $sH5_3^{N1}$. (A) Ten chickens per group were immunized twice with purified $sH5_3^{N1}$ protein preparations produced in HEK293T cells (293T) or HEK293S GnTI(-) cells (GnTI). As a control, chickens were mock-treated (PBS). Blood samples were taken three weeks after the first and after the second vaccination. HI titers against 8 hemagglutinating units (HAU) $sH5_3$, produced in HEK293S GnTI(-) cells, in serum for each bird are shown. (B) Six mice per group were immunized twice with $sH5_3^{N1}$ protein preparations produced in HEK293T cells (293T), HEK293T cells treated with KIF (KIF) or HEK293S GnTI(-) cells (GnTI). Blood samples were taken three weeks after the second immunization. HI titers against 8 hemagglutinating units (HAU) $sH5_3$, produced in HEK293S GnTI(-) cells, in serum for each mouse are shown. (A-B) Bars represent the geometric means per group. Significant differences between groups are indicated (A; Student's t-test, B; ANOVA), with *, ** and *** corresponding to $p < 0,05$, $p < 0,01$ and $p < 0,001$, respectively. (C) Purified $sH5_3^{N1}$ protein preparations were analyzed by SDS-PAGE followed by western blotting. When indicated the proteins were treated with EndoH or PNGaseF prior to electrophoresis. The recombinant proteins were detected using a mouse anti-Strep-tag antibody (Schmidt and Skerra, 2007).

To further confirm the different glycosylation states of our H5 preparations, we analyzed the interaction of $sH5_3^{N7}$ with the dendritic cell C-type lectin DC-SIGN, which is known to interact with carbohydrates carrying terminal mannose moieties (Wang et al., 2008). After coating of HA in 96 well plates, binding of DC-SIGN to HA was determined using an ELISA-type assay. While no appreciable DC-SIGN binding was observed with HA carrying complex glycans or single GlcNAc residues, binding to HA proteins' carrying terminal mannoses was readily observed (Fig. 2B). The extent of binding to DC-SIGN appeared to be proportional to the number of mannose moieties exposed on the HA protein.

As mammalian cell-derived HA trimers were found to induce higher levels of neutralizing antibodies than similarly produced monomeric HA protein (Weldon et al., 2010), we also analyzed the oligomeric nature of the purified HA proteins produced with the different expression systems by using Blue Native PAGE. As shown in Figure 2C, all proteins migrated at the high position in the gel that corresponds to their trimeric nature (Cornelissen et al., 2010). When the H5 preparations were heat-denatured, the initially trimeric HA species dissociated in monomers.

Mass spectrometry

Finally, glycans attached to the $sH5_3^{N7}$ proteins were analyzed using mass spectrometry. To this end, glycans were enzymatically released from the glycoprotein using PNGaseF and analyzed after purification and labeling by MALDI-TOF-MS. The monoisotopic mass of each of the peaks within the resulting spectra were used to assign a monosaccharide composition to each signal, and to deduce a putative structure as indicated in Figure 3. The glycans derived from HEK293T cell-produced HA (Fig 3A) gave rise to a relatively complex mass spectrum, with major signals suggesting the presence of di-, tri- and tetra-antennary complex-type glycans with *N*-acetyllactosamine [Gal(β1-4)GlcNAc(β1-)] antennae. Signals representing extensions of these glycans with one or more *N*-acetylneuraminic acids and/or carrying a fucose at the reducing end GlcNAc were also observed. These putative assignments were supported by β-galactosidase treatment, which led to the loss of all non-sialylated galactoses, thereby confirming the number of antennae in each glycan and the absence of possible linear *N*-acetyllactosamine repeats (Figure S1). For example, the AA-labeled fucosylated tetra-antennary glycan of composition F1N6H7 observed at m/z 2637.2 $[M-H]^-$ disappeared after β-galactosidase treatment, giving rise to a new signal at m/z 1988.5 representing the F1N6H3 species formed by the loss of four galactose residues from the four *N*-acetyllactosamine antennae in the non-treated glycan.

The spectrum of the released N-glycans of HA produced in HEK293T cells treated with KIF (Fig 2B) showed only one peak at m/z 2002.5 $[M-H]^-$ which corresponded to the high mannose N-glycan carrying 9 mannoses, in line with the inhibitory effect of KIF on glycan-processing. The spectrum of the HEK293S GnTI(-) cell-produced HA also gave only one high-abundant signal at m/z 1354.3 $[M-H]^-$, corresponding to a high mannose peak carrying 5 mannose-moieties (Fig 2C). This is in agreement with the expectations, since the absence of GnTI activity prevents the addition of GlcNAc to the α1-3 linked core Man, thereby blocking further processing of the glycan. The majority of the N-glycans released from S2 cell-produced HA were of the paucimannosidic-type (Fig 2D). Major signals with composition F1H3N2 and H3N2 were observed at m/z 1176.5 $[M-H]^-$ and m/z 1030.4 $[M-H]^-$ respectively. In addition, some minor signals were observed at m/z 1014.4 $[M-H]^-$ (F1H2N2), m/z 1233.5 $[M-H]^-$ (H3N3) and at m/z 1354.5 $[M-H]^-$ (H5N2). This observation is in line with previously reported N-glycan structures on recombinant proteins expressed in S2 cells (Kim et al., 2005).

HA-glycosylation-dependent induction of antibodies against $sH5_3^{N7}$

After having analyzed the glycosylation states of the different $sH5_3^{N7}$ protein preparations, the HA

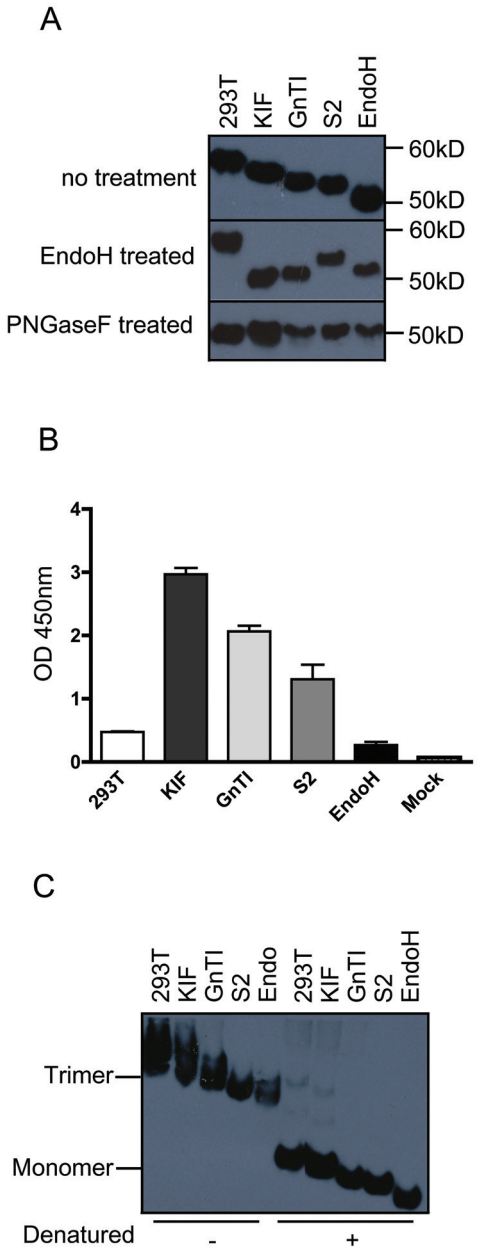

Figure 2. Analysis of different $sH5_3^{N7}$ preparations. $sH5_3^{N7}$ proteins produced in HEK293T cells (293T), HEK293T cells treated with KIF (KIF), HEK293S GnTI(-) cells (GnTI), insect S2 cells (S2), or HEK293S GnTI(-) cell-produced $sH5_3^{N7}$ treated with EndoH (EndoH) were purified and analyzed. (A) The HA proteins were analyzed by SDS-PAGE followed by western blotting. The recombinant proteins were detected using a mouse anti-Strep-tag antibody (Schmidt and Skerra, 2007). When indicated samples were treated with PNGase F or EndoH prior to electrophoresis. (B) Binding of Fc-tagged DC-SIGN (2.5 μg/well) to wells coated with the different HA preparations (5μg/well). Mock indicates that no HA protein was coated. Binding of DC-SIGN was detected using HRP-labeled goat-anti-human IgG. (C) Blue native-PAGE analysis of the HA protein preparations. The position in the gel of the trimeric, and monomeric protein species is indicated, as well as when samples were denatured by heating them for 1' at 95°C prior to electrophoresis.

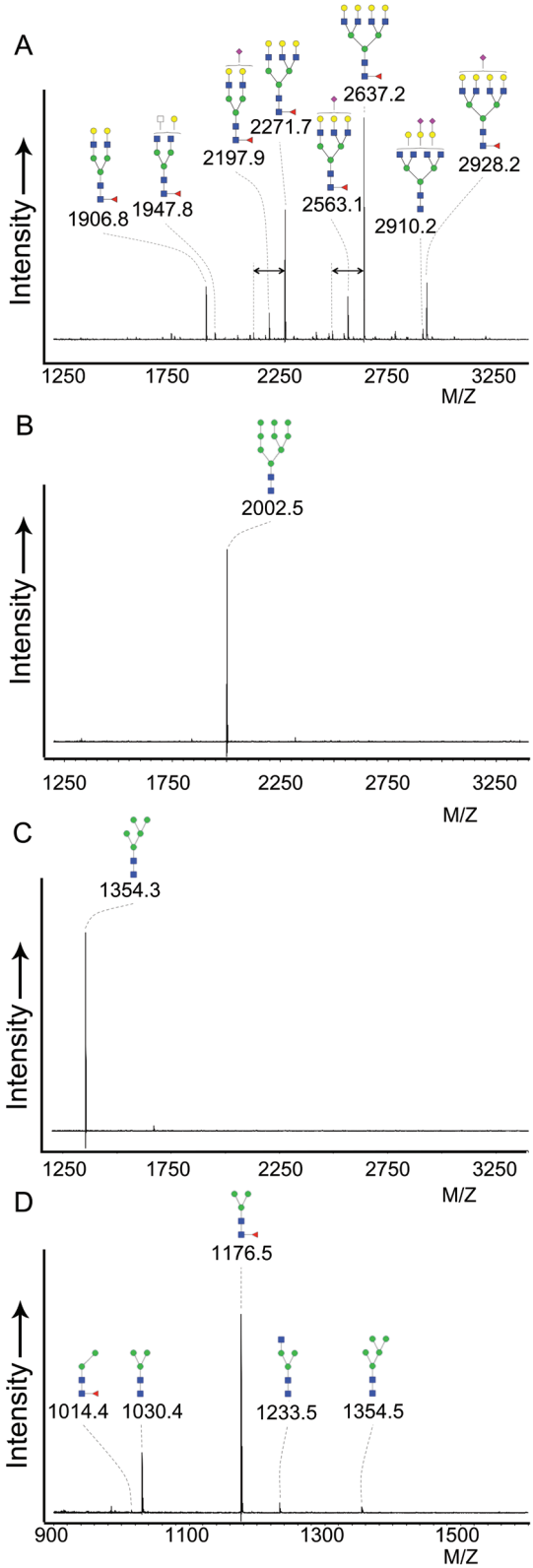

Figure 3. Mass spectrometry analysis of different $sH5_3^{N7}$ preparations. MALDI-TOF-MS of the N-glycans of purified $sH5_3^{N7}$ protein preparations produced in (A) HEK293T cells (293T), (B) HEK293T cells treated with KIF (KIF), (C) HEK293S GnTI(-) cells (GnTI), and (D) insect S2 cells (S2), N-glycans were released by PNGase F, labeled with anthranilic acid and analyzed in negative-ion reflectron mode. All signals are labeled with monoisotopic masses and structures deduced from this mass. In the case of HEK293T sample (A) only the 10 signals with the highest abundance were labeled with assigned structures, these structures were confirmed by beta-galactosidase treatment, in each case confirming the number of terminal galactose residues present (Fig S1). Double-headed arrows indicate a difference in fucose content. Red triangle, fucose; purple diamond, *N*-acetylneuraminic acid; yellow circle, galactose; blue square, *N*-acetylglucosamine; green circle, mannose, white square, N-acetylhexosamine.

proteins were tested for their ability to induce HI antibodies. To this end, mice were immunized twice with the different HA preparations, after which the HI serum antibody titers were determined. The results are shown in Figure 4. In agreement with the previous experiment, mice vaccinated with HA proteins carrying terminal mannose moieties displayed significantly lower HI antibody titers than mice immunized with HA carrying complex glycans. No apparent differences were observed in the HI antibody titers induced by HA proteins containing glycans with 9, 5, or fewer mannose moieties. Removal of the high-mannose glycans from the HA proteins produced in HEK293S GnTI(-) cells by EndoH-treatment, appeared to result in higher HI antibody titers, although this difference was not significant. When the data were pooled based on the absence (HEK293T cell-produced HA and EndoH-treated HA) or presence (HA produced in HEK293T cells treated with KIF, HEK293S GnTI(-) cell-produced HA, and S2 cell-produced HA) of terminal mannose moieties, again significantly lower HI antibody titers were observed for the mice immunized with HA proteins carrying mannose moieties (Fig. 4B).

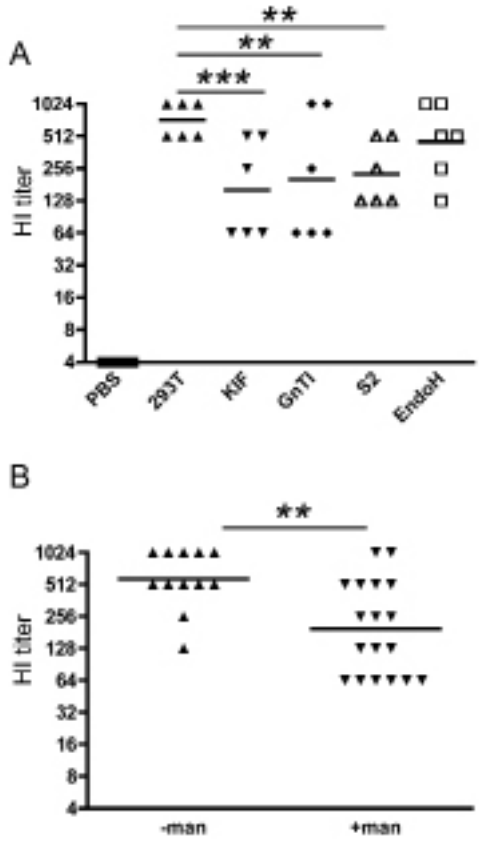

Figure 4. HA-glycan-dependent induction of antibodies against $sH5_3^{N7}$ .Six mice per group were immunized twice with $sH5_3^{N7}$ protein preparations produced in HEK293T cells (293T), HEK293T cells treated with KIF (KIF), HEK293S GnTI(-) cells (GnTI), insect S2 cells or with HEK293S GnTI(-) cell-produced $sH5_3^{N7}$ treated with EndoH (EndoH). Blood samples were taken three weeks after the second immunization. HI titers against 4 HAU $sH5_3$, produced in HEK293T cells, in serum for each mouse are shown (A). (B) Graph shows the data pooled on the basis of the absence (-man; HEK293T cell-produced HA and EndoH-treated HA produced in HEK293S GnTI(-) cells) or presence (+man; HA produced in HEK293T cells treated with KIF, HEK293S GnTI(-) cell-produced HA, and S2 cell-produced HA) of terminal mannose residues in the $sH5_3^{N7}$ protein preparations, in which data are shown. Bars in the scatter dot plots represent geometric means per group. Significant differences between groups are indicated (A; ANOVA, B; Student's t-test) with *, ** and *** corresponding to $p < 0{,}05$, $p < 0{,}01$ and $p < 0{,}001$, respectively

Discussion

Recombinant soluble trimeric HA proteins provide an attractive new vaccination approach against IAV. The glycosylation state of the recombinant HA proteins is known to be affected by the specific expression platform used. Therefore it is of interest to study to what extent the immunogenicity of recombinant soluble HA proteins is influenced by their glycosylation state. In this study we utilized three different cell lines and different glycan modifying enzymes to produce different sets of soluble trimeric H5 antigens that only differ in their glycosylation state. HA proteins produced in HEK293T cells were shown to contain different complex glycans. When the HEK293T cells were treated with KIF, the HA proteins only carried high mannose N-glycans carrying 9 mannoses. HA proteins produced in HEK293S GnTI (-) cells were shown to contain only high mannose glycans containing 5 mannose moieties, while insect S2 cell-produced HA contained different types of paucimannosidic glycans. Treatment of HA protein produced in HEK293S GnTI(-) cells resulted in HA proteins carrying single GlcNAc residues. Chickens or mice immunized with these different purified HA preparations displayed different levels of HI antibody titers, indicating that the glycosylation state of recombinant HA antigens indeed affects their immunogenicity and is a factor of significant importance when developing vaccines based on recombinant HA proteins, and, probably more general, vaccines based on glycoprotein antigens.

Our results indicate that HA proteins carrying terminal mannose moieties induce lower HI antibody titers in chicken en mice than HA proteins carrying complex glycans. In a previous study, Wei and coworkers (2008) reported that recombinant HA proteins produced in mammalian cells are comparable or slightly better in eliciting neutralizing antibodies than their insect cell-derived counterpart (Wei et al., 2008). However, the immunogenicities of their different HA preparations were not compared directly within one animal experiment. Furthermore, a different adjuvant, mouse strain and HA dose were used as compared to our study. Consistent with our results, also for human immunodeficiency virus (HIV) gp120 it has been observed that removal of terminal mannose moieties, which are abundantly present on gp120 proteins expressed in mammalian cells, increased its immunogenicity (Banerjee et al., 2009). In another study, the antibody response against gp120 was shown to be enhanced by occluding the mannose moieties on gp120 with griffithsin (Banerjee et al., 2011). It was demonstrated that the mannose moieties of the N-linked glycans of HIV gp120 induce immunosuppressive responses in dendritic cells, which correlated with DC-SIGN expression on these cells (Shan et al., 2007). As the HA preparations carrying terminal high mannose moieties exhibited efficient binding to DC-SIGN in *in vitro* experiments, a similar mechanism may apply for soluble trimeric HA antigen preparations. Alternatively, a role for other mannose-binding lectins, for example in clearance of antigens containing terminal mannose containing glycans, can not be excluded (Lee et al., 2002).

Removal of high mannose glycan side chains by treating H5 preparations with EndoH appeared to increase the immunogenicity of the recombinant proteins, resulting in antibody titers similar to those obtained after immunization with HA proteins carrying complex glycans. Wang and coworkers (2009) recently reported that antiserum raised against H5 protein bearing only single N-linked GlcNAc side chains showed stronger neutralization of IAV than antiserum elicited against HA containing complex glycans (Wang et al., 2009). In addition, mice vaccinated with HA protein containing the single GlcNAc were better protected against a lethal challenge than mice that received the HA protein with the complex glycans. When compared to our results, the observed differences may be attributed to the differences in HA, adjuvant, mouse strain or amount of HA used. Indeed, mice vaccinated with our HA preparations were fully protected against a lethal H5N1 challenge (Cornelissen et al., 2010), while a maximum of only 40% of the vaccinated mice were protected in the study of Wang and coworkers (2009).

Our results show that the immunogenicity of recombinant HAs is affected by their glycosylation state and thus by the expression system used to generate these antigens. Importantly, HAs carrying terminal mannose moieties, such as those produced in insect cells, were shown to induce lower HI antibody titers when compared to other HA preparations. Although removal of almost the complete N-glycan side chain did not result in higher antibody titers against the homologous HA, it will be of interest to study whether antisera raised against such preparations are more broadly reactive as glycosylation may shield conserved epitopes on the HA protein (Chen, Ma, and Wong, 2011; Das et al., 2010).

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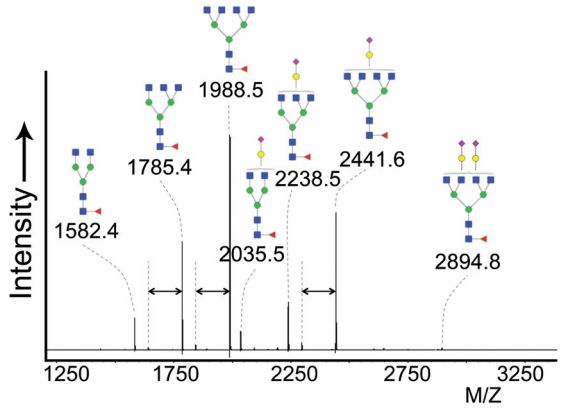

Figure S1 MALDI-TOF-MS of the AA-labeled released N-glycans of $sH5_3$ produced in HEK293T cells, after Jackbean beta-galactosidase treatment. Anthranilic acid-labeled glycans (4µl) were treated with beta-galactosidase from Jackbean (45mU, Prozyme, Hayward, CA) in 60µl 250mM sodium citrate buffer for 24hr at 37°C. Digestion products were purified using Ziptip C18 (Millipore, Billerica, MA) following the manufacturers instructions. Glycans were eluted directly to a MALDI-target plate with 10mg/ml DHB in 50% AcN containing 0,1%TFA and analyzed in the negative-ion reflectron mode. Signals were measured in negative-ion reflectron mode. Monoisotopic masses and deduced structures are indicated for the 10 signals with the highest abundance. Compared to the spectrum before treatment (Fig. 3) a clear loss of all galactoses not carrying N-acetylneuraminic acid is visible, indicating a high abundance of terminal galactose. Double headed arrows indicate a difference in fucose content. Red triangle, fucose; purple diamond, N-acetylneuraminic acid; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

Summarizing discussion

Influenza A viruses (IAVs) continue to pose a serious threat to public health. Seasonal influenza epidemics may affect up to 15% of the population and result in more than 500.000 deaths worldwide per year (http://www.who.int/mediacentre/factsheets/fs211/en/). In addition, new animal-origin influenza viruses may emerge that have acquired potent human-to-human transmissibility, as illustrated just recently by the swine origin new pandemic H1N1 virus in 2009 (Dawood et al., 2009). While the disease caused by this virus has mostly been mild, with a mortality rate comparable to previous seasonal viruses, other pandemic viruses, the 1918 H1N1 virus in particular, were much more devastating, causing millions of deaths (Taubenberger and Morens, 2006). The last decade, the highly pathogenic avian influenza H5N1 virus has raised serious concerns. Although this virus has not yet evolved the ability to efficiently spread among the human population (Uyeki, 2008), it frequently infects humans with a case fatality rate of ~60%.

The hemagglutinin (HA) protein plays crucial roles in the early stages of virus infection as it binds to sialic acid receptors, which results in endocytic uptake of the virions, and is responsible for the low pH-induced fusion of viral and cellular membranes (Skehel and Wiley, 2000). Because of its role in sialic acid receptor binding, it is the most important determinant of virus tropism (Matrosovich et al., 2000). In addition, the HA protein is also the major immunogen. Antibody levels against HA correlate with protection against infection and disease (Osterhaus, Fouchier, and Rimmelzwaan, 2011). In this thesis I have focused on these two key features of the HA protein. In this final chapter I will discuss the results of my studies in a broader perspective.

Recombinant HA proteins as attractive vaccination antigens

Vaccination against influenza virus is the most effective way to prevent or limit disease. Conventional influenza vaccines consist of viruses propagated either in embryonated eggs or in cell cultures, after which they are inactivated and partly purified. The development, registration and production of these vaccines takes a relatively long time, while in addition safety, efficacy and production issues have to be taken into account (Ellebedy and Webby, 2009). As protective immunity against influenza virus infection and disease is primarily conferred through HA via the induction of anti-HA antibodies, recombinant purified HA proteins may constitute an attractive alternative vaccination approach. Indeed, promising results have been obtained with recombinant full-length HA proteins expressed in insect cells with the baculovirus system (Treanor et al., 2007). However, as the full-length HA proteins are membrane-anchored, they may not retain their native membrane-bound structure upon purification. On the other hand, simple removal of the transmembrane domain will result in monomeric HA proteins, which probably do not provide a good representation of the native antigenic epitopes.

To overcome these issues we decided to produce recombinant, soluble, stable HA trimers that are secreted from cells, by replacing the transmembrane anchor of HA with a GCN4 trimerization motif. In **Chapters 4, 5 and 6** we evaluated the vaccine potential of these recombinant soluble trimeric HA proteins produced in mammalian cells. In these recombinant proteins the transmembrane domain and cytoplasmic tail has been substituted by a trimerization motif and a strep-tag, resulting in soluble trimeric proteins that can be easily purified form the cell culture supernatant. A double immunization of chickens, mice, ferrets or swine with the HA trimer preparations was shown to protect against a homologous (lethal) challenge with IAV, including the highly pathogenic avian influenza (HPAI) virus H5N1. Vaccination with the soluble trimers induced strong antibody responses, resulted in diminished virus replication and excretion upon challenge, and decreased the clinical effects of infection.

In **chapter 4** we show that while a double immunization was needed to protect all mice against a

lethal H5N1 challenge, a single dose (2 μg) resulted in only 40% survival, in correlation with the anti-HA titers in the sera of the animals. Interestingly, immunization of chickens, which have a much higher body weight, with a single dose of 2 or 10 μg resulted in 90 or 100% survival, respectively, suggesting that the HA trimer preparations are more effective in conferring protective immunity in chickens than in mice. Although the reason for this apparent discrepancy is not known, our results show that single immunization with soluble recombinant trimeric HA is sufficient to protect chickens against HPAI virus H5N1. The efficacy of our HA vaccine preparations was also demonstrated by the absence of viral RNA in the protected birds, which would imply that a single immunized flock can pose a barrier against further spread of the virus. This may be a valuable feature, when the virus transmission cycle needs to be interrupted as soon as possible and the risk for humans to the potentially zoonotic HPAI needs to be limited to the upmost extent.

Our results are in agreement with other studies that demonstrate the ability of recombinant trimeric soluble HA proteins to induce neutralizing antibodies (Wang et al., 2009; Wei et al., 2008; Weldon et al., 2010), and to (partially) protect against a challenge (Wang et al., 2009; Weldon et al., 2010). Interestingly, recombinant soluble HA trimers stabilized by the presence of a foldon or GCN4 trimerization motif proved to be superior antigens when compared to their monomeric counterparts. Cleaved monomers failed to induce significant neutralizing antibodies against H5N1 virus, even though anti-H5 antibodies were detected by ELISA (Wei et al., 2008). Similarly, recombinant soluble trimeric HA induced much stronger protective immune responses than the monomeric form of HA (Weldon et al., 2010). This difference in the ability to raise neutralizing antibodies between monomers and timers was suggested (Wei et al., 2008) to be due to the preferential induction of antibodies against epitopes present in the monomeric form and not in the trimer, similar to observations with human immunodeficiency virus type 1 (HIV-1) gp120 monomers and trimers (reviewed in (Douek, Kwong, and Nabel, 2006)). Alternatively, native epitopes are presented in the trimeric protein, while the monomeric HA may presents epitopes that are associated with the altered low-pH conformation of the HA protein (Weldon et al., 2010).

Whereas vaccination with recombinant soluble HA was needed and sufficient to reduce virus replication, immunization with recombinant soluble NA by itself already markedly reduced body weight loss and lung pathology (**chapter 6**). Obviously, optimal protection was achieved by the combination of the two antigens. Immunization with NA does not induce neutralizing antibodies, but may restrict viral replication allowing infection but not disease (infection-permissive immunization; reviewed by (Johansson and Brett, 2007)). In agreement with our results, several studies have shown that immunization with NA engenders protective immunity (Johansson, 1999; Johansson and Brett, 2008; Johansson and Kilbourne, 1994; Johansson, Pokorny, and Tiso, 2002), not so much against infection as against symptoms of influenza.

Co-administration of recombinant soluble tetrameric neuraminidase (NA) proteins to the HA preparations enhanced the HA-specific immune response (**chapter 6**) and markedly decreased the clinical effects in ferrets after challenge with the new pandemic H1N1 virus. The mechanism by which the NA antigen affects the immune response to the HA protein is not yet understood, but may possibly be related to the enzymatic activity of the recombinant soluble tetrameric NA protein preparation, as neuraminidase treatment is known to enhance the capacity of resting B cells to stimulate the proliferation of T cells (Bagriacik and Miller, 1999). Furthermore several immune cells use sialic acid-mediated signal transduction pathways (Chen et al., 2011; Collins et al., 2006).

While the conventional inactivated (subunit) IAV vaccines are administered without adjuvants, our highly purified HA (and NA) preparations need adjuvants for the induction of a detectable immune response, at least in ferrets. Two types of adjuvants were used in our studies: Stimune

(also known as Specoll), a water-in-oil adjuvant, consisting mainly of markol52 (mineral oil), span 85 and tween85 (**chapter 4 and 5**) and ISCOM Matrix M (**chapter 6**), a formulation based on immune-stimulating complex technology. Stimune, the strong adjuvant properties of which have been described for several animal species (Leenaars et al., 1994), is approved for animal but not for human use. ISCOMS are cage-like structures with a diameter in the order of 40nm, consisting of cholesterol, phospholipids and purified saponins (Rimmelzwaan and Osterhaust, 2001). Antigens can be incorporated in the structure of ISCOMs, but this is not essential. ISCOM Matrix M has also not yet been approved human use, although clinical trials are underway.

In another study, in which soluble HA trimers were used as a vaccine preparation in Balb/c mice, much less protection against a challenge with H5N1 was observed after two immunizations (Wang et al., 2009) than in our study (**chapter 4**), even though the mice received a 10 times higher dose (20 µg) of a very similar HA protein. The difference most likely relates to the different adjuvants used (aluminum hydroxide [Alum] vs Stimune). Alum is known to induce low antibody titers when used with subunit vaccines (Bresson et al., 2006). Indeed, when combined with our HA preparations, the immune stimulating properties of Stimune were far greater than those of Alum (unpublished results). Further testing will be required to determine the extent to which other adjuvants can improve the immunogenicity of recombinant HA proteins. Adjuvants may differ not only in their potency, but may also affect stability of the HA trimer. Remarkably, Weldon and coworkers (Weldon et al., 2010) were able to protect mice by vaccinating them twice with relatively low amounts (3 µg) of soluble trimeric HA preparations in the absence of adjuvants. This observation may be somehow related to the different HA protein (derived from the H3N2 virus A/Aichi/2/68) used.

N-linked glycosylation is essential for proper folding, oligomerization and transport of the HA protein (Hanson et al., 2009). In addition, oligosaccharides on HA proteins may also shield epitopes (Skehel et al., 1984) or serve as a target for recognition of IAV by the innate immune system (Reading et al., 2007; Vigerust and Shepherd, 2007). In **chapter 7** we analyzed to what extent the the glycosylation state of the recombinant HA preparations affects the HA-specific antibody response. Our results show that HA proteins carrying terminal mannoses induce lower antibody titers than HA proteins carrying fully glycosylated N-linked side chains or single GlcNAc residues. Very similar results have been obtained for HIV-1 gp120 protein. Also for this protein, removal of mannose moieties increased its immunogenicity (Banerjee et al., 2009; Banerjee et al., 2011). It was demonstrated that the mannose moieties of the N-linked glycans of HIV gp120 induce immunosuppressive responses from dendritic cells, which correlated with DC-SIGN expression on these cells (Shan et al., 2007). As our HA preparations carrying terminal mannoses demonstrated efficient binding to DC-SIGN in *in vitro* experiments, a similar mechanism may apply for these proteins. Alternatively, a role for other mannose-binding lectins, for example in clearance of antigens containing terminal mannose containing glycans cannot be excluded (Lee et al., 2002). Regardless of the responsible mechanism, our results indicate that mammalian cell-produced HA preparations carrying complex glycans are better immunogens than their insect cell-derived counterparts with paucimannose N-linked side chains.

Acquisition of N-linked glycosylation sites near antigenic sites may sterically prevent antibody binding to its epitope (glycan shielding). The HIV gp160 protein provides a clear example of hyperglycosylation as an effective immune escape mechanism (Pejchal et al., 2011). Glycan shielding of epitopes has also been demonstrated for HA (Das et al., 2011; Munk et al., 1992; Skehel et al., 1984). However in contrast to the HIV gp160 protein, the HA proteins are not hyperglycosylated (Das et al., 2010; Vigerust et al., 2007). This is probably due to interference of HA glycosylation with receptor binding (de Vries et al., 2010; Ohuchi et al., 1997; Wang et al., 2009) thereby creating a fitness barrier to accumulating glycosylation sites and providing a ready explanation for the paucity

of N-linked glycan side chains on HA when compared for example with HIV gp160 (Das et al., 2011). Yet, Wang and coworkers (2009) recently reported that mice vaccinated with HA preparations containing a single GlcNAc residue at each glycosylation site (monoglycosylated HA) were better protected against a lethal challenge than mice that received HA proteins carrying complex glycans (fully glycosylated HA). However, similar H5 preparations did not differ in their immunogenicity in our hands (**chapter 7**). The reason for this discrepancy is not known, but may be attributed to the different H5 protein, adjuvant, mouse strain or amount of HA used. Experiments are currently underway to repeat and extend our observations by directly comparing H5 antigens that differ in the number of N-linked side chains (4 vs 5). In addition, we will analyze whether antisera raised against monoglycosylated HA preparations are more broadly reactive as glycosylation may shield conserved epitopes on HA (Chen, Ma, and Wong, 2011; Das et al., 2010).

Our results indicate that recombinant soluble oligomeric glycoprotein preparations provide an attractive alternative vaccination approach, which is likely to be amenable to the envelope glycoproteins of other viruses. In addition, the recombinant protein approach allows the easy modification of these proteins with the aim of enhancing their immunogenicity. This may for example be achieved by fusing the recombinant proteins directly to co-stimulatory molecules (cis-adjuvant approach; (Melchers et al., 2011)). The recombinant proteins may also be fused to other functional moieties such as linkers that enable the tight binding to gram-positive enhancer matrix (GEM) particles (Audouy et al., 2007). The novel adjuvant GEM particles are produced from the nonpathogenic food-grade bacterium *Lactococcus lactis*, by heating in acid. The resulting non-living peptidoglycan particles were shown to act as potent adjuvants for intranasal immunization (Saluja et al., 2010a; Saluja et al., 2010b). The addition of linkers to the recombinant proteins enables the direct binding to GEM particles, thereby avoiding additional purification steps. Such GEM-recombinant protein preparations have recently been shown to induce high anti-HA antibodies titers after mucosal immunization of mice (unpublished results). Studies are underway to evaluate the protective efficacy of such preparations.

The recombinant proteins may also be modified to confer broader protection. An attractive approach appears to be the generation of so-called 'headless' HA proteins that are comprised of the conserved stalk region (Ekiert et al., 2011), but lack the globular head, which is much more prone to antigenic variation. Vaccination of mice with headless HA elicited immune sera with broader reactivity than those obtained from mice immunized with a full-length HA (Steel et al., 2010), in agreement with the hypothesis that the globular head domain of an intact HA molecule inhibits recognition of the stalk region by immune cells, either through steric shielding or as a result of immune dominance of the membrane-distal portion of the protein (Sagawa et al., 1996; Steel et al., 2010). The potential significance of the HA stem region as an immunogen is also demonstrated by other reports that show the ability of antibodies targeting the HA stalk domain to neutralize virus infectivity (Corti et al., 2011; Ekiert et al., 2011).

The future success of the recombinant protein vaccination approach critically depends on the production costs per dose of antigen. Thus, the expression levels achieved with our production system, transient transfection of mammalian cells, which range from 5-20 µg/ml, will need to be increased. In addition, to be able to rapidly react to the changing demands of different HA's, expression platforms will be required by which the proteins can be produced within a short period. This will necessitate the generation of systems in which HA genes can be efficiently and reproducibly integrated chromosomally in the production cell line. The feasibility of such systems for recombinant soluble HA proteins has recently been demonstrated (Lu et al., 2011).

Recombinant HA proteins as tools to study virus-receptor interactions
In addition to their ability to protect animals after immunization against infection with IAV, the recombinant soluble trimeric HA proteins also turned out very useful to study IAV-receptor interactions. The advantages of using recombinant HA preparations rather than intact virus preparations are obvious (Rillahan and Paulson, 2011). Intact virus preparations have biosafety issues, while the growth of viruses in cell culture or eggs may result in adaptive mutations (Gambaryan, Robertson, and Matrosovich, 1999). The production of recombinant genes can be achieved relatively easily once the sequence of the viral genome has been determined. In addition, the recombinant proteins can be readily mutated using site-directed mutagenesis, without the need for the laborious reverse genetics approaches that are required for intact viruses. A drawback of the recombinant HA approach may be related to the difference in valency when compared to the intact viruses. Whereas the multivalent interactions between HAs in the viral envelope and sialylated glycans on the host cell membrane provide the high avidity binding, single HA proteins bind their ligands with very low affinities in the milimolar range (Shriver et al., 2009; Viswanathan et al., 2010). This may be partially overcome by pre-complexation of the recombinant trimers using primary and secondary antibodies (Srinivasan et al., 2008). However, to what extent differences in receptor binding observed between different recombinant soluble trimeric proteins translate to differences in receptor binding in the context of a virus particle remains to be elucidated.

We analyzed to what extent receptor binding by HA was affected by the glycosylation state of the recombinant protein. Different glycosylation states were obtained by using different HA expression platforms or by treatment of HA with glycosidase (**chapter 2**). The results indicate that HA proteins that only differ in their glycosylation state possess different receptor fine specificities. Sialylated HA proteins demonstrated narrow receptor specificity. In agreement with previous studies (Ohuchi, Ohuchi, and Matsumoto, 1999; Wang et al., 2009), removal of sialic acids was needed for efficient receptor binding. Desialylated HA trimers demonstrated similar receptor fine specificity as HA proteins produced in 293S cells lacking a functional N-acetylglucosaminyl transferase I protein. HA proteins produced in these 293S GNT1(-) cells carry high mannose rather than complex glycans. However HA proteins with smaller glycan side chains, either by expression of HA in insect cells (**chapter 2**) or by enzymatic truncation (Maley et al., 1989; Wang et al., 2009), displayed more promiscuous receptor binding profiles, by gaining avidity, but losing specificity. As a consequence, differences in receptor binding that could be detected using the HA preparations carrying high-mannose glycans, could not be observed with the insect cell-derived HAs (**chapter 2**).

The observation that the length of the glycan side chain (**chapter 2** and (Wang et al., 2009)]) affects receptor binding is in agreement with previous observations that show that HA-receptor binding is influenced by the absence or presence of N-linked glycans adjacent to the receptor site (Das et al., 2011; Ohuchi et al., 1997). The HA proteins used in our study both carried N-linked side chains at their tips close to the receptor binding site. N-linked glycans on envelope glycoproteins may be advantageous to viruses as they may shield off antibody epitopes. However, in the case of the HA protein they appear to come at a cost (Das et al., 2010)]; see also above), and are therefore limited in their occurence despite decades of virus evolution (Sun et al., 2011; Vigerust and Shepherd, 2007; Wei et al., 2010). On the other hand, the deletion or introduction of N-linked glycosylation side chains may be one of the mechanisms by which IAVs modify their receptor binding, thereby enabling a switch in host tropism.

In **chapter 2 and 3** glycan array technology was applied to determine the receptor fine specificity of HA proteins derived from animal and human viruses. One of the outcomes of these studies is the observation that binding of HA is not only determined by the linkage of the terminal sialic acid to the vicinal galactose, but is also affected by other characteristics of the glycan structures. Thus,

while H2 derived from a herring gull virus preferred binding to type 1 chain glycans (Neu5Acα2-3Gal**β1-3**GlcNAc), H7 derived from a turkey virus more readily bound to type 2 glycans chains (Neu5Acα2-3Gal**β1-4**GlcNAc). These results are in agreement with those of others who showed that IAVs derived from different avian species may differ in their receptor-binding specificity by also recognizing the inner parts of the α2-3 sialylated glycans (Gambaryan et al., 2005). Also H1 proteins derived from swine or human viruses that only bound to α2-6-linked sialic acids showed additional preferences for particular glycan structures as these H1 proteins generally bound more readily to GlcNac-Gal repeats (poly-LacNac) terminating in α2-6 linked SIA. These glycans were also found to be important for infection with swine H3N2 viruses (Bateman et al., 2010) and were proposed to be important functional receptors for human H1N1 viruses (Chandrasekaran et al., 2008). The molecular basis for specificity towards SIA containing poly-LacNac repeats is not well understood (Xu et al., 2011). It might be explained by the topology of the glycan when bound by HA. While the SIA residue is fixed relative to the HA receptor binding site in all available co-crystal structures, additional sugars beyond the SIA adopt two distinct three-dimensional topologies, depending on the linkage of SIA to the galactose (Chandrasekaran et al., 2008; Viswanathan et al., 2010). While α2-3-linked sialylated glycans or short oligosaccharides containing α2-6-linked SIA typically adopt a cone-like topology, longer α2-6 oligosaccharide motifs such as 6′-sialyl-polylactosamine (Neu5Acα2-6[Galβ1-4GlcNAcβ1-3]2-) adopt an umbrella-like structure. This umbrella conformation provides optimal contact of the fourth and fifth glycan residues (Gal-GlcNac) with the 190-helix of the receptor binding site, thereby explaining the preferential binding of HA proteins to these glycans (Gamblin et al., 2004).

Comparison of the HA protein derived from the swine origin new pandemic H1N1 virus with a closely related H1 protein derived from a swine virus showed dramatic differences in their receptor-binding properties (**chapter 3**). While new pandemic H1 protein exhibited hardly any binding to different substrates, the swine protein efficiently bound to a number of α2-6 sialyl glycans. These differences were mapped to residues at position 200 and 227, mutation of which was shown to have a synergistic effect on binding. While the residue at position 227 is part of the receptor-binding site, this is not the case for the residue at position 200. The importance of this latter residue for receptor binding is probably explained by this residue making a potential hydrogen bond with residue 191 of the 190-helix of the receptor- binding site. This hydrogen bond is lost when the Thr residue is substituted by Ala. Why this substitution results in increased receptor binding is not yet understood, but it is in agreement with a recent other study (Xu et al., 2011). In addition, the reciprocal mutation (A200T) was observed after in vitro selection with NA inhibitors, which often results in mutations in HA that weaken cell attachment (Baz, Abed, and Boivin, 2007). The residue at position 227 has been proposed to form an interaction network with the residues at position 219 and 186. This network has been suggested to be disturbed in new pandemic H1 protein (Maines et al., 2009; Viswanathan et al., 2010), which may partly explain its low avidity receptor binding. The E227A mutation may have a positive effect on the interaction network, resulting in increased receptor binding. Changing the residue at position 219 into a lysine, thereby creating a theoretically more stable network, also resulted in increased SIA binding (Jayaraman et al., 2011).

Whether and to what extent the identity of the residues at position 200 and 227 contribute to efficient spread in the human population is not yet clear. While both Ala and Thr are observed at position 200 in new pandemic H1N1 viruses that are currently circulating, the residue at position 227 is invariably a glutamic acid. Remarkably, more than 95% of the swine H1 influenza viruses and dead-end human infections with H1 zoonotic viruses contain an alanine at this position. Furthermore, the glutamic acid is also invariably found in the seasonal human H1N1 isolates since 1978, suggesting that this residue is somehow important for optimal interaction of human IAVs with their host. Introduction of the E227A mutation in the new pandemic H1N1 virus was recently shown to negatively affect

viral growth in human airway epithelium, although respiratory droplet transmission in ferrets was not appreciably affected (van Doremalen et al., 2011). Interestingly, introducing a lysine at position 219 increased respiratory droplet transmission in a ferret model (Jayaraman et al., 2011). However, also this substitution has not yet been observed in the field. Substitution of the other residue in the putative interaction network (S186P) has been observed in several field strains (Strengell et al., 2011). This mutation, which only caused a minor antigenic change, has been observed in mouse-adapted new pandemic H1N1 virus and has been shown to contribute to virulence in mice (Ilyushina et al., 2010; Ye et al., 2010). It will be of interest to systematically study the effect of these mutations on receptor binding, replication and transmission.

Understandably, much research, including this thesis, has been focused on the interaction of HA with sialic acid receptors, as this interaction is a main determinant of virus tropism and of the ability of some IAVs to cross the host species barrier. Recent developments, including glycan array and recombinant protein technology have moved the field forward, although many questions remain to be answered. One of the neglected areas of research appears to be the interdependence of HA avidity and specificity, and the enzymatic activity of the NA protein. So, while the last few years have resulted in much information regarding the receptor usage of HA, much less is known about the enzymatic activity of the NA protein. The enzymatic activity of NA (i.e. substrate specificity) needs to match the activity of HA (i.e. ligand specificity), to achieve efficient viral infection and replication (Gulati et al., 2005; Mitnaul et al., 2000; Shtyrya et al., 2009; Wagner et al., 2000). Although the structure of several NA subtypes has been determined in complex with ligands, and the mutations that result in drug resistant viruses shave been mapped, the determinants of NA substrate specificity remain essentially unknown (Li et al., 2011; Pourceau et al., 2011). A recombinant protein approach as described in this thesis may also be very useful to study/analyze the activity of the NA protein.

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Nederlandse samenvatting

Influenza A virussen veroorzaken ziekte bij mens (griep) en dier. Van oorsprong komen deze virussen voor bij vogels, maar sommige influenza a virussen zijn in staat om andere dieren en mensen te infecteren. Humane virussen veroorzaken de jaarlijkse seizoensgebonden griepepidemieën. Influenza A virussen kunnen ook een grieppandemie veroorzaken, wanneer de meeste mensen niet over een bestaande afweer tegen het betreffende virus beschikken. De laatste pandemie werd veroorzaakt door het nieuwe pandemische H1N1 virus, in de volksmond beter bekend als het "Mexicaanse griepvirus".

Het influenza A virus bevat een gesegmenteerd RNA genoom van negatieve polariteit. Het RNA genoom is omgeven door het virale membraan dat drie envelop eiwitten bevat, waaronder het hemagglutinine (HA) en neuraminidase (NA). Deze twee eiwitten bepalen het subtype van het virus. Er zijn 16 HA (H1-16) en 9 NA (N1-9) varianten bekend. In wild water vogels komen alle verschillende HA en NA subtypes voor, bij mensen daarentegen worden alleen de H1N1, H2N2 en H3N2 subtypes gevonden. HA bindt aan siaalzuurbevattende receptoren en is na opname van het viruspartikel in de cel ook verantwoordelijk voor het fuseren van het virale membraan met dat van de gastheercel, waardoor het RNA genoom in het cytoplasma vrijkomt. De functie van NA is complementair aan die van HA. Het NA klieft siaalzuurreceptoren, waardoor nieuwgevormde virussen vrijkomen van de gastheercel en niet aggregeren.

De specificiteit van de interactie van HA met siaalzuur is een belangrijke determinant van het tropisme van het virus. Terwijl virussen die mensen kunnen infecteren een voorkeur hebben voor siaalzuren die via een α2-6 linker gebonden zijn aan het volgende suikerresidu, prefereren vogelvirussen α2-3 gebonden siaalzuren. Bovendien blijken de verschillende influenza A virussen nog andere, meer subtiele voorkeuren voor specifieke siaalzuurbevattende receptoren aan de dag te leggen. Daarnaast is HA het belangrijkste immunogeen van het virus en correleert de gastheers antilichaamrespons tegen HA met bescherming tegen virusinfectie.

In dit proefschrift wordt het onderzoek beschreven naar het gebruik van recombinante HA eiwitten om aan de ene kant de receptorvoorkeur van verschillende virussen en aan de andere kant de toepasbaarheid van deze eiwitten als vaccin te onderzoeken. Aangezien HA een membraangebonden eiwit is, een eigenschap die het opzuiveren van functionele eiwitten bemoeilijkt, werd gekozen voor het produceren van oplosbare HA eiwitten. Hiertoe werd het transmembraandomein vervangen door een trimerisatie motief, zodat het oplosbare HA zijn trimere, functionele vorm behoudt. Vervolgens werd onderzocht of de functionaliteit van HA wordt beïnvloed door het productiesysteem en dus door verschillen in de N-glycosylering van het eiwit. Onze resultaten laten zien dat dit inderdaad het geval is. Korte suikerketens, als gevolg van expressie in insectencellen, leiden tot betere binding van HAs aan siaalzuurbevattende structuren. Gedetailleerde analyse van het bindingsprofiel aan verschillende siaalzuurbevattende suikers van de HAs met behulp van zogenaamde 'glycan arrays' liet vervolgens zien dat deze verbeterde binding wel ten koste gaat van de specificiteit van binding. Echter, subtiele verschillen in siaalzuurbinding tussen HAs afkomstig van verschillende virussen konden niet gedetecteerd worden met HAs geproduceerd in insectencellen, maar wel na gemaakt te zijn in zoogdiercellen.

Na de uitbraak van het "Mexicaanse griepvirus" werd het zoogdier-expressiesysteem vervolgens benut om te onderzoeken in hoeverre het HA van het nieuwe pandemische virus afwijkt van een zeer vergelijkbaar HA afkomstig van een varkensvirus. Het HA van het humane virus bleek een veel zwakkere receptorbinding te hebben dan het HA van het varkensvirus. Uitgebreide analyse van hybride en mutant eiwitten leidde tot de identificatie van twee aminozuurveranderingen, op positie

200 en 227, in HA die verantwoordelijk zijn voor de gevonden verschillen. Opvallend genoeg wordt slechts één van deze veranderingen gedetecteerd in de huidige circulerende humane H1N1 virussen.

Omdat er behoefte is aan verbeterde griepvaccins, werden de recombinante, trimere, oplosbare HA eiwitten ook getest op hun potentieel om te fungeren als vaccin. Hierbij werden verschillende HA eiwitten getest in verschillende diermodellen. Kippen en muizen bleken volledig beschermd tegen een dodelijke infectie met het hoog pathogene H5N1 virus na vaccinatie met H5 eiwit in aanwezigheid van adjuvans. Terwijl één vaccinatie voldoende was om kippen volledig te beschermen, was er een tweede vaccinatie nodig voor volledige bescherming van muizen. Kippen die beschermd waren scheidden geen virus uit. Dit suggereerd dat het mogelijk is, in het geval van een uitbraak, verdere verspreiding van het virus te voorkomen. Vervolgens werd recombinant H1 eiwit getest in een varkens- en frettenmodel, waarin de dieren geïnfecteerd werden met het nieuwe pandemische virus uit 2009. Een tweevoudige vaccinatie met dit eiwit had een sterk remmend effect op de replicatie en uitscheiding van het virus in varkens. Ook in fretten werd de replicatie van het virus sterk geremd na vaccinatie met recombinant H1 eiwit. In deze studie werden fretten ook gevaccineerd met recombinant tetramere, oplosbare NA eiwitten. Vaccinatie met NA had weinig effect op de vermeerdering van het virus, maar verminderde wel in sterke mate de ziekte symptomen als gevolg van de virus infectie. Optimale bescherming werd dan ook geïnduceerd door vaccinatie met beide antigenen. Bovendien had gelijktijdige toediening van NA en HA had een positief effect op de HA-specifieke antilichaamrespons.

Ten slotte werd onderzocht in welke mate de glycosylering van HA van invloed is op de HA-specifieke antilichaamrespons na immunisatie met recombinant H5. Door H5 tot expressie te brengen in verschillende cellen in de aan- of afwezigheid van enzymen die de modificatie van de suikerketens beïnvloeden werden H5 preparaten verkregen die alleen verschillen in hun N-glycosylering. HA eiwitten met eindstandige mannose residuen aan hun suikerketens bleken beduidend minder immunogeen te zijn dan HA eiwitten met complexe suikers of HA eiwitten waar de suikerketens van verwijderd waren. HA eiwitten geproduceerd in zoogdiercellen zijn dus superieure antigenen vergeleken met vergelijkbare eiwitten gemaakt in insectencellen.

Het onderzoek zoals beschreven in dit proefschrift laat zien dat recombinante, trimere, oplosbare HA eiwitten excellente hulpmiddelen zijn om influenza A virus-receptor interacties te bestuderen. Bovendien vormen deze eiwitten een aantrekkelijk alternatief voor bestaande griepvaccins.

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Curriculum Vitae

Robert Paul de Vries was born in Naarden, The Netherlands on May 26, 1983. He graduated from the secondary school in 2002 at, “Laar & Berg” where he obtained his VWO diploma. In September that year he started his biomedical studies at the University of Amsterdam. In 2006 Robert started his 9-month internship at the Amsterdam Medical Centre at the department of experimental virology, under the supervision of Dr. Rogier Sanders. At the beginning of 2007 Robert did a 6-month internship in San Diego California USA at the Torrey Pines Institute for Molecular studies under the supervision of Dr. James Binley. In October that year Robert received his Masters Degree and started his PhD in November at Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases & Immunology, Virology Division. Here he studied receptor binding en immunogenic properties of the influenza A virus hemagglutinin under the supervision of Dr. Xander de Haan and Prof. Peter Rottier. This thesis describes the results obtained during his PhD. In anticipation of defending his PhD Thesis, Robert de Vries started his postdoctoral research at the department of chemical physiology at the Scripps Research Institute in San Diego under supervision of Prof. James Paulson.

List of publications

1. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120.
Crooks ET, Moore PL, Franti M, Cayanan CS, Zhu P, Jiang P, de Vries RP, Wiley C, Zharkikh I, Schülke N, Roux KH, Montefiori DC, Burton DR, Binley JM.
Virology. 2007 Sep 30;366(2):245-62. Epub 2007 Jun 19.

2. The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity.
de Vries RP, de Vries E, Bosch BJ, de Groot RJ, Rottier PJ, de Haan CA.
Virology. 2010 Jul 20;403(1):17-25. Epub 2010 May 2.

3. A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1.
Cornelissen LA, de Vries RP, de Boer-Luijtze EA, Rigter A, Rottier PJ, de Haan CA.
PLoS One. 2010 May 14;5(5):e10645.

4. Recombinant soluble, multimeric HA and NA exhibit distinctive types of protection against pandemic swine-origin 2009 A(H1N1) influenza virus infection in ferrets.
Bosch BJ*, Bodewes R*, de Vries RP*, Kreijtz JH, Bartelink W, van Amerongen G, Rimmelzwaan GF, de Haan CA, Osterhaus AD, Rottier PJ.
J Virol. 2010 Oct;84(19):10366-74. Epub 2010 Aug 4.
*Contributed Equally

5. Only Two Residues Are Responsible for the Dramatic Difference in Receptor Binding between Swine and New Pandemic H1 Hemagglutinin.
de Vries RP, de Vries E, Moore KS, Rigter A, Rottier PJ, de Haan CA.
J Biol Chem. 2011 Feb 18;286(7):5868-75. Epub 2010 Dec 20.

6. Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus.
Loeffen WL, de Vries RP, Stockhofe N, van Zoelen-Bos D, Maas R, Koch G, Moormann RJ, Rottier PJ, de Haan CA.
Vaccine. 2011 Feb 11;29(8):1545-50. Epub 2011 Jan 8.

7. A stabilized HIV-1 envelope glycoprotein trimer fused to CD40 ligand that activates dendritic cells.
Melchers M, Matthews K, de Vries RP, Eggink D, van Montfort T, Bontjer I, van de Sandt C, David K, Berkhout B, Moore JP, Sanders RW.
Retrovirology. 2011 Jun 20;8(1):48.

8. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity.
Ambepitiya Wickramasinghe I, de Vries RP, Grone R, de Haan CA, Verheije M.
J. Virol. 2011 Jun 22 doi:10.1128/JVI.05112-11

9. Influenza A virus entry of cells lacking sialylated N-glycans
de Vries E, de Vries RP, Wienholts MJ, Floris CE, Jacobs MS, van den Heuvel A, Rottier PJ, de Haan CA *Submitted for publication*

10. Glycosylation-dependent immunogenicity of recombinant soluble hemagglutinin.
de Vries RP, Smit CH, Rigter A, Cornelissen LA, Eggink D, Sanders RW, Hokke CH, Rottier PJ, de Haan CA *Manuscript in preparation*