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IMMUNODOMINANCE OF ANTIGENIC SITE B IN RECENT CIRCULATING H3N2 INFLUENZA VIRUSES

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IMMUNODOMINANCE OF ANTIGENIC SITE B IN RECENT CIRCULATING H3N2 INFLUENZA VIRUSES

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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Dedicated to my beloved father Ivan Dmitrievich Popov.

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ABSTRACT

Type A influenza viruses cause seasonal epidemics and global pandemics driven by antigenic drift in the viral surface glycoproproteins, hemagglutinin (HA) and neuraminidase (NA). Each year in the United States more than 200,000 people are hospitalized and 20,000-36,000 people die from flu-related complications (CDC, 2011). Subtype H3 HA has accumulated 20.4% amino acid sequence changes in HA1 in the 42 years since it appeared in humans in 1968. Five antigenic sites, A-E, were mapped on the surface of 1968 and 1971 HAs by escape mutant analysis and competition assays using monoclonal antibodies (mAbs), but there are no detailed mapping studies of recent H3 HAs. The presence of five antigenic sites on HA would appear to mean that host immune response might be limited to only the most immunodominant antigenic site on HA. The goal of our research is to determine if there is immunodominance of particular antigenic sites and whether this immunodominance changes from year to year due to accumulation of mutations in antigenic sites during antigenic drift.

To test the immunogenicity of antigenic sites in modern H3N2 viruses we analyzed the epitopes of two human monoclonal antibodies, E05 and 1_C02, specific to HA1 of vaccine strain A/Wisconsin/67/05 (H3N2) and Wisconsin-like influenza A/Oklahoma/309/06. To partially map the epitopes of E05 and 1_C02 we used mutants of HA of Wisconsin-like influenza A/Oklahoma/309/06 (H3N2) in antigenic site A (NNES121-124ITEG, N126T, N133D, TSSS135-138GSNA, K140I, RSNNS142-146GPGSG), and antigenic site B (HL156-157KS, KFK158-160GST, NDQI189-192QEQT, A196V). To estimate the immunodominance of antigenic sites in recent H3N2 influenza virus HA we studied the binding of antibodies in human plasma after

seasonal vaccination in 2006-2007 (H3N2 component A/Wisconsin/67/05) and after vaccination in 2008-2009 (H3N2 component A/Uruguay/716/07) to wild type and mutant recombinant HA proteins. We used the same panel of mutant HAs to determine the specificity of polyclonal antibodies in postvaccination human plasmas. Our results suggest that antigenic site B is immunodominant over antigenic site A in recent H3N2 viruses.

To confirm the immunodominance of antigenic site B in recent influenza viruses we isolated escape mutant viruses of A/Perth/16/08 selected with post-vaccination human plasmas in season 2010-2011 (H3N2 component A/Perth/16/09 from two individuals. Both contained two mutations (H156Q and G186V) in antigenic site B of HA and there was no mutation in antigenic site A of HA. Our results suggest that antigenic site B of A/Perth/16/09 is immunodominant in both subjects. Together with serum reactivities against mutant recombinant HAs, these results confirm that antigenic site B has been immunodominant over site A in H3N2 viruses at least since 2005 even though there have been changes in both sites A and B between A/Wisconsin/67/05 and A/Uruguay/716/07 and between A/Uruguay/716/07 and A/Perth/16/09.

The understanding of immunodominance of antigenic sites of HA may allow to predict the antigenic drift and design the vaccine ahead to influenza epidemics.

SPECIFIC AIMS

Five antigenic sites were mapped on the surface of 1968 and 1971 HAs by escape mutant analysis and competition assays using monoclonal antibodies (mAbs), but there are no detailed mapping studies of recent H3 HAs. It has been observed that new epidemic strains arise due to changes in only one or two antigenic sites, implying that not all antigenic sites are equally important. **The goal of this study is to determine if there is immunodominance of particular antigenic sites and whether this immunodominance changes from year to year.** The specific aims are:

Aim 1. Identify the antigenicity of antigenic site A and antigenic site B on modern H3 HAs

To address this aim we investigated mutants in antigenic site A (NNES121-124ITEG, N126T, N133D, TSSS135-138GSNA, K140I, RSNNS142-146PGSG), and antigenic site B (HL156-157KS, KFK158-160GST, NDQI189-192QEQT, A196V) of HA of Wisconsin-like influenza A/Oklahoma/309/06 with human monoclonal antibodies 1_C02 and E05 made against the H3 vaccine component in season 2006-2007 A/Wisconsin/67/05. I measured the Affinity of binding of monoclonal antibodies 1_C02 and E05 to wildtype and mutant HAs.

Aim 2. Define the dominance of antigenic site A or antigenic site B in circulating viruses in season 2006-2007 and season 2008-2009

To obtain experimental data if there is dominance of antigenic sites on modern H3 HAs, we measured the antibodies in plasma of human subjects who received the 2006-2007 trivalent subunit influenza vaccine (H3 component

A/Wisconsin/67/05) or the 2008-09 formulation (H3 component A/Uruguay/716/07). Plasmas were tested against expressed HA of Wisconsin-like influenza A/Oklahoma/309/06 and site-directed mutants in antigenic site A (NNES121-124ITEG, N126T, N133D, TSSS135-138GSNA, K140I, RSNNS142-146PGSG), and antigenic site B (HL156-157KS, KFK158-160GST, NDQI189-192QEQT, A196V) with "native ELISA" analysis.

Aim 3. Selection of escape mutant viruses with polyclonal antibodies in post-vaccination human plasmas

We isolated escape mutant viruses of A/Perth/16/09 selected with post-vaccination human plasmas. The sites of mutation in escape mutant HAs showed that the dominant polyclonal antibodies are directed to antigenic site B on HA.

A better understanding of immunodominance of antigenic sites may allow prediction of future antigenic drift and assist in vaccine strain selection.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

A history of influenza virus and flu pandemics

Influenza epidemics are common annual event. Influenza epidemics are unpredictable in time and severity. However, there are features that make influenza epidemics more likely (reviewed in (138)). First, epidemics occur in winter months when it is cold and a higher humidity. Secondly, epidemics are first seen in Southern Hemisphere countries, and later spread to Europe and North America in winter months of these areas. Thirdly, epidemics are likely to occur when a variant virus appears that shows antigenic changes from previous strains, and cross-reacting antibodies, gained by previous infection or vaccination, is low (224). Because of nonspecific symptoms and lack of features specific only to influenza, it is very difficult to discern the history of this disease. Influenza was likely to exist since ancient time. In 412 BC Hippocrates described an epidemic with sweatiness and illness and modern doctors believe that was influenza. The Italians used the expression “ex influential colesti”, believing that they were under some celestial influence that was responsible for the epidemic badly affected the city of Florence in 1387. Villaini and Segui modified the term, and it became known as “una” influenza (130). The French referred to influenza as “ the grippe”, suggesting the acute onset of influenza in which the patient suddenly was seized or gripped by disease. The major pandemic in the 16th century that affected human populations is considered unquestionably as influenza. Caius, the English physician described a

"sweating disease" in 1551 characterized by headache, fever and myalgia that killed some patients in hours but lasted only a few days in survivors. It is believed that Caius described the influenza (137).

According to dictionary definition, a pandemic is simply a widespread epidemic. When we refer to influenza, a pandemic means a worldwide epidemic caused by a new influenza virus (17).

The greatest pandemic happened in the last century in 1918 and was known as Spanish flu. In the USA Spanish flu killed 550,000 people (0.5% of the population); a number ten times greater than that of Americans killed in the First World War. In a few places, such as villages in Alaska, Spanish flu killed more than 25% of the population. Globally, the influenza pandemic of 1918 killed 20-40 million people and is estimated to have sickened 50% of the population. The 1918 influenza killed more people in less time than any other disease (11, 55, 205).

After the 1918 pandemic there were two major global pandemics of influenza in the 20th century: in 1957 Asian influenza virus (H2N2), and in 1968 the Hong Kong (H3N2). In 2009 WHO declared a global outbreak of an H1N1 virus derived from swine and this pandemic was named as swine-origin H1N1 pandemic (**Fig. 1**) (22). The worldwide epidemic H1N1 that occurred in 1977 is not considered a pandemic by WHO because the virus was not novel. The highly pathogenic avian influenza H5N1 is emerging since 1998. Due to the high lethality and virulence of "bird flu" the H5N1 virus is a potential threat in the world.

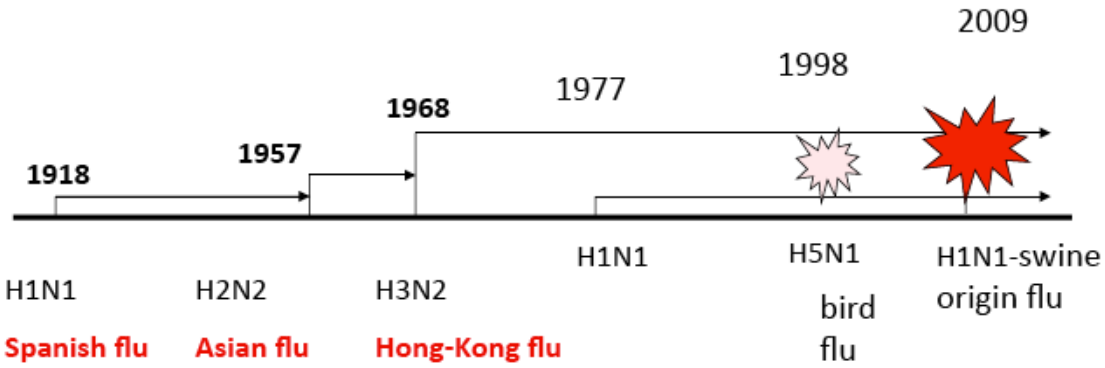

Fig. 1. Flu pandemics since 20th century.

Viral isolation

Influenza viruses as a "filterable agent" were isolated from chickens suffering from fowl plague in 1901 (55). Much later, in 1955, it was shown that this "filterable agent" was type A influenza virus (202). Human influenza virus was first isolated in Wilson Smith's laboratory in 1933 (166, 177). Smith inoculated ferrets with nasopharyngeal washes from humans with influenza. The inoculated ferrets became infected and that infection spread to other animals in the cage. The viral isolate is denoted A/Wilson-Smith(WS)/33 H1N1 influenza virus. The full nomenclature for influenza isolate includes the type of virus, the host of origin (except for human), place and year of isolation, and subtype. For example, A/Aichi/2/68 (H3N2) is a type A of influenza virus of H3N2 subtype isolated in Aichi, Prefecture (Japan), from human patient #2 in 1968.

Type B influenza virus was isolated in 1940 (60) and type C was isolated in 1947 (189).

Influenza is one of the most studied of viruses and virus disease. Influenza virus is a research interest of scientists, epidemiologists, physicians, and the pharmaceutical industry. Despite that, little is done to change the course of influenza infection by a new virus as was seen in 2009 (4).

Influenza virus

Classification

Influenza viruses belong to the family *Orthomyxoviridae.* Orthomyxoviruses contain segmented, linear, and negative-sense, single-stranded RNA genome and a lipid envelope. Orthomyxoviruses are divided into three types A, B and C based on the antigenic properties of the viral nucleoprotein. The number of RNA segments is eight in influenza viruses A and B, seven in influenza virus C. Each RNA segment encodes at least one protein: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1 – F2 (PB1-F2) (55). Influenza B and C viruses infect humans, causing mild illness. By contrast, the influenza A viruses cause disease in domestic poultry, horses, pigs as well as humans. Influenza viruses are maintained in a widespread natural reservoir in wild waterfowl and shorebirds. Influenza viruses periodically emerge as antigenically novel viruses through genomic re-assortment and this has become the definition of "pandemic influenza".

Influenza A viruses are sub-divided into subtypes based on the antigenic identity of two glycoproteins exposed on the virion's lipid envelope, the hemagglutinin (HA) and the neuraminidase (NA); to date these are subtypes H1 to H16 of the hemagglutinin (HA) and N1 to N9 of neuraminidase (NA) although an H17 has been recently proposed (191). Influenza A viruses H1N1 and H3N2 along with type B viruses are currently circulating in the human population and these are the antigens in the annual trivalent vaccines. No antigenic subtypes have been identified in the influenza B and C viruses.

Viral replication cycle

The influenza virus life cycle can be divided into the several stages: 1. Binding to cell surface receptors; 2. entry into host cell; 3. entry of vRNPs into the nucleus; 4. transcription and replication of the viral genome; 5. export of the vRNPs from the nucleus and translation of viral proteins; 6. assembly and budding at the host cell plasma membrane; 7. release or budding of virion particles (**Fig. 2**).

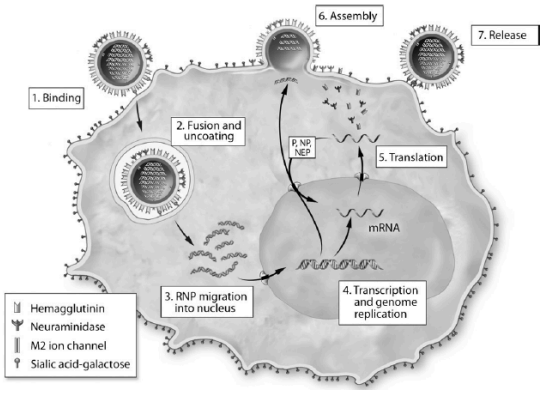

Fig. 2. The influenza A virus replication cycle (From (19) with permission).

In the life cycle of influenza virus the surface glycoprotein hemagglutinin (HA) plays a major role in recognition of cell surface receptors, fusion with cell membrane and as a component in assembling and budding of viruses.

Viral structure

Despite its importance as a pathogen, little is known about the virus structure. Influenza virions are pleomorphic. Influenza virus exibits intristic structural variability (pleimorphy). Particles can exists as spherical virions (approximately 100 nm in size) or filamentous (elongated) particles with length more than several μm. Both vary in size. Filamentous particles are commonly found in original human isolates and early egg passages (174). On the other hand, laboratory adapted strains are predominantly spherical due to rapid growth of the small spherical particles (42). The viral structure may correlate with its infectivity and pathogenicity but the mechanisms are not known.

Cryo-electron microscopy (cryo-EM) has been used to image influenza virions at neutral pH (28, 61, 227) and low pH (139, 164, 181). However, the structures from slices ~100nm of all levels of cryo-EM studies were irregular.

Cryo-electron tomography (cryo-ET) has been used to reconstruct three-dimensional images of individual particles of influenza virus and show that virions are highly redundant (79). In this study the viral particles were classified into five classes: four spherical and one elongated, distinguished by features of RNA organization, matrix layer. The five classes are: 1. Spherical, abundant particles with an evident matrix layer and disordered arrangement of RNPs; 2. Elongated particles; 3. Particles that lack matrix layer and have jumbled RNPs; 4. (With

matrix); 5. (No matrix) and condensed cores in slices appeared as parallel arrays of seven to eight rods. The elongated particles share the feature of class I, except their RNPs are aligned to the long axis of the elongated virion.

Despite difference in structures, most virions have three common features: 1. virions are covered with glycoprotein spikes; 2. there are two layers of equal density and thickness underlying the spikes; the first layer is lipid bilayer and second is matrix protein monolayer; 3. virions contain RNPs.

The structure of glycoproteins

In negative strained specimens and cryotomograms, two types of spikes are seen on the surface of all influenza viral particles. Trimeric spikes are identified as the HA. Other spikes are square or mushroom shape and belong to the tetrameric NA. Glycoproteins on the surface are packed closely and irregularly. But the distribution of HA and NA over surface appear to be not entirely random. Clusters of HA spikes are abundant. Single NA spikes surrounded by HA spikes (**Fig. 3**).

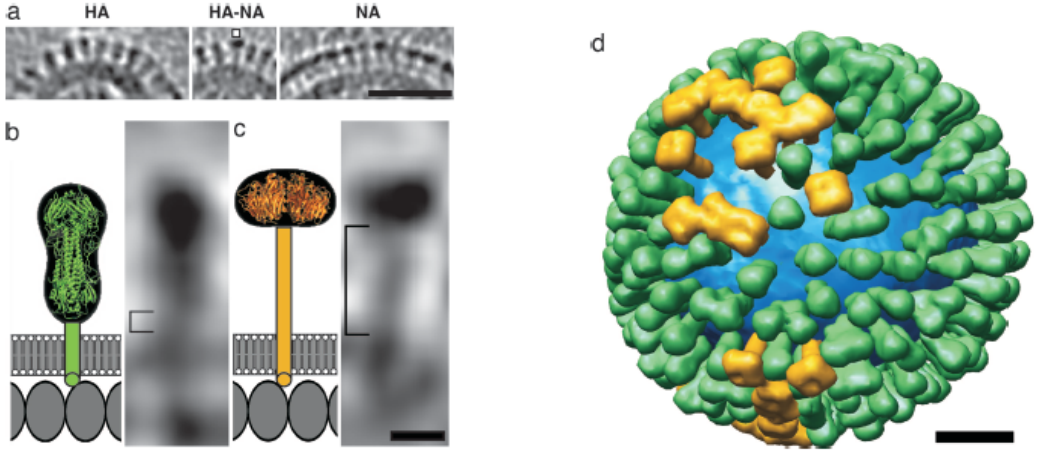

Fig 3. HA (a, b) and NA (a, c) structure resolved by cryo-ET and its distribution on the viral surface (79). (d) Model of distribution of HA (green) and NA (gold) on the single influenza virion. The lipid bilayer is blue. Scale is 20nm. Figure reproduced with permission.

More recently, cryo-ET of individual virions was used to solve the virion structure at low pH compared to neutral pH (59). In this study the irreversible structural changes in HA on the viral surface, early stage of fusion process observed in individual virion after transferring to pH 4.9 are characterized. It was shown that the low pH first affects the HA that became disorganized on the surface and irreversible structural changes of HA were seen after longer incubation. Additional major changes were observed in virion size and shape: disappearance of elongated virions, coagulation of matrix protein and RNPs and resolving of M1 (**Fig. 4**).

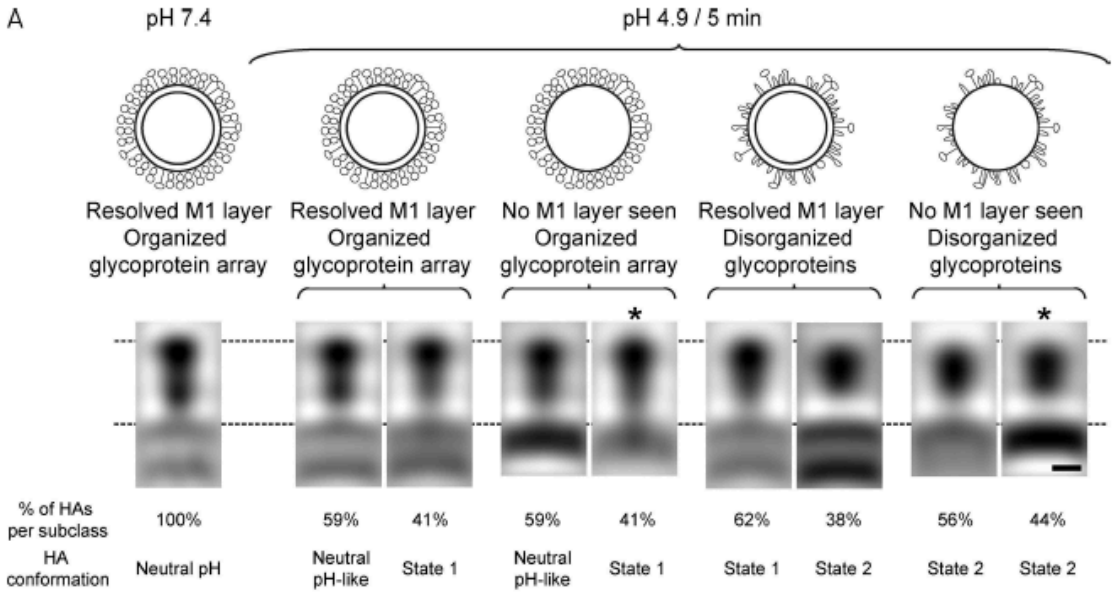

Fig. 4. Subtomogram averaging showing altered conformations of HA ectodomains. Schematic diagram of analyzed virion types (79). Reproduced with permission.

The formation of filamentous virion is not completely understood. The M1 and M2 proteins have been proposed to be a determinant in the formation of filamentous

virion (142, 143). Immunofluorescence microscopy was used to study the budding of H3N2 A/Udorn/72 virus and formation of filamentous particles in different types of cells. The effect of the inhibitory 14C2 antibody directed against the N-terminal extracellular domain of the M2 protein on viral morphology, assembly, and release was studied. It was shown that filamentous particle formation was inhibited in cells treated with anti-M2 14C2 antibody but not by anti-NA antibodies. Analysis of the protein compositions of spherical and filamentous particles showed that the NP:M1 or NP:HA ratios in filamentous particles were significantly lower than in spherical particles, but the filaments have higher levels of NP per particle. These results indicate that the filamentous morphology of the A/Udorn virus depends on the matrix (M1) and/or M2 proteins.

On the other hand the low energy state of membrane vesicles corresponds to spherical shape. It is likely that the disappearance of elongated virions at low pH corresponds to their converting to spherical shape. If the shape of elongated or filamentous virions is determined by the membrane-lining lattice of M1 protein the relaxation of M1-envelope interactions as well as M1-M1 interactions would cause the formation of spherical morphology in response to low pH (79, 80).

The RNPs are packed quite densely in virions. Based on most observations RNPs are visualized individually only in class II virions (elongated). Lengths of RNPs vary from ~24nm up to ~100nm. Each RNP makes a contact with matrix at one or both ends (79).

The arrangement of RNPs varies with the morphology and size of virion. In elongated viral particles RNPs are localized making a parallel bundle. All RNPs are

associated with the matrix at one end of the virion. In spherical particles the contact points of the various RNPs complexes on the matrix are not clustered but are distributed around the interior surface. Elongated and spherical particles were resolved by cryo-ET. However, it not found the correlation of the structure and viral infectivity.

The data presented in analysis of individual virions by cryo-ET show that there are several sequential changes inside of the virion induced by low pH (59) and these changes are related to the RNP complex. A summary of these events are given in **Fig. 5**. At low pH 4.9 the RNP complex condenses onto the M1 layer (step 2) and, finally, is no longer seen (step 3). At neutral pH the RNP complex condenses to two separate poles.

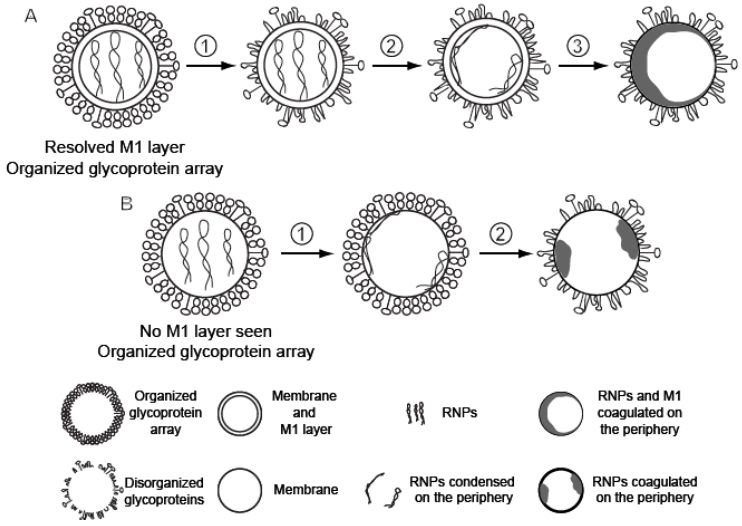

Fig 5. Schematic of sequential changes in influenza virions induced by low pH. A. incubation at pH 4.9 (step 1) adherence of RNPs to the periphery (step 2), the condensation of RNPs and M1 into a single coagulate (step 3). B. In neutral pH virions lacking an M1 layer, organized glycoproteins, RNPs move to the periphery (step 1) a disordering of the glycoprotein array and condensation of the RNPs at the periphery (step 2) (59). Reproduced with permission.

Condensation of RNP prevents the infectivity of influenza virus. This implies, that the structural changes in HA and RNP are connected. HAs should complete the conformational changes to fuse with endosome membrane in order to avoid the RNP condensation and release RNP complex into cell cytoplasm.

Thus, analyses of viral structure show that: 1. There are two different shapes of influenza virus, spherical and filamentous. 2. There are common features of all virions. These common features are: all virions are covered with two shapes of spikes belonging to HA and NA; 3. Virions are enveloped by a lipid bilayer; the segmented ssRNA genome is located inside the virion. 4. Cryo-electron microscopy investigations show dramatic changes in influenza virion between neutral pH and low pH due to structural rearrangement of both RNP and HA. The changes in HA structure will be discussed in the Chapter Hemagglutinin.

Vaccines

Flu remains a serious disease despite the availability of vaccines against influenza virus. Each year in the United States more than 200,000 people are hospitalized and 20,000-36,000 people die from flu-related complications (56). H1N1 and H3N2 along with type B viruses are currently circulating in the human population and these are the antigens in the trivalent vaccines. The most dangerous side of influenza infections is complications that result from or follow the initial viral infection. These include viral pneumonia and bronchitis, secondary bacterial pneumonia, bronchiolitis or myocarditis and pericarditis that may lead even to death. Vaccination against influenza reduces the number of influenza illness cases and prevents post-influenza complications and (29).

Current Influenza Vaccines

Currently, vaccination is the main option to reduce influenza disease burden. In the USA both live-attenuated and inactivated influenza vaccines are approved. Trivalent vaccines contain two A types of influenza, H1N1 and H3N2, and a B virus component. The formulation of trivalent vaccine is updated each year and these changes are due to antigenic drift of the strain circulating in the human population. The viruses are adapted to grow in embryonated eggs, which produce high yields of virus. Due to concerns that egg adaptation can change antigenic properties, efforts are being made to grow vaccine viruses in mammalian cell culture. The efficacy of the vaccines for influenza depends on choosing the right strains. Antigenic "match" of vaccine component is a primary factor for effective vaccines against circulating viruses in human population.

On the other hand the effectiveness of vaccines also depends on individual factors of subjects such as their immune status, and age. Studies show that inactivated vaccines give a protective antibody titer in approximately 70-80% of healthy people under 65 (136). For individuals over 65, this number is lower 30-40%, but the current inactivated vaccines, nevertheless, protect up to 80%, and so prevent high risk of death to the elderly. Thus, annual vaccination against flu has potential benefits in preventing disease, hospitalization and even death.

A major limitation of current vaccines against influenza is that growth of the virus is time consuming. To obtain the vaccine for a new viral strain takes six to eight months, and so, in a pandemic situation, the new virus spreads around the world in this time (**Fig. 6**). Production of large amounts of vaccine during

epidemics is limited by availability of eggs and sometimes delayed by difficulties in adapting the virus to grow in eggs. In 2009 such a situation happened with swine-origin H1N1 (2).

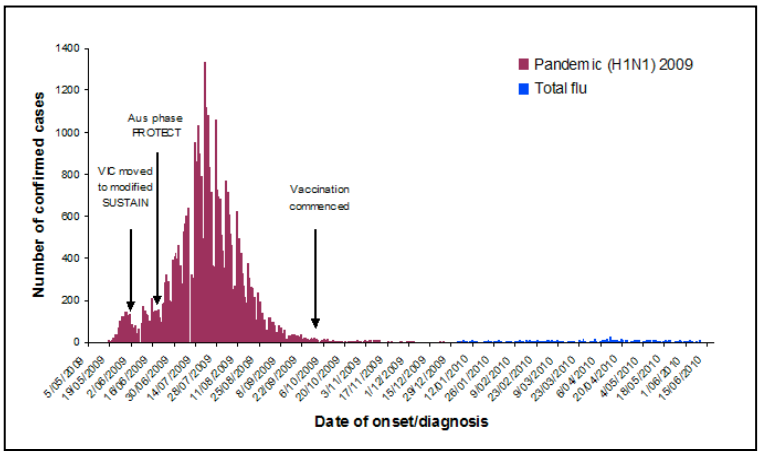

Fig. 6. Laboratory confirmed cases of pandemic (H1N1) 2009 and total influenza in Australia, to 2 April 2010 (2). The epidemic was over before the vaccine became available.

Cold-adapted influenza virus vaccines (LAIV)

Cold-adapted influenza virus vaccines also known as a live attenuated vaccines (LAIV) have been used for many years in Russia and are recommended for use in children without evidence of side effects. In addition, there is no evidence of the spreading of vaccine strains within Russia or in the world after LAIV vaccination (204).

LAIV is delivered by nasal spray. This route of introduction has advantages over more costly injection using needles. In certain age groups (the elderly, and children from 6 months to 9 years) LAIV is more protective than inactivated vaccines and even if a vaccinated person becomes infected, the incidence of secondary bacterial infections and use of antibiotics are decreased (122).

Thus, live attenuated vaccines have high potential as efficient and economical vaccines.

Prospective vaccines

1. Genetically engineered live influenza virus vaccines

Creation of attenuated influenza vaccines by introduction of site-specific changes into the genome is a prospective approach to modify the vaccines (53, 62). It was shown that mutations in the NA promoter region of influenza A virus attenuates the pathogenesis in mice. Another example is engineered changes in PB2 (polymerase) gene (134). By mutating specific amino acids in the PB2 polymerase subunit, two temperature-sensitive (ts) attenuated viruses were obtained. These strains might have potential as live attenuated vaccines.

Other examples of candidate engineered vaccines are LAIV vaccine expressing mutant NS1 genes and vaccine using the replication-defective influenza viruses.

Following viral infection the host usually responds by IFN (interferon proteins) expression. NS1 is an internal protein encoded by the smallest gene segment. It was shown that NS1 has IFN-antagonist activity (63). Genetically changed NS1 protein that increase IFN induction may allow effective immune response without causing illness.

Another approach is a replication-defective vaccine strain by elimination of the M2 gene. Such strain grows efficiently in tissue culture but not in mice (206).

2. DNA vaccines

DNA vaccination is a vaccination with plasmid DNA encoding one or more

influenza virus proteins. The data from DNA vaccination to influenza are limited, and most investigations are done with mice, chickens and ferrets (100, 111). One of the concerns of DNA vaccination is a packaging system or vector to transport of desired DNA to desired cell type. Plasmid DNA delivery attached to charged molecules as liposome or calcium salts is inefficient. Systems for DNA transport including retrovirus, vaccinia virus, adenovirus is proposed. However, concerns about infectivity restrict the use of viral vectors in human vaccines.

Novel adjuvant approaches

Adjuvanted influenza vaccines have been licensed in Europe and named as "virosomal" vaccines. This type of vaccine includes the *Escherichia coli* heat labile toxin or synthetic adjuvants such as MF59 within lecithin vesicles to improve the immunogenicity of the trivalent viral components. Such influenza vaccines show high protection with lower amounts of antigen needed. Such vaccines may also be used intranasally (69).

Another type of vaccines is the killed and inactivated influenza virus vaccines prepared for intramuscular injection with liposome–like complex of viral particles and liposomes named as immune stimulating complexes (ISCOMS) This type of vaccine has been shown to be effective on mice (152) but is not licensed in humans.

Universal vaccines against influenza

One candidate for a broad-spectrum vaccine against influenza is the HA stem (mostly HA2) that is considerably conserved between HAs. The receptor binding HA1 globular domain of HA shows much higher sequence variability relative to the fusion active HA2 subunit. Several studies suggested that HA2-

directed antibodies can protect mice against influenza (70, 129, 175). Recently, neutralizing human monoclonal antibodies (Abs) that bind in the stem domain were shown to cross-react and neutralize several subtypes of viruses and provide broad range protection (51, 186, 226). These antibodies act by targeting the HA2 region of the HA molecule and presumably prevent the conformational changes of HA at low pH, thus blocking fusion of viral and cell membranes. There are several attempts using recombinant expressing soluble HA2 domain in the absence of HA1 produced at low pH. The stalk region of HA was successfully expressed on the surface of mammalian cells and also on HIV gag viral-like particles (180). However, there is difficulty in expressing only HA2 domain because it does not fold correctly. In the study (27) the authors demonstrated a successful design of a soluble immunogen that largely consists of an HA2 subunit in its neutral pH conformation. The HA2 subunit was expressed in *E. coli* in the presence of parts of the HA1 domain that were included for correct folding of HA2. The expression system in *E. coli* has the advantage of rapid and cheap production of large quantities of HA2 subunit but the disadvantage of not adding the sugar chains to HA2.

The alternative to targeting a conserved subunit may be the construction of a "generic" HA, based on a set of conserved amino acids such as those in the receptor binding site, that could be used as a immunogen in vaccines. Another vaccine approach could be the creation of a specific prospective HA based on prediction of immune selection of HA. The knowledge of immunodominance of antigenic sites of HA gives the opportunity for such predictive vaccine design.

Not only HA can be used in creation of immunogen. Neirynk et al. (121) proposed to generate an universal vaccine against influenza A virus by using the extracellular domain of the M2 channel protein to the hepatitis B virus core protein. However, such vaccines lacking HA may not represent an improvement compared to current vaccines. This may be due to immunodominance of HA over other viral proteins in influenza virus.

Surface proteins

Three proteins are exposed on the surface of influenza virus, M2 channel, neuraminidase and hemagglutinin. Surface proteins of influenza virus are target for neutralizing antibodies.

M2 channel

Viral infection involves the delivery of transcriptionally active viral vRNPs to the nuclei of infected cells. This process triggers the expression and replication of the virus genome and, as a consequence, the budding of viral particles from infected cells (87). The delivering of viral RNPs is a multiple step process that begins with HA attachment to a cell receptor. Following attachment, the virus enters the cell by receptor-mediated endocytosis. The low pH 5-6 of endosomes interior activates a structural changes in the HA which induces the fusion between viral and endosome membranes. Acidification of the virion interior by H^+ ion flow through the M2 ion channel in the viral membrane causes dissociation of the M1 protein from the RNPs and, subsequently, migration of free RNP to the nucleus (120, 135).

The M2 ion channel protein is the third membrane protein exposed in the viral surface that can be as a target for neutralizing antibodies. M2 channel protein contains only ~1% of total viral protein which is equal ~ 23-60 copy per virion (229). M2 is a 97–residue membrane protein that has its amino- and carboxy-terminal ends directed towards the outside and inside of virus respectively. M2 protein is a homotetramer in native state and contains three structural domains: an amino-terminal extracellular domain (23 amino acid residues), trans-membrane domain (TM) (19 residues) and cytoplasmic domain (54 residues). The sequence of the extracellular domain of M2 is highly conserved even between types of viruses (49) giving the opportunity to create a broadly-neutralizing antibodies (vaccine) directed to M2 (132).

M2 is present at low copy number of molecules on the virion surface, the M2 is shielded by NA and HA (103) perhaps explaining why antibodies against M2 proteins are at low or undetectable levels in most exposed individuals (24).

The structure of M2 channel and models of mechanism of the function and inhibition by drugs were recently proposed by Schell and Chou based on NMR (155) and other hand by Stouffer et al/ DeGrado based on 3D crystallography analysis (184) were published in the same issue of *Nature* in 2008.

Recent solid state NMR spectroscopy (ssNMR) investigation of TM structure of M2 channel with amantadine show that there are two sites of binding of drugs (33). The high affinity drug binding site is occupied by a single drug molecule and localized in N-terminal channel surface which is inside of M2 channel. The low affinity binding site, is localized on the C-terminal surface of

channel. The second site was observed only at high concentration of drug on lipid bilayer. The X-ray crystallization model had the drug binding inside the channel.

Thus, the structure and mechanism of M2 remains under investigation whereas the role of M2 protein as a target for neutralizing antibodies is minimal. At the same time, the M2 channel is a target of antiviral drugs against influenza (7).

Neuraminidase

Neuraminidase (NA) is one of the two major glycoproteins on the viral surface.

~ 5% of total influenza protein composition (79) belongs to NA. NA is the second most abundant glycoprotein of influenza virus. NA of type A and B viruses cleave the α–ketosidic linkage between terminal sialic acid and an adjacent sugar residue. NA is considered to be a receptor-destroying enzyme (74).

The NA is a tetramer that is exposed on the viral surface with box-shaped head (10x10x6 nm) (8, 43, 196). The three-dimensional structure of NA has been solved for influenza A and B types. The fold of type A and B NA are almost identical despite homology of only 28% (30, 43, 196). The tetrameric NA protein has a circular four-fold symmetry. Each polypeptide monomer folds into six antiparallel β–sheets. The head is centrally attached to a stalk that is attached to the membrane by an N-terminal hydrophobic sequence. Consequently, its carboxyl terminus is localized outside (8). The stalk region is flexible in length and sequence. In contrast to cytoplasmic region of HA, the six amino-acid tail is highly conserved among all NA subtypes of influenza virus A (25, 26). But it was found that deletion of five residues does not affect the viability of virus. Mutant viruses lacking the NA or the

NA cytoplasmic tail become more filamentous (88). The stalk region of NA also can be reduced, interchanged with a foreign sequence or deleted completely (34, 112). “Short NA stalk” viruses are sometimes accompanied by mutations in HA1 that could affect receptor binding. This suggests that the balance between HA and NA is critical for efficient influenza virus replication (14, 25, 26, 117) although this may not always be the case (78). NA deficient viruses may be replicated in the cell culture. NA deficient viruses contain the internal deletions of NA leaving regions encoded cytoplasmic and trans-membrane domains and part of stalk (75, 109, 110). Mutations incorporated in NA-deficient viruses have also indicated the importance of a balance between NA and HA functions (85). Whether these NA-deficient mutants exist in infected hosts is not known.

The catalytic active sites of NA are localized in head domains of tetrameric NA. Sialic acid cleaved from the cellular receptor is bound in a large pocket surrounded by nine acidic residues, six basic residues and three hydrophobic residues of each NA monomer. Each residue in the NA catalytic pocket is strictly conserved in influenza viral NA. NA of type B can functionally replace the NA of type A suggesting conserved catalytic activity of NAs (68).

Antigenicity of NA

Antibodies against NA are characterized but they do not block the attachment of virus to cell. NA antibodies are less abundant than anti-HA antibodies. It is true that HA is a major target against immune system. But anti-NA antibodies block infection as shown by selection of escape mutants (76, 151, 207, 209). *In vitr*o

assay data show that anti-NA antibody inhibit NA activity (76, 125, 208) leading to the conclusion that anti-NA antibodies bind to epitopes surrounding the enzyme active site. In the three-dimensional structure of NA, the sites of escape mutations are localized in loops surrounding the active site pocket (Fig. 7). X-ray structures of complexes of NA bound to monoclonal antibody Fabs show the complete epitope maps of two antibodies to N9 and one to N2 (194, 200).

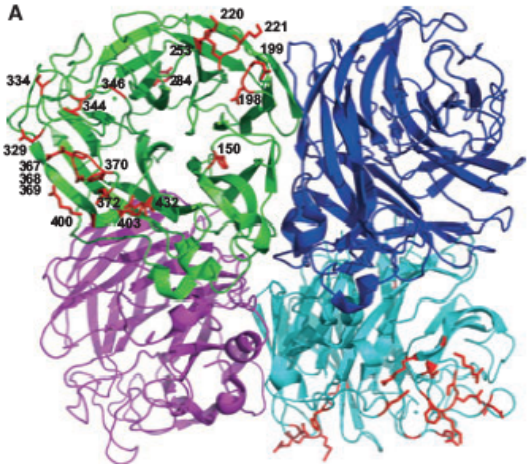

Fig. 7. Escape mutations in NAs surround the active site pocket. NA is a tetramer. All known escape mutations are marked in the globular domain of NA (green) monomer (Figure from (6) reproduced with permission).

Hemagglutinin

Three-dimensional structure

An H3 HA was the first membrane glycoprotein structure (**Fig. 8**) to be solved by crystallography (222).

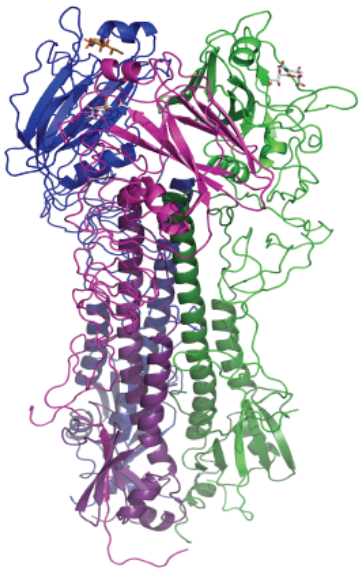

Fig. 8. The crystal structure of HA A/Aichi/2/68 is a trimer. Each monomer is colored. Figure was made from PDB ID 2VIR using PyMol (Schrödinger, LLC).

Now X-ray crystallographic structures of H3N2 HA have been determined for the three different conformations: bromelain-cleaved soluble HA (BHA) of A/Aichi/2/68 (219, 222) which represents the conformation of cleaved HA, then uncleaved HA0 precursor (38) and fragments of low pH-treated BHA.

The crystal structure of H3 HA shows a trimer that measures ~13.5 nm long from the viral, envelope membrane to its tip and 1.4-4 nm in cross section.

Each of the three subunits comprises two chains HA1 and HA2, after proteolytic cleavage of the precursor protein HA0. As a result the molecule of HA contains six chains that are three HA1 and three HA2.

Each monomer of HA is folded into two structurally distinct domains, a globular domain (head) and elongated domain (stalk). The globular head is entirely composed of HA1 residues (328 aa) and contains an eight-stranded antiparallel β-

sheet motif termed as a "jelly roll". A short α-helix (the "190" helix) separates strands 3 and 4 of the "jelly roll". The residues from one side of the 190 helix and from residues near the top of the jelly roll form a pocket that is the receptor binding site in each monomer of the HA molecule. The receptor–binding site is surrounded by highly variable loop structures. The binding sites of neutralizing antibodies against influenza viruses are mapped within these variable loops.

The HA2 subunit (221 aa) creates two anti-parallel α-helices that form part of the elongated domain, or stalk, of the HA. One of the α-helices is longest known in globular proteins (~7.5 nm).

The HA1 and HA2 chains of each monomer are connected by single disulfide bond (HA1 14- HA2 137) (222).

A hemagglutinin trimer is stabilized by interactions between three major HA2 α-helices. These interactions form a triple–stranded coiled coil in the interior of the trimer. The N-terminal (top) half of the coiled-coil superhelix is tightly packed with several non-polar residues in van der Waals contact around the 3-fold axis. The C-terminus end of the superhelix expands away from the axis with polar and charged residues from each monomer experiencing electrostatic repulsion from like residues in the other monomers.

In the middle of stalk the cleavage site between HA1 and HA2 is located. In uncleaved HA0, eight residues that immediately surround the cleavage site, including five hydrophobic residues of HA2, are directed away from the molecules into aqueous solvent and form a surface loop in the middle of the stalk. After

cleavage the carboxy end of HA1 becomes exposed on the trimer 2.2 nm distant from the N-terminal end of HA2 due to significant conformation change of the HA0. The Hydrophobic N-terminus of HA2 becomes buried in the trimeric structure filling the cavity. The cleaved HA is presumable metastable.

Disulfide bonds in HA

HA native structure is a stable structure. It is well established that disulfide bonds significantly contribute the stability of protein structures in native state. The importance of individual cysteine residues during folding and assembly in vivo of influenza virus HA was tested by substitution mutagenesis Cys-Ser (159). In H3 HA there are several cysteines involved in disulfide bonds. It was shown that a) cysteine residues in the ectodomain are essential for efficient folding of HA and for stabilization of folded HA; b) cysteine residues in the globular domain are likely to form disulfide bonds rapidly and directly, without involvement of intermediate, nonnative linkages; c) cysteine residues in the stalk form a specific disulfide bond separated with partner residue by hundreds of amino acids including other cysteine residues.

There are two opposite ideas how cysteine bonds can be created. First of them is stated that disulfide bonds can be created spontaneously at the certain redox conditions and specifically when the protein conformation favors them (47). According to this idea disulfide bonds will be created after correct folding of the protein. Another idea is that disulfide bonds can be formed at an intermediate state of a local region of a protein to lock a region into a particular folded state (94).

Both scenarios predict that “non-native” disulfide bonds may be formed

during protein folding (46). However, investigations suggest that the protein contains only native disulfide bonds (36).

The crystal structure of H3 HA shows six disulfide bonds (222). Four of them are within HA1, one is within HA2 and one connects HA1 and HA2. Comparison of the amino acid sequences of HA from 12 influenza virus subtypes shows that the positions of the 12 cysteines in the ectodomain are completely conserved, suggesting the importance of these residues in forming the disulfide bonds essential for the folding of HA.

Intensive investigation of the cysteines involved in three disulfide bonds in the H2N2 A/Japan/305/57 HA: Cys4 and Cys462; Cys42 and Cys273; Cys55 and 67, has been reported (160). This investigation of the effect of serine substitutions suggests that the major effect of the loss of a disulfide bond is destabilization of the HA conformation leading to increasing protein misfolding.

Cys 55 and Cys 67 are very close in polypeptide chain and not separated by other cysteine residues as Cys 4 and Cys 42. The bridge between Cys 55 and Cys67 seems likely to form directly and rapidly. Single mutations of both cysteines are severely defective in folding and transport suggesting the importance of Cys 55 and Cys 67 for HA folding.

Assembly of influenza HA trimer and its role in intracellular transport

Hemagglutinin of influenza virus is an integral membrane glycoprotein. It is co-translationally inserted into the endoplasmic reticulum as a precursor, HA0, and is transported to the cell surface via the Golgi complex. Detailed investigation of assembly and transport of HA of influenza virus at the molecular level was done

(44). The HA0 is synthesized and glycosylated in the endoplasmic reticulum (ER) (116, 147). Then HA0 is transported in the same way as cellular plasma membrane proteins through the Golgi complex to plasma membrane. HA0 is modified by attachment of oligosaccharide side chains to the glycosylation sites. The location and number of glycosylation sites are not conserved among HAs of different strains and subtypes. In addition to the post-translational glycosylation, there are other post-translational modifications of HA. The three cysteine residues in the carboxyl-terminal region of HA2 are acylated with palmitic acid (154) by thioester linkage (197).

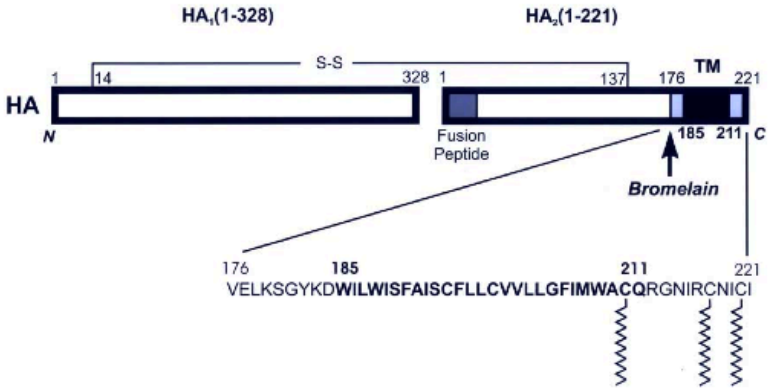

Fig. 9. Schematic structure of the influenza A/Aichi/2/68 HA. Three palmitic acid residues covalently linked in 210, 217 and 220 to C-end of HA2. TMD is 185-211 aa residues. Figure from (99) with permission.

In **Fig. 9** (101) the schematic structure of H3 HA is presented. Three-dimensional structural information is not available for the trans-membrane C-terminal anchoring peptide (185-211 TMD) in the viral membrane. Peptide (176-221 HA2) was found to be palmitoylated on three conserved cysteine residues (182) in positions 210, 217 and 220 (101). The trans-membrane domain has crucial

role at a late stage of membrane fusion (157). HA TMD secondary structure, orientation, oligomerization and interactions with lipids are very poorly understood.

There are only few studies of the function of HA TMD published (101, 102, 157). A synthetic analogue of the full-length HA TMD was reconstructed into phospholipid bilayer (157).

The results of studies by MS show that HA is palmitoylated at the two cysteines in its cytoplasmic tail and stearolyted at the cysteine located in trans-membrane domain (**Fig. 10**).

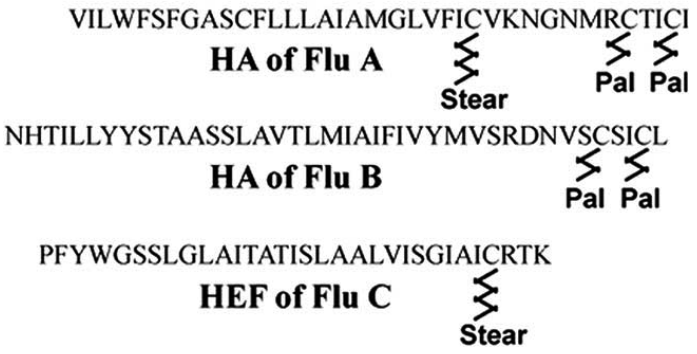

Fig. 10 (from (102)**. The putative TMDs (embedded in lipids) and CTs (cytoplasmic tail) of HA of influenza A and B virus and HEF of influenza C virus** with palmitate (Pal) and stearate (Stear) attached to individual cysteine residues are shown. Three cysteines of HA2 are modified in H3 influenza viruses. Reproduced with permission.

Function of palmitoylation and stearatoylation of HA TMD

All hemagglutinin glycoproteins of influenza viruses are S acylated at certain cysteines, but differ in the number and location of their acylation sites (199). HA of all subtypes of influenza virus A is acylated at three highly conserved cysteine residues (555, 562 and 565) (101, 102, 182, 198).

It was shown that the hydrophobic modification is essential for influenza

virus replication. Depending on the virus strain, virus mutants with more than one acylation site deletion showed dramatically decreased growth or could not be created at all by reverse genetics (37, 96, 234). For the H3 HA subtype, the data are consistent. Palmitoylation of HA is not required for fusion activity (168, 182). However, for other subtypes, including H1, H2 and H7, the fusion activity of non-palmitoylated HA varied between reports (95, 96). It was shown that there is no difference in the ability of nonpalmitoylated H3 HA to induce fusion whether expressed alone or in the context of virus (37). One possible explanation for the discrepancy in the impact of palmitoylation mutants on fusion between HA subtypes is might be that HA palmitoylation may affect the kinetics of fusion differently among subtypes.

HA in membrane fusion and viral entry

The fusion of influenza viruses to the endosomal membrane is mediated by the HA (214). Therefore, HA is required for viral and cell membrane fusion and entry into the cell. Cleavage of HA0 generates the C-terminus of HA1 (328 aa of HA0) and N-terminus of HA2. This process of exposure of N-terminus of HA2 is crucial for infectivity of influenza virus (218). Arg329 exposed after cleavage at the C-terminus of HA1 is removed by a cell carboxypeptidase (65). Mutation of HA R329Q prevents the cleavage into HA1 and HA2 suggesting that R329 is critical for tryptase recognition and cleavage (38). Cleavage occurs at the cell surface or on released viruses. For HA0 of the subtypes H1, H2 and H3 cleavage may be mediated by the serine protease, tryptase Clara of the bronchiolar epithelium. This enzyme recognizes the Q/E-X-R sequence found at the cleavage sites of the HA.

The sequence of 19 aa fusion peptide is crucial for HA-mediated fusion. Mutations within the fusion peptide decrease or abolish fusion activity of HA.

The next crucial process required for viral infection is the low pH-induced conformational changes of HA (170, 215). The three major refolding events are: 1. An extended loop and short α helix become an extension of the central triple-stranded coiled coil in native HA (76-105 aa extended to 38-105 aa) (**Fig. 11**).

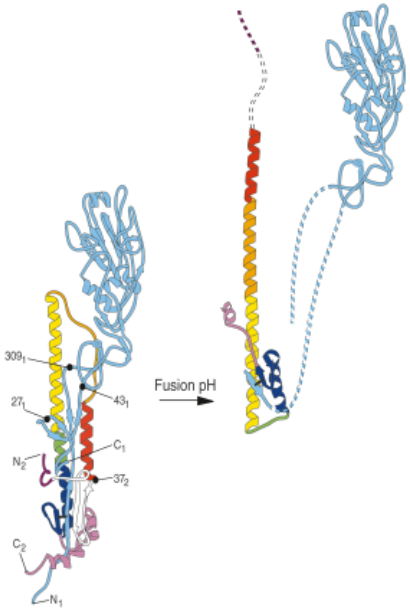

Fig. 11. Conformational change in the HA occurs at low pH to convert it into the fusion-competent state. Rearrangement of helices in HA2 at pH 5-7 (173) with permission.

2. The middle of the long α helix of native HA2 forms a reverse turn. 3. Amino acids 141-175 located in C terminal refold to a small β sheet hairpin. The overall effect of this refolding is to deliver the fusion peptide toward to target membrane and to bend the molecule in half so that the fusion peptide and the viral membrane anchor are near the same end of the elongated molecule of HA.

The mechanism of membrane fusion was intensively investigated and it is believed that several steps are involved as described in **Fig. 12.** (213). The HA2 N-terminal fusion peptide is the most highly conserved region in HA. From mutant analysis it is known that the length of fusion peptide is crucial for fusion activity and two charged residues of HA2 are essential. The region 1-22aa of HA2 is inserted into the membrane for fusion.

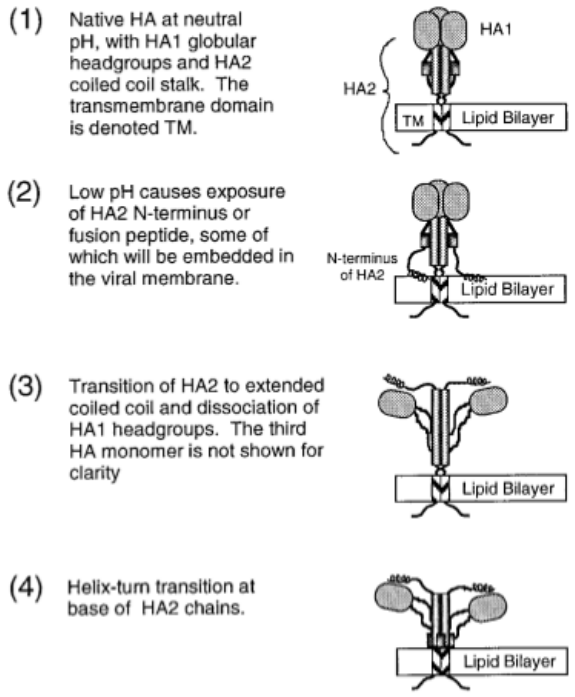

Fig. 12. Steps in HA-mediated membrane fusion. (From (213). Fusion of HA with cell membrane contains several steps. Step1. Transmembrane domain is recognized TM. Step 2. Exposure of N-terminal fusion peptide. Step. 3. Transition of HA2 and dissociation of HA1 headgroups. Step. 4 Helix-turn transition of HA2. Reproduced with permission.

Glycosylation

HA as well as NA is a glycosylated protein. Both the globular head and stem regions contain N-linked oligosaccharide chains. Glycans on the surface of HA are attached to Asn residues of the site motif Asn-X-Ser/Thr, where X is any amino acid except proline. Not all sites can be glycosylated. Glycosylation may be inhibited by combinations of Asn-X-Ser or when the glycosylation site follows specific amino acid sequences.

The appearance and disappearance of oligosaccharides on the globular domain has been observed during antigenic drift of influenza A/H3N2 viruses from 1968 to 2012. Loss and gain of glycosylation sites on HA and NA sometimes have dramatic effects on the biosynthesis, stability and function of viral HA and NA. Glycosylation sites on HA1 of H3 subtype have been progressively increased from three to eleven in 2012 since appeared in human circulation in 1968. During 43 years of circulation (1968-2012) loop A of H3N2 viruses gained four glycosylation sites (at 122, 126, 133, 144 residues). This is only loop in the surface of H3 HA with multiple oligosaccharide chains. Other two new sites for glycosylation are within antigenic site E and antigenic site D. Glycosylation at 165 is localized on the top of globular domain near the 190 helix of site B. Thus, recent circulating H3N2 human isolates accumulated as many as eleven potential glycosylation sites on the HA1 of HA during antigenic drift (231) but it is not known whether all of these potential sites are glycosylated or not.

The progressive increase in N-linked glycosylation sites observed over time in H3 HA1 does not appear to be a general feature of influenza virus evolution and was not observed in human influenza B or in avian H3.

The stem region of HA of 1968 Hong Kong influenza virus contained five glycosylation sites (Asn residues 8, 22, 38, 81 and 285 of HA1). These are strictly conserved among all human H3 HAs (5, 173).

Functions of glycosylation

The loss and gain of glycosylation sites from HA as well as NA could have effect on expression, stability, and functions of viral glycoproteins. It was shown by site-directed mutagenesis that some glycosylation sites are important for expression of functional NA (150). Oligosaccharides affect a number of biological functions of viral HA. Glycosylated sites near the HA receptor binding pocket affect its ability to recognize sialic receptors on the cell surface as well as altering preference for binding to the type of receptors. It was shown that mutants that lacked an oligosaccharide chain on the top of the HA molecule (at 165) (**Fig. 13**) differed from parent viruses in their ability to recognize specific cell surface receptors (86, 118). Mutant viruses lacking glycosylation at 165 and/or at 246 were different compared to wild type viruses in their ability to agglutinate red blood cells (81, 195). The effects of the additions of oligosaccharides at 165, 63, 126, 122, 246, 133 and removal at 81 that observed during antigenic drift in 1975 (drift of A/Aichi/2/68 to A/Victoria/3/75) on the biological activities of H3 HA was investigated in report (5) (**Fig. 13**).

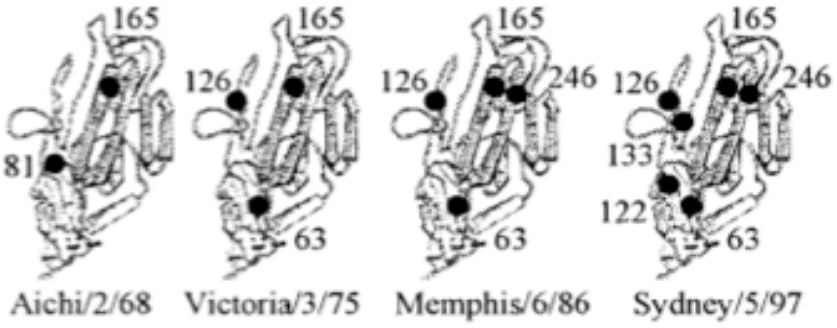

Fig. 13. Schematic drawing of the globular head of influenza A/H3N2 virus HA, showing the change in N-glycosylation sites among representative isolates (from (5)) reproduced with permission.

In this study seven H3 HA mutants in the globular domain (G1-G6) were made with additions of glycosylation sites on the globular domain of HA that were observed in natural isolates during antigenic drift in H3 HA from 1968 to 1997. Mutant HAs with glycosylation sites mimic the HAs of isolates A/Aichi/2/68, A/Victoria/3/75, A/Memphis/6/86 and A/Sydney/5/97.

The conclusion of this study was that addition of oligosaccharide chains drastically reduced the reactivity of six human plasmas against A/Aichi/2/68. An additional conclusion was that increasing the number of glycans decreases the binding to cell surface receptors but increases the ability to escape from neutralizing antibodies. That means that evolution of H3 virus moves towards survival against neutralizing antibodies but the price for that is lower binding to cell receptors.

The biological effect of gradually increased number of HA oligosaccharides on virulence is investigated. In one study (188) glycosylations at 165 and/or 246 were studied. Deletions of both sites were associated with high weight loss and death of all infected mice. Viruses lacking glycosylation at either 165 or 246 induced disease and death at 10^5 PFU but not at a lower level. The 165 glycosilation mutant caused death at lower titer than that 246 mutant viruses, demonstrating that a particular site of N-glycosylation is associated with enhanced viral virulence in mice. These data were consistent with previous reports (81, 141). In a second study (201), the effect of gradually increasing the number of oligosaccharide chains on virulence was investigated. It was shown by mouse lungs histopathology, as well as vitality, that increasing the extent of HA glycosylation decreased the viral virulence.

It was well established that glycosylation can modulate recognition of HA by antibodies. There are several lines of evidence suggesting that the number, structure and location of the oligosaccharides affect immunogenicity of HA. Oligosaccharide chains create a shield around the globular domain of HA and masking of the protein surface by the additions of carbohydrates may be more effective than single amino acid changes in HA sequence (158, 171). Variation in number and location of glycosylation sites during antigenic drift has been suggested to prevent the recognition of major antigenic sites by neutralizing antibodies. Authors Skehel et al, 1984, (171) provided the first evidence using a mAb-selected variant HA with addition of oligosaccharide chain at aa 63. In this

study mutant viruses of the A/Hong Kong/1968 were selected in the presence of neutralizing antibody specific to HA. A single amino acid substitution Asp63 to Asn was found in the HA. This mutation generated a site for glycosylation Asn-Cys-Thr at aa 63-65 and preventing the binding of a neutralizing antibody. Addition of tunicamycin to the growth medium resulted in a virus that was once again bound by the antibody. However, it is now clear that the added glycan sites in loop A do not block it, since antigenic mutations (including escape mutations) have still been seen despite all the extra glycan chains.

Thus, HA is synthesized as a precursor HA0 in ER and modified during transport by glycosylation and adding of palmytate and stearate fatty acids to individual cysteines. HA0 is cleaved by trypsin-like protease into HA1 and HA2 on cell surface. HA1 and HA2 are connected with cysteine bond. Non-reversible refolding of HA at low pH is crucial for infectivity of influenza virus. H3 HA is crystallized as a trimer. Each monomer contains globular domain and elongated stalk. The correctly folded, co-translationally modified native structure of HA as a major antigen recognized by immune system will be discussed.

Immunodominance

To begin, what is the immunodominance? There are several definitions found in the dictionaries. Immunodominance is “The dominance of an antigen (over all others) in its ability to produce an immune response” or “the epitope on a molecule that provokes the most intense immune response (225) or “the property of an antigenic determinant that causes it to be responsible for the major immune response in a host” (1), or the “degree to which a subunit of an antigenic

determinant is involved in binding or reacting with antibody" (3). A complex protein antigen has multiple potential determinants to induce an immune response. However, certain determinants on native proteins preferentially invoke an antibody response (228).

There two sides of the immunodominance phenomenon: immunogenicity versus antigenicity. Immunogenicity refers to the ability to induce an immune response, epitope of antibody, whereas antigenicity refers to the ability to be recognized by the immune system. An epitope is defined as those amino acids that make contacts with antibody. An antigenic site is a group of overlapping epitopes recognized by antibodies. These properties do not necessarily coincide (128).

Immune epitopes can be subdivided into two structural categories: continuous (linear) and discontinuous (conformational) (73). All T cell epitopes are linear while antibody epitopes can be either linear or discontinuous. However, even with a continuous epitope the antibody binds with high affinity to one preferred conformation (156), while MHC molecules force the peptide into the preferred binding conformation. Direct evidence for the structural recognition of peptides by antibodies was obtained by investigating the peptides of the same amino acid sequence but different conformations (221). Another study of binding of antibodies to peptides with the same amino acid sequence but synthesized from amino acids in L- or D- conformation show that antibodies bind to only one chemical structure of peptide, showing that the interaction involves more than the side chains (108).

An assembled epitope consists of amino acids far apart in the polypeptide chain but folded together in the three dimensional structure to form a surface binding site. In other words, antigenic determinants must be conformational (21). The properties that have been considered to influence immunogenicity of epitopes are accessibility, hydrophilicity and mobility.

Accessibility is clearly essential if an antibody is to bind to a protein epitope. Accessibility algorithms are useful when the 3D structure is known to determine the surfaces where antibodies might bind.

Hydrophilicity. In aqueous environments the protein must be folded to bury hydrophobic residues and expose hydrophilic residues on the surface. Several studies proposed the idea that the antigenic sites might be predicted from primary sequence analysis (82, 83). In very early studies (162, 163) it was shown that charge can be important in antigen-antibody interactions. But several studies showed that hydrophobic and especially aromatic residues are important for binding with antibodies (20, 162, 165). For example, immunochemical analysis of binding and antigenic specificity of antibodies to tobacco mosaic virus protein showed the specificity to Ala-Thr-Arg. The importance of hydrophobic interactions in binding to antibodies was tested using short peptides with Tyr in center (20). In a study of the antigenicity of myoglobin-related peptides it was concluded that the antibody binding properties of the 30 or so small peptides (two-seven residues) are dominated by their cationic and/or hydrophobic properties (131, 165).

In conclusion, while antibody-binding sites are largely hydrophilic because they are on protein surfaces, hydrophobic interactions are seen to also play a role in antigen-antibody complexes.

Mobility of an antigenic site.

The idea that mobility contributes to immunodominance came from studies of protein-protein interactions. Mobility was attractive for its theoretical implications but the predictions have not held up to practice. This factor was considered promising for production of short peptide vaccines. But to date there is no licensed vaccine created by short peptides.

Mobility of portions of a protein is measured by B values in x-ray crystallographic data. B values are available only for relatively high-resolution structures. From studies of synthetic peptides binding to antibodies specific to native structure of protein it was concluded that short peptides must be in motion to bind to the epitope of the antibody (187). But such binding is very weak compared to the whole protein and this conclusion can be made regarding the specificity of antibodies for peptides but not to the immunodominance of peptides to produce neutralizing antibodies.

Another approach was used to test the importance of mobility of antigenic sites. Westhof et al. (212) compared the mobility of segments of polypeptide chain of tobacco mosaic virus (TMV) protein with known antigenic sites. This analysis showed that six of the seven sites showed high mobility. However, another study found that antibodies against native proteins bind better to polypeptides with low

mobility (9). Thus, the idea that mobile sites of a protein are more immunogenic than sites with low mobility is not supported by the evidence.

The response of the immune system to a molecule with multiple epitopes gives rise to the phenomenon of immunodominance; some antigenic areas are more immunogenic than others. Here I am going to evaluate the data from studies of complex antigens. Influenza hemagglutinin as a model for immunodominance of antigenic sites is poorly understood and investigated.

But in pathogens such as foot and mouth disease virus, Hepatitis A and Hepatitus B virus, and the bacteria *Echinococcus granulosus* the existence of immunodominant sites of the proteins has been shown.

Immunodominance of antigenic sites of Foot-and-mouth disease (FMDV) viral capsid protein

FMDV causes highly infectious and economically devastating disease of livestock. There are seven major serotypes of FMDV, and each serotype includes a large spectrum of subtypes and variants (18). The antigenic heterogeneity is one of the major obstacles to control FMDV by vaccination (146). By the mid-1970s researchers had developed information concerning the virus capsid structure and determined that one of the capsid proteins, VP1, had a prominent surface loop (149). Based on this information a new strategy developed for vaccine against FMDV. Novel vaccines were based on recombinant protein and peptides with highly immunogenic properties of VP1.

Several antigenic sites were identified in FMDV in serotype C: 1. Major site, named as A, localized at the disordered loop of VP1; 2. the carboxy-terminus of VP1; 3. Complex site, named as D, contained residues of several proteins VP1, VP2, VP3. It is known that antigenic site A contains highly conserved RGD motif and this motif is involved in cell receptor recognition site using integrins as receptors (114).

It was shown that neutralizing antibodies in a host vaccinated with virus are directed to antigenic sites and a quantitative analysis of antibodies directed to three antigenic sites was made. The result of that study was direct evidence of immunodominance of antigenic site A in VP1 (149). Indirectly, it was shown that specific removal of the surface loops comprising antigenic site A and C by trypsin treatment of intact virions led to a substantial decrease of immunogenicity and antigenicity (185). Peptides derived from antigenic site A of VP1 induced neutralizing antibodies. It was concluded that only specific regions are essential for the immunizing potency of the VP1.

Direct evidence of immunodominance of antigenic site A in VP1 was shown by immunoglobulin fractionation from blood samples of swine vaccinated with virus, tested against synthetic peptides from antigenic sites A and C coupled to Sepharose beads (115). However, all attempts to make a vaccine based on site A peptides have failed. Although site A is clearly involved in inducing neutralizing antibodies, it does so effectively only in the context of whole virus.

Echinococcus granulosus

The existence of immunodominance was analyzed using four monoclonal antibodies defining overlapping epitopes in Antigen B (AgB) (71), the major antigen of *Echinococcus granulosus*, the organism that causes cystic hydatid diseases. AgB is a 120 kDa protein composed of multimers of homologous but different 8 kDa subunits (AgB8-1, AgB8-2, AgB8-3) (169). The immunotest reagents to diagnose *Echinococcus granulosus* are based on the high immunogenicity of AgB (15). Antibodies detected by ELISA in patient sera were largely targeted against N-terminal extension of AgB8 domains (16). But among all AgB8 domains AgB8-1 exhibits the highest diagnostic sensitivity and specificity, higher even than native AgB (72). This finding was used to make monoclonal antibodies against a 38-mer synthetic peptide of the N-terminal extension of AgB8-1. The monoclonal antibodies were used to identify critical residues of the immunodominant region of *E. granulosus* (71). The epitopes of these mAbs all include the consensus sequences EVKYFER (residues 17-24) within the N-terminal extension of the AgB8-1 subunit.

It was shown using the estimated model structure of the 17-24 peptide. The antibodies recognize the same region. But the epitopes of antibodies are different (145) (**Fig 14**).

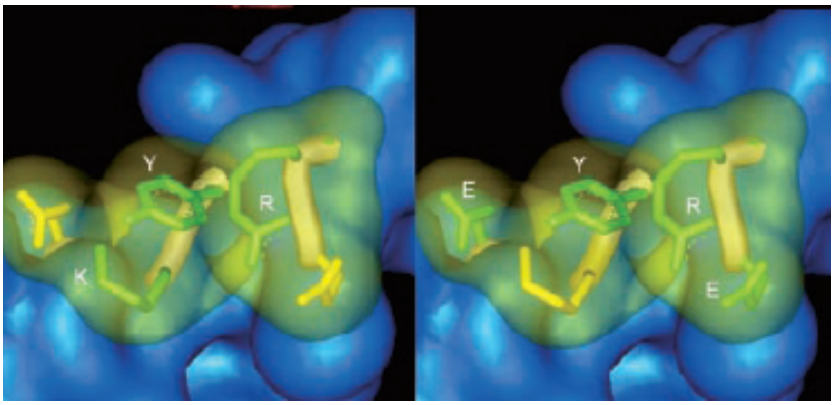

Fig 14. Putative model of sites KYX_{2-3}(R/K) and EG(Y/W)PERY presented in (71). According to this model the main contact residues of four mAbs form a contiguous surface. Reproduced with permission.

The main information from this data are: 1. AgB is an example of protein in which residues 17-24 within the N-terminal extension are highly immunogenic. This site is immunodominant. 2. mAbs directed to AgB have different epitopes. All epitopes are within one region but different sequence. Model structure suggests that the epitopes are structural. 3. Elimination of side chain of the residue required for contact with antibody or conformational change of recognition site abolishes the recognition of antigenic site.

Immunodominance of antigenic sites in Hepatitis protein

There are several types of hepatitis viruses. The development of vaccines against HAV and HBV viruses has been aided by biological assays highly protective of immunity.

HAV

The hepatitis type A virion is icosahedral and the capsid is comprised of three structural proteins (VP1, VP2 and VP3). VP1 is a abundant surface protein.

Denaturation of the virus leads to loss of antigenicity suggesting that antigenic sites are strictly conformationally dependent as measured by monoclonal antibodies specific to intact HAV (84). To identify the immunogenic sites of VP1 in HAV the panel of neutralizing antibodies was evaluated by competition immunoassays. Characterization of these antibodies and related escape mutants suggested existence only a single neutralization immunogenic site on the HAV and PV1 is a major polypeptide against neutralizing antibodies. In study of covalently cross-linking monoclonal antibodies to native HAV it was confirmed that the only major antigenic site is located in VP1.

HBV

There are three surface proteins of hepatitis B viral envelope. Small surface protein (sHBsAg) is a major structural surface protein and is composed of 226 amino acids. The sHBsAg amino acid sequence contains a highly conformational, hydrophilic domain from 100 to 160 amino acids and this region is named "a" determinant. For hepatitis B virus it is known that antibody to the immunodominant "a" determinant of small surface antigen is highly protective (220).

The reagents of diagnostic assays against HBV are directed to "a" determinant. The structure of "a" determinant is stabilized by a backbone of conserved disulfide-bonded cysteine residues. The working model of "a" determinant was solved based on data using conformational peptides and phase display experiments (39, 140) (**Fig. 15**).

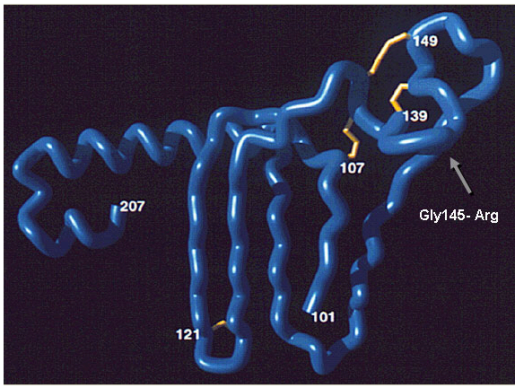

Fig 15. Gly/Arg 145 mutant in the projecting amino acid 139–147 antigenic loop of the "a" determinant. This mutant produces false-negative results in some commercial assays (39). Reproduced with permission.

The key feature of this model is a large loop stabilized by a backbone of conserved disulfide-bonded cysteine residues between 108-138 and finger-like projection stabilized by other disulfide bond between 121-124 cysteine residues. Mutagenesis analysis of the 108-138 cysteine residues cause reduced antigenicity and decreasing of the expression level of protein (92) but the mutants in 121-124 cysteine residues are similar to wild type (12).

The model includes other loop structures stabilized between cysteine pairs 136–149 and 139–147. The human immune response to HBsAg is primarily directed against disulfide-bonded conformational structures of the "a" determinant. The changing of these conformational sites not only can result the decreasing of viral infection neutralization but also can affect the detection by diagnostic assay (90, 113). Described data suggest that loop (aa139-147) in the structure of HBsAg is immunodominant.

HEV

There is no vaccine approved against HEV. However, the possibility of the creation and efficacy vaccine against hepatitis E was published (167, 230). Recently, immunodominant and conformational epitopes to the capsid protein of HEV were investigated using monoclonal antibodies (50). The candidate for immundominance in HEV was found to be the major structural and capsid protein PORF2 (193). Recombinant adenovirus expressing the ORF2 (112-660aa) antigen (56 kDa) of hepatitis E virus (HEV) was created to evaluate immunization effect in BALB/c mice (50).

In the past few decades, enormous progress regarding the development of vaccines against viral hepatitis was made. More specifically, safe and effective vaccines against hepatitis B have been available since 1988. A similarly safe and effective vaccine against hepatitis A became available in 1995. Recently, very promising results have been announced for a prospective vaccine against hepatitis E (167, 233). This form of viral hepatitis is responsible for epidemics of severe and sometimes fatal illness in less developed countries. There are no vaccines against HCV. But in both cases of vaccines against HAV and HBV were created using information of immunodominant sites in surface proteins. What determine the immunodominance of surface protein or some specific part of it to be immunodominant? It is little known about structures of immunodominant determinants in hepatitis viruses. Only model for “a” determinant for HBsAg is proposed. There are no structural data for vaccine candidates against HAV and

HEV.

Immunodominance of antigenic sites described in pathogens play major role for creation of vaccines. The epitopes of investigated neutralizing antibodies are conformational. The changes in the antigenic sites abolish the binding of antibodies.

Immunodominance in hemagglutinin of H3N2 influenza virus

Protection against influenza virus infection is mediated by neutralizing antibodies directed against HA. There are five antigenic sites A-E mapped on the surface of HA (Fig. 16). The immunodominance in HA of H3N2 influenza virus is described in Chapter II, in the Introduction of submitted paper.

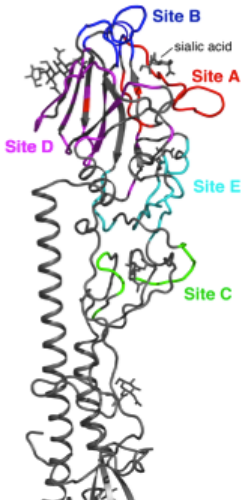

Fig. 16. Antigenic sites A-E on the surface of H3 HA of A/Aichi/68. Each antigenic site is colored.

CHAPTER II
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Immunodominance of Antigenic Site B over Site A of Hemagglutinin of Recent H3N2 Influenza Viruses

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ABSTRACT

H3N2 influenza viruses have now circulated in the human population for 43 years since the pandemic of 1968, accumulating sequence changes in the hemagglutinin (HA) and neuraminidase (NA) that are believed to be predominantly due to selection for escape from antibodies. Examination of mutations that persist and accumulate led to identification of antigenically significant mutations that are contained in five antigenic sites (A – E) mapped on to the H3 HA. In early H3N2 isolates, antigenic site A appeared to be dominant while in the 1990s site B seemed more important. To obtain experimental evidence for dominance of antigenic sites on modern H3 HAs, we have measured antibodies in plasma of human subjects who received the 2006-07 trivalent subunit influenza vaccine (H3 component A/Wisconsin/67/05) or the 2008-09 formulation (H3 component A/Uruguay/716/07). Plasmas were tested against expressed HA of Wisconsin-like influenza A/Oklahoma/309/06 and site-directed mutants in antigenic site A (NNES121-124ITEG, N126T, N133D, TSSS135-138GSNA, K140I, RSNNS142-146PGSG), and antigenic site B (HL156-157KS, KFK158-160GST, NDQI189-192QEQT, A196V). "Native ELISA" analysis and escape mutant selection with two human monoclonal antibodies demonstrated that antibody E05 binds to antigenic site A and 1_C02 binds to site B. We find that most individuals, after vaccination in seasons 2006-07 and/or 2008-09, showed dominance of antigenic site B recognition over antigenic site A. A minority showed dominance of site A in 2006 but these were reduced in 2008 when the vaccine virus had a site A mutation.

A better understanding of immunodominance may allow prediction of future antigenic drift and assist in vaccine strain selection.

INTRODUCTION

Influenza viruses are major pathogens that cause seasonal epidemics and global pandemics. Each year in the United States more than 200,000 people are hospitalized and 20,000-36,000 people die from flu-related complications (56). Due to rapid accumulation of mutations to escape host defense mechanisms, the vaccine components must be frequently updated to protect the human population against influenza. There are three types of influenza viruses, A, B and C. Type A viruses are divided into subtypes according to cross-reactivity of sera with viral surface glycoprotein antigens; to date these are subtypes H1 to H16 of the hemagglutinin (HA) and N1 to N9 of neuraminidase (NA) although an H17 has been recently proposed (192). H1N1 and H3N2 along with type B viruses are currently circulating in the human population and these are the antigens in the trivalent vaccines. HA is involved in two steps of the process of influenza infection. It binds the virus to sialic acid residues of glycoproteins or perhaps glycolipids that act as receptors on host cells then, following endocytosis, HA mediates the fusion of viral and cellular membranes to allow release of the viral genome-polymerase complex into the cell (reviewed by Skehel and Wiley (173)). Neutralizing antibodies directed against the hemagglutinin are considered the most protective against influenza virus infection and vaccine responses are most commonly tested by hemagglutination-inhibition assays.

To escape from neutralizing antibodies produced in response to infection and, most recently, mass vaccination, changes in HA have accumulated in a process named antigenic drift over the 43 years since the H3N2 subtype of influenza virus was first isolated from humans in 1968. From 1968 to 2010 there have been 108 amino acid changes identified at 63 residue positions in HA1 (total length 328 amino acids) in the major epidemic strains and most of these changes are considered to result from antigenic drift because the majority (85.5%) are clustered into regions called antigenic sites. "Antigenic site" was an operational term introduced by Gerhard and Webster (66) to describe specificities of monoclonal antibodies (mAbs). Antibodies that competed with each other for binding were considered to bind the same antigenic site. Webster and Laver identified four antigenic sites on the surface of H3 HA (A-D) by competition assays (210) and Skehel identified a fifth antigenic site, E (172). Each antigenic site contains many epitopes, structurally defined as the amino acids on the antigen that contact amino acids of the antibody (106). Competition between antibodies that bind the same site suggested that epitopes in the same site are physically overlapping but are distinct, and no one antibody molecule binds to the whole of an antigenic site. Evidence for the location of epitopes came from characterization of escape mutants, selected by mAbs, that contain single amino acid substitutions that reduce binding of the mAb to undetectable levels (35, 105, 172, 211, 217). The three-dimensional structure of A/Aichi/2/68 X-31 HA (222) showed the location of escape mutations selected by monoclonal antibodies. Assuming that the amino acid that changes in an escape mutant is within the epitope, there was now an indication of where the antigenic

sites are located. Wiley and Wilson (217, 219) took into account the sites of all known escape mutations and their corresponding antigenic site assignment, together with changes in naturally circulating viruses from 1968 to the mid-1980s, to suggest the physical boundaries of sites A-E on H3 HA, and they compiled a directory of amino acids in each of antigenic sites A-E (Figure 1A) (217). This is the map we and others have been using but it important to stress that (i) most aspects of this map were not experimentally confirmed and (ii) we do not know if this map, which was developed based on 1968 and 1971 isolates, applies to currently circulating viruses. Certainly many of the amino acids on the Wiley and Wilson list appear to be important in antigenic drift when studied phylogenetically (31) or experimentally (153).

Some mutations in HA1 created new sites for attachment of oligosaccharide chains and since the beginning of the Hong-Kong pandemic period in 1968 the number of N-linked glycan attachment sites in HA1 has increased from three to eleven. Accumulation of oligosaccharide chains in antigenic sites has been suggested to contribute to immune evasion (161, 172, 201, 203).

Antibodies directed against the head of the HA are the most abundant of the neutralizing antibodies after vaccination. X-ray structures of complexes of mAb Fabs bound to HA show how antibodies can block binding of the viral HA to sialic acid receptors on host cells (97, 98, 216). Antibodies were shown to bind near the sialic acid binding site or somewhat distant but in an orientation where the Fc portion would obstruct binding to cellular receptors (23, 57, 58, 97). Monoclonal

antibodies specific to the more conserved stem domain of H1N1, H3N2 and H5N1 viruses have been described in recent studies (45, 51, 52, 186, 226). These antibodies have broad neutralizing activity between viral subtypes and apparently act by blocking the conformational change that leads to fusion (186). The broadly neutralizing antibodies that bind to the stem region have not been detected as a significant component of the antibody repertoire but they were found to be induced by a "headless" construct (179). At this time vaccine production is still re-tooled annually according to new antigenic variants that are altered in the traditional neutralizing antigenic sites in the receptor binding domain of HA1.

Antibodies with flat binding surfaces cannot penetrate the receptor binding site of HA (148) but they can sterically block receptor attachment by binding to epitopes on the loops surrounding the sialic acid binding site (219, 222). The problem is that mutations in these loops can abrogate antibody binding without affecting HA function. One crystal structure shows an anti-H1 antibody in which the CDR3 loop is long enough to enter the receptor binding site, giving a degree of cross-reactive neutralization among seasonal H1N1 viruses (216), but in most cases the virus can easily escape antibody neutralization.

The presence of five independent antigenic sites on HA would appear to mean that at least 5 amino acid sequence changes would be needed for a new antigenic variant to emerge. In the early years of H3N2 circulation, this appeared to be the case (219) but in recent years the changes that necessitate a change in

vaccine strain have been fewer in number. The host immune response may be limited to only the most immunodominant antigenic sites of HA.

Early studies of antigenic sites on HA of A/Memphis/1/71 suggested that antigenic site A was immunodominant (104). In this study only variants changed in antigenic site A were discriminated by polyclonal antiserum. However, a rabbit was immunized with a mAb-selected escape mutant of A/Memphis1/71 that had the change G144D in HA1 and the serum was absorbed with wild type virus so that only antibodies against the new epitope would remain. There were not any; the changed epitope was not immunogenic, indicating a change in immunodominance. Studies by Temoltzin-Palacios and Thomas (176, 190) showed that the neutralizing Ab response of CBA/Ca mice is focused on a few regions of the HA1 subunit after intranasal infection with A/Aichi/2/68 X-31 virus. Sequence analyses of variant viruses isolated after a second infection of mice showed that 60% of analyzed viral HAs had a G158E mutation and 17% contained a D61N mutation. It was concluded that antigenic sites B and E are immunodominant in mice infected with the X-31 virus. Six polyclonal human plasma samples collected in 1976 with hemagglutinin-inhibition activity against Aichi/68 showed decreased binding to mutants in antigenic site A (5) which correlates with the data obtained with mouse mAbs specific to site A discussed above. A computational study suggested that site A was immunodominant in 1968-1971 and 1989-1995 while site B was dominant in 1972-1987 and 1996-2003 (133). Studies with human sera have given mixed results of clear immunodominance of site A in 1991 (123) and part of site B in

1998-99 sera (153) but no clear dominance was seen in a study of sera collected in 2004 (126) .

Overall, the phylogenetic analyses (31, 32) and serum studies (123, 153) suggest that sites A and B are the most important in directing antigenic drift of H3N2 human viruses, and so we have investigated the immunogenicity of antigenic sites A and B of recent H3 HAs. We mapped the binding of two human monoclonal antibodies to wild type A/Oklahoma/309 HA and mutant HAs derived from it, and we tested the reactivity of polyclonal antibodies in human plasma samples after seasonal vaccination in 2006 (H3N2 2006-07 component A/Wisconsin/67/05) and/or after vaccination in 2008 (H3N2 2008-09 component A/Uruguay/716/2007), to wild type HA and mutants in antigenic sites A and B. Our results indicate that most neutralizing antibodies in human plasma against both vaccine strains A/Wisconsin/67/05 and A/Uruguay/716/07 are directed to antigenic site B. We conclude that antigenic site B is immunodominant over site A in recently circulating H3N2 viruses and that site B mutations may drive the next antigenic drift.

RESULTS

Design of mutants

For this study we made mutations in antigenic sites A and B in HA1. To test if mutations accumulated since 1968 have changed the map of antigenic sites on

HA and, second, to map epitopes of monoclonal antibodies made against a recent virus, we mutated the sequence of HA1 of a local Wisconsin-like virus, A/Oklahoma/309/2006 (H3N2), to those amino acids in HA1 of the earliest human H3N2 virus A/Aichi/2/1968. The mutations are summarized in **Table 1.**

Expression of HA in the Bac-to-Bac® expression system

Initially we expressed HA in a full-length form in mammalian cells, but the level of HA on the cell surface was too low to reliably quantify antibody binding. We therefore used a Baculovirus expression system with a synthetic codon-optimized gene that deleted the transmembrane domain and included a trimerization sequence (183). HA expression from the synthetic gene was greater than 100 fold more than full-length HA expressed in mammalian cells, and >90% of baculovirus-expressed HA was secreted into the supernatant.

Based on this we made the HA mutants (**Table 1**) in the baculovirus system. All the mutants were expressed (**Figure 2**). We quantitated wild type and mutant HAs by Western blot analysis using a commercial anti-HA tag antibody taking advantage of the HA-tag sequence (YPYDVPDYA) that is conserved in all H3 HAs (**Figure 3**).

We confirmed the correct folding of wild type and mutant HAs expressed from baculovirus using trypsin digestion; trypsin cleaved the expressed HA0 into HA1 and HA2 while misfolded HA would have been degraded into small peptides (64, 67).

Effect of mutations in HA of A/OK/309/06 on binding to human monoclonal antibodies

We measured the affinity of binding of purified human monoclonal antibodies (hmAbs) 1_C02 (D1-7) and E05 (D3-4) (223) to wild type and mutant HAs using a native ELISA, capturing the HA by its His6-tag to avoid denaturation (**Table 2**). The V_H and V_L genes for hmAb E05 and 1_C02 were from single B cells of patients vaccinated with trivalent subunit vaccine containing H3N2 components A/California/7/04 and A/Wisconsin/67/05 X-161b) respectively. HmAbs 1_C02 and E05 bind intact virions of A/Wisconsin/67/05 X-161b and Wisconsin-like isolate A/Oklahoma/309/06 with high affinity but show little or no binding to related H3N2 viruses when tested in a native ELISA (**Table 3**). Our ELISA results with recombinant mutant HAs show that the binding site of 1_C02 is site B. We found that mutations in antigenic site B decrease the affinity of binding of mAb 1_C02 to HA from a Kd of 1 nM in wild type to 6 nM in mutant KFK158-160GST while there was no detectable binding to HL156-157KS. There was no significant change in binding to NDQI186-190QEQT and A196V. No mutations in antigenic site A affect the binding of 1_C02. We found that E05 binds to site A. Mutations K140I and RSNNS142-146GPGSG in antigenic site A abolished the binding to E05 but other mutations in antigenic site A NNES121-124ITEG, N126T, N133D and TSSS135-138GSNA had no effect. No mutations in antigenic site B affected the binding of E05. Our results (**Table 2**) show that the epitopes of human mAbs E05 and 1_C02 are contained in antigenic sites A and B respectively (**Figure 1B**). It is important to emphasize that these epitopes do not encompass the whole of each

antigenic site, which would be physically impossible given the size of an antibody footprint compared to the large surface areas assigned to sites A or B.

Selection of escape mutants with human mAbs

We selected escape mutant viruses after growth of A/Wisconsin/67/05-X161b virus in the presence of antibody 1_C02 or E05. An escape mutant virus selected by 1_C02 (EM1_C02) contains a substitution F159S in antigenic site B of HA1. Two escape mutants selected by E05 had amino acid substitutions at 140 (K140E and K140T) in antigenic site A. The binding constants (Kd) are included in **Table 3**. These results confirm the binding of hmAb E05 to an epitope within site A and of 1_C02 to an epitope within site B.

Reactivity of wild type and mutant HAs with polyclonal antibodies in human plasma after vaccination

To test antibodies in human plasma after vaccination we used the same panel of mutants. If antibodies in human plasma are dominantly expressed against epitopes in antigenic site A or B we will observe reduced binding of sera to mutants in this antigenic site. We used 18 human plasma samples collected 6 weeks after vaccination in Fall 2006 (H3 component A/Wisconsin/67/05) and 11 vaccinated in Fall 2008 (H3 component A/Uruguay/716/07). We tested binding of plasma antibodies to wild type and mutant baculovirus-expressed HA, captured on His-tag antibody plates to ensure preservation of native structure. The overall avidity of

binding ($Kd_{apparent}$) of plasma antibodies after vaccination in 2006 or 2008 are shown in **Figure 4** and **Supplementary Tables S1** and **S2**. There is a wide range of avidities among the different plasmas (**Figure 4, panels A** and **B**) and so we normalized the results relative to wildtype and looked for significantly reduced binding (Kd increased ≥30%) to mutants in site A or site B or both (**Figure 4, panels C** and **D**).

The results are summarized in **Table 4**. Twelve of 18 subjects vaccinated in 2006 showed reduced binding to site B mutants; 11 of these 12 showed significantly reduced affinity to the mutant KFK158-160GST. Only 7 of 18 subjects showed reduced binding to site A mutants; 6 recognizing the mutation K140I. After vaccination in 2008, 7 of 11 subjects discriminate site B mutants, and all 7 recognized the KFK158-160GST mutant. Only 2 of the 11 showed reduced binding to site A mutants. Six subjects were vaccinated in both seasons. All 6 showed reduced binding to site B mutants in 2006 and 4 of 6 in 2008. Four of the six showed reduced binding to site A mutants in 2006 but none in 2008.

Human plasma antibodies after vaccination against A/Wisconsin/67/05 or A/Uruguay/716/07 have low binding to escape mutant virus EM 1_C02

We measured binding of post-vaccination plasma samples (15 from 2006 and 11 from 2008 vaccinations) to A/Wisconsin/67/05 virus and escape mutants derived from it using the native ELISA technique, this time capturing virus by binding to sialylated glycans on turkey erythrocyte ghosts used to coat the wells.

Only virions with native HA will attach to the sialic acids. A/Wisconsin/67/05 and A/Uruguay/716/07 viruses and escape mutants derived from Wisconsin/05 virus were titrated with plasma dilutions to generate binding curves and estimate the overall apparent affinity ($Kd_{apparent}$). Our results show that on average antibodies in human plasma samples in both seasons have reduced binding to escape mutant virus EM1_C02 but not to EM E05 (**Figure 5**). These data are in accord with the results of testing human plasma antibodies against mutant HAs (**Figure 4**) in that antibodies in the majority of human plasmas bind to epitopes within antigenic site B and only a minority to antigenic site A after vaccination in 2006 and even fewer in 2008.

DISCUSSION

In the early years after the emergence of H3N2 viruses in humans multiple sequence changes were observed from one epidemic isolate to next. During the first 10 years (1968-1979), 33 amino acid changes accumulated in HA1 (10.1%) but the vaccine was changed only 3 times. In recent years the vaccine has been changed more frequently even though there are fewer changes in circulating viruses (40). For example, the only consistent differences between Wisconsin-like viruses and Brisbane/Uruguay-like viruses are G50E in site E and K140I in site A (**Table 5**). These two changes were sufficient for the H3N2 component of vaccine to be changed for season 2008-09.

Antigenic map of H3 HA

Wiley and Skehel proposed a list of amino acids contributing to each of the five antigenic sites A - E (**Figure 1A**) (217), but most of these have not been experimentally tested. The PDB database contains 4 crystal structures of mAbs bound to X-31 HA (PDB IDs 2VIR, 1QFU, 1E08, 1KEN) and while these confirm the locations of immunogenic regions, there is some overlap of the classical 5 sites; for example, antibody HC19 contacts 5 amino acids assigned to Site A as well as 5 listed in Site B (97, 98). As antigenic drift proceeded from 1968 to 2011, the amino acid sequence changes are clustered, but not exclusively, into the proposed antigenic sites, but there are no structural maps of neutralizing epitopes on the receptor binding domain of newer H3 viruses. We used the panel of site A and B mutants to partially map the epitopes of the hmAbs.

The E05 epitope

Mutations K140I and RSNNS142-146GPGSG in antigenic site A eliminate the binding of E05 but other mutations in antigenic site A as well as mutations in antigenic site B have no effect (**Table 2**). Two escape mutants selected with E05 were isolated; both had changes at 140 (K140T and K140E). The E05 epitope therefore appears to be centered around amino acids 140-146. ELISA data of E05 binding to H3N2 viruses shows high affinity binding to Wisconsin/67/05 and Oklahoma/309/06, low affinity binding to California/7/04 and no binding to A/Beijing/89, A/Panama/99, A/Wyoming/03, A/Uruguay/716/07 or A/Perth/17/09 (**Table 3**). Uruguay/07 and Perth/09 viruses share a mutation K140I in HA1

(**Figure 1B**), and this is likely to be the reason why E05 does not bind to them. Wyoming/03 HA has a mutation N145K compared to Wisconsin/05 HA which is likely to be the reason for non-binding of E05.

The low binding of E05 to A/California/07/04 is not immediately explained. The only difference in site A between California/04 and Wisconsin/05 viruses is N122D with loss of an N-linked glycan, but this glycan site is present in Oklahoma/309/06, which binds E05 with high affinity when HA is expressed in insect cells or the virus is grown in mammalian cells. It is possible that the glycan added in mammalian or insect cells does not interfere with E05 binding but the longer complex glycan on the egg-grown California/07/04 virus blocks E05 binding. We have not been able to adapt A/Oklahoma/309/06 in chicken eggs to test this. In any case, our data suggest that the epitope of neutralizing antibody E05 is centered on the site A loop of the HA.

The 1_C02 epitope

Antibody 1_C02 binds to A/Wisconsin/67/05 and A/Oklahoma/309/06 viruses, but not California/07/04 or Uruguay/716/07. An escape mutant virus selected by 1_C02 (EM1_C02) contains a single substitution, F159S (**Figure 1B**), in antigenic site B. ELISA analysis of mutants in site B shows that mutation HL156-157KS dramatically decreases the binding of 1_C02, but the adjacent mutation KFK158-160GST, that includes F159S, results in only a six fold decrease in affinity. 1_C02 binds to mutant NDQI189-192QEQT and A196V as well as to

wild type. To try to understand these effects of mutations, we used SWISS-MODEL (13, 93) to model the structure of A/Oklahoma/309/06 HA and its mutants on to the crystal structure of H3 HA of A/California/04 determined recently in our laboratory. In California/04, H156 is stacked against F159 and also makes contact with T196. F159 is present in Wisconsin/05 and Oklahoma/309/06 but the change T196A may alter the interactions in this region. In the energy-minimized model of A/Oklahoma/309/06, F159 is in the same orientation as in California/07/04, but the orientation of the side chain of F159 is changed in the HL156-157KS mutant. We propose that re-orientation of F159 in B-loop in the structure of HA disrupts the binding of mAb 1_C02. Perhaps the increased flexibility of GST158-160 compared to KFK allows antibody 1_C02 to bind while it cannot when only F159 is changed, as in the escape mutant. Sequence changes that might explain why mAb 1_C02 does not bind A/California/07/04 are N188D, S193F, T196A and D225N. The only change that could explain why A/Uruguay/716/07 does not bind 1_C02 is L194P (Table 5). It seems most likely that antibody 1_C02 interacts with the face of the 156-loop that is distal to the 190-helix, and that mutations at F159 and L194 alter the conformation so that 1_C02 cannot bind although these side chains may not be in direct contact with antibody.

From these results we conclude that the assignment of antigenic sites A and B originally made on the X-31 structure apply, at least approximately, to modern H3 HAs. Crystal structures of antibody complexes are needed to fully define the E05 and 1_C02 epitopes but to date we have not obtained suitable crystals.

Antibodies in human plasma samples after vaccination show decreased binding to mutant HA KFK158-160GST

Thirty years ago, Webster made a large panel of mAbs against the HA of A/Memphis/1/71 virus and used these to select escape mutants (104, 211). The results led to the identification of four antigenic sites (210) with the fifth added by Skehel (172). Although the escape mutants showed dramatic loss of binding when tested with mAbs used for their selection and other mAbs that recognize the same antigenic site, most of the variants showed no difference when tested with polyclonal mouse, rabbit or ferret hyperimmune sera. The exceptions were mutants with changes at 140 and 145 (104). The conclusion was that site A was immunodominant in the early H3N2 viruses. We have made a detailed study of recognition of sites A and B after 2006 and 2008 vaccinations. Of 18 subjects who were given trivalent influenza vaccine in 2006-07, 72% showed antibodies against sites A, B or both and of these, 66% showed reduced avidity against site B mutants and 39% against site A mutants. Plasmas of eleven subjects vaccinated in 2008-09 were tested, including six subjects who were vaccinated in both seasons. 64% showed reduction of binding to site B mutants and only 18% against site A. Most of the site A response in 2006-07 was to the K140I mutant. In 2008-09 the Uruguay vaccine component has the K140I mutation so it is not surprising that no subjects vaccinated in 2008 discriminated the K140I mutant. The KFK158-160GST mutant was recognized by 11 of the 12 site B responders in 2006 and 7 of 7 in 2008 (**Table 4**). There were no changes in this region of HA1 between Wisconsin/05 and

Uruguay/07 viruses, but our results suggest this is a prime candidate position for antigenic drift. Indeed, in the following vaccine strains, A/Perth/16/2009 and A/Victoria/361/2011, there is a mutation of K158N. Okada et al. (127) described a panel of mAbs generated by phage-display from a single donor that included clones that bound to site C of earlier viruses, but for 1997 and 2003 viruses most clones bound site B. Ohshima et al. found antibodies made by phage display bound a wide variety of epitopes (126), showing that there are antibodies directed to the minor antigenic sites that might select mutations in sites C, D and E but to spread, these would need to be in addition to the immunodominant site B. HA of A/Perth/16/09 compared to A/Uruguay/716/07 viruses contains mutations in antigenic site B (K158N, N189K) but also sites A (S138A) and D (K173Q). Other changes in the Perth/09 vaccine strain in site A (N144K) site D I214S, site E (E62K) and site B P194L are not found in other strains co-circulating with Perth/16/09 or subsequent isolates and so seem not to be antigenically important. Our results suggest that in recent years, antigenic site B has been immunodominant over site A but it appears that the few people with dominant site A antibodies in 2006 allowed escape and spread of the Uruguay/Brisbane viruses because of the K140I mutation. In 2008 there was clear predominance of antibodies against site B in the population, predictive of the site B change K158N that was later seen in the Perth16/09 and Victoria/361/11 epidemic strains.

MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from all human subjects and the study was approved by the Institutional Review Boards of the University of Oklahoma Health Sciences Center and Oklahoma Medical Research Foundation.

Viruses and cells

The viruses used in this study were PR8 reassortants A/California/07/2004 (CDC#2005712034), A/Wisconsin/67/2005-X161b, and A/Uruguay/716/2007 X-175, all obtained from CDC, and A/Oklahoma/309/2006, a Wisconsin-like H3N2 isolate. Viruses were grown in embryonated chicken eggs or in Madin-Darby canine kidney (MDCK) cells in DMEM: Ham's F12 medium (1:1) with ITS+ (BD Biosciences) and trypsin added as previously described (109). *Spodoptera frugiperda* Sf9 insect cells (Invitrogen, Cat. No 11496-015) were used as the host for baculovirus expressing wild type and mutant HAs. Sf9 insect cells were grown in BD BaculoGold^{TM} TNM-FH Insect medium (BD Biosciences).

Antibodies and selection of escape mutants

Human monoclonal antibodies E05 (D3-4) and 1_C02 (D1-7) have been described (223). The VH and VL genes were isolated from single B cells of subjects vaccinated with the 2005-06 or 2006-07 trivalent subunit influenza vaccine, respectively and cloned into expression vectors to produce IgGs. The expressed antibodies were purified on Protein A columns. Escape mutants were selected by incubating A/Wisconsin/67/05-X161b with 1_C02 or E05 mAb and inoculating MDCK cells in a 6-well plate with varying dilutions of the antibody-virus mixture. Escape mutants that grew out were passaged three times at limiting dilution then the HA1 region was sequenced.

Plasmid construction and mutagenesis

The HA gene of A/Oklahoma/309/06 was cloned into plasmid pCAGGS/MCS (99) as described (78). For transient expression of wild type and mutant HA proteins, HeLa or COS cells were grown in DMEM containing 10% supplemented calf serum (HyClone), 1% glutamine, 1% sodium pyruvate and 1% PKS (penicillin, streptomycin, kanamycin sulfate). Wild type and mutant HA plasmid expression vectors were transfected into the mammalian cells using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instruction. To quantify the binding of HA and mutants to human monoclonal antibodies 1_C02 and E05 (223) and antibodies in human plasma samples after vaccination we used a Baculovirus expression system. An insect cell codon-optimized HA gene of A/Oklahoma/309/06 was synthesized by GeneArt (Regensburg, Germany). The construct included the N-terminal gp67 secretion signal peptide, then residues 1-512 of HA, the trimerization foldon sequence (183), thrombin cleavage site and His6-tag. Wild type and mutant HA were cloned into the pFastBac™ expression vector for expression in the Bac-to-Bac® Baculovirus expression system (Invitrogen).

To introduce mutations into antigenic sites A and B the QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies) was used. Mutagenic oligonucleotides were named according to the original amino acid(s), their position, and the residue(s) that are changed. The following mutagenic oligonucleotides were used:

Antigenic site A:

forw Bac NNES121-124ITEG:

GCACCCTCGAGTTCATCACCGAGGGCTTCAACTGGACCGGTG

forw Bac N126T: ACAACGAGTCCTTCACCTGGACCGGTGTCAC

forw Bac N133D: CCGGTGTCACCCAGGACGGCACCTCCTCCTC

forw Bac TSSS135-138GSNA:

GGTGTCACCCAGAACGGCGGCTCCAACGCTTGCAAGCGTCGTTCCAC

forw Bac K140I: CCTCCTCCTCTTGCATCCGTCGTTCCAACAACTC

Forw Bac RSNNS142-146GPGSG:

CACCTCCTCCTCTTGCAAGCGTGGTCCCGGCAGCGGCTTCTTCTCCCGTC

TGAACTGG

Antigenic site B:

forw BacHL156-157KS:

GTCTGAACTGGCTGACCAAATCGAAGTTCAAGTACCCCGCTC

forw Bac KFK158-160GST:

AACTGGCTGACCCACCTGGGGTCCACGTACCCCGCTCTGAACGTG

forw Bac NDQI189-192QEQT:

GCACCACCCCGGCACCGACCAAGAGCAGACCTTCCTGTACGCTCAGGC

forw Bac A196V:

CCAGATCTTCCTGTACGTTCAGGCTTCCGGTCGTATC

All mutations were confirmed by sequence analysis of the whole HA1 coding region.

Expression of wild type and mutant HAs

To produce recombinant baculovirus containing wild type and mutant HAs, SF9 insect cells were transfected with the pFastBac™ HA construct using Cellfectin®II reagent (Invitrogen™) according to the manufacturer's protocol. After 72 hours the supernatant containing P1 viral stock was checked for expression of HA protein by Western blot analysis. Positive P1 viral stocks were used to generate high-titer P2 baculoviral stocks that were used for large scale (200 ml) production of HAs. HAs were purified on a nickel column and tested for correct folding and processing into HA1 and HA2 by trypsin digestion.

Quantification of expressed wild type and mutant HAs

HAs expressed in the Baculovirus system were quantified by a Western immunoblotting assay. Protein samples were loaded after boiling in 2x loading buffer (10 M urea, 4% SDS, 2% β-mercaptoethanol, 112.5 mM Tris pH 6.8, 0.01% bromphenol blue) and run in 12% SDS-PAGE. Proteins were electroblotted to Immobilon™-P transfer membrane (Millipore) in buffer (200mM CAPS pH 11.0, 10% v/v methanol) for 2h. To detect HAs we used goat polyclonal anti-HA tag (YPYDVPDYA, conserved in H3 HA) antisera (Novus Biologicals®). The blots were developed with 10 ml bromochloroindolyl phosphate and nitroblue tetrazolium substrate solution (Sigma). The bands were scanned and quantitated

using ImageQuant software (Molecular Dynamics) and amounts of HA were determined from a standard curve of GST-HA tag (YPYDVPDYA) fusion protein.

To measure the amount of HA that was correctly folded we used a native enzyme-linked immunosorbent assay (ELISA). HA (50 μg) expressed from SF9 cells was captured on His-Tag® Antibody Plate wells (Novagen®) and incubated overnight at 4ºC. Wells were washed 3 times with PBS, then serial dilutions of human monoclonal antibodies or heat-inactivated plasma were added to the wells and incubated at room temperature for 1 hour. After washing in PBS, alkaline-phosphatase-conjugated goat anti-human polyvalent immunoglobulin (α, γ and μ-chain specific) secondary antibody (Sigma#3313) was bound for 1 hour, the wells washed free of unbound conjugate and ρ-nitrophenyl phosphate substrate (Sigma #104) added. The color was developed at room temperature for 1 hour and absorbance was read at 405 nm. Antibody affinities were calculated by nonlinear regression (Y=Bmax x X/(Kd + X)) (GraphPad Prism software,) of ELISA curves plotted from eight dilutions of antibody or human plasma and represented as the dissociation constant (Kd) calculated in terms of binding sites (half-IgG). For human plasma, this is an overall or apparent Kd. A Kd increase greater than or equal to 1.3 is considered a significant reduction of binding of antibodies in human plasma samples.

Native ELISA to quantitate antibodies against viruses

We used turkey red cell ghosts solubilized with β-octylpyranoside to capture native virions for ELISA as previously described (54). The turkey red blood cells were purchased from Lampire Biological Laboratories, Inc.

ACKNOWLEDGMENTS

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FIGURES

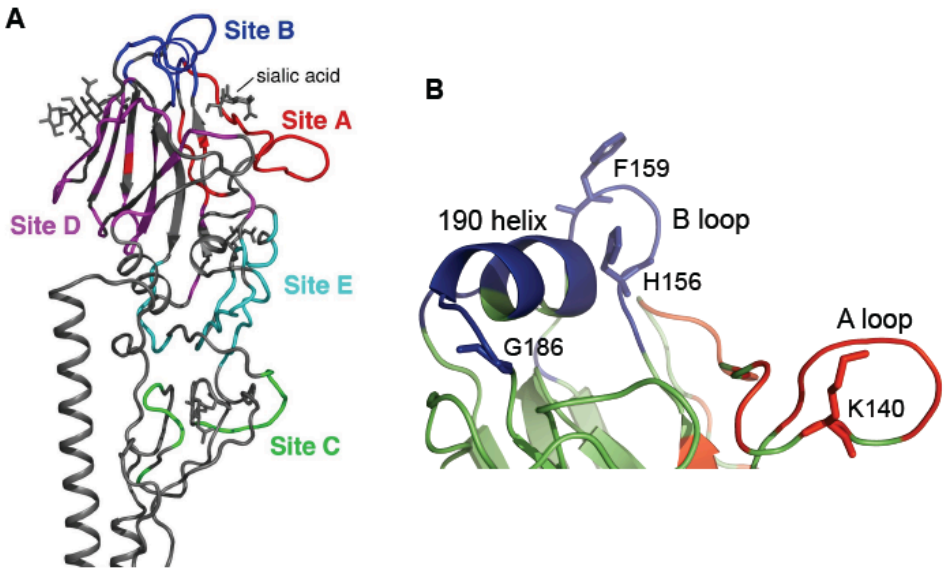

Figure 17. Antigenic structure of H3 HA. Five antigenic sites A-E are mapped on the HA1 surface of H3N2 influenza viruses. (**A**). Antigenic site A (red color) and antigenic site B (blue color) are localized on the top of HA around the receptor binding pocket. (**B**). The "190 helix" and "B loop" create antigenic site B. The "A loop" is a part of antigenic site A. Figure 1A was made from PDB ID 2VIR (58) using PyMol (Schrödinger, LLC). Figure 1B was made from an Oklahoma/309 HA structural model made by SWISS-MODEL.

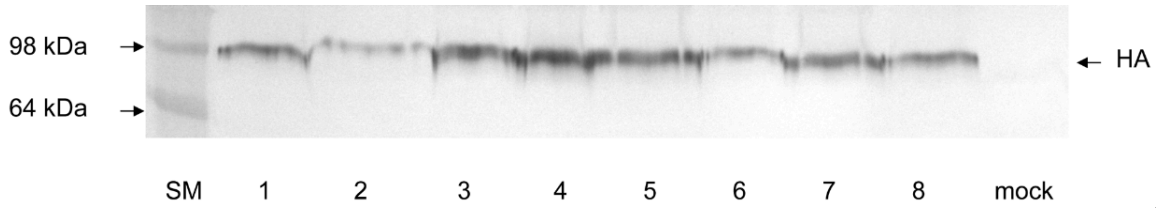

Figure 18. Expression of wild type and mutant HAs in the baculovirus system.
1. Wild type 309 HA 2. Mutant HL156-157KS 3. Mutant KFK158-160GST 4. NDQI189-192QEQT 5. A196V 6. N133D 7. TSSS135-138GSNA 8. K140I.
Supernatant (25 µl) from a 25cm^2 flask of Sf9 cells infected with the recombinant baculoviruses expressing wild type and mutant HA was loaded on a 12% SDS-polyacrylamide gel. HA was visualized by immunoblotting assay with anti-HA tag (YPYDVPDYA) polyclonal antiserum.

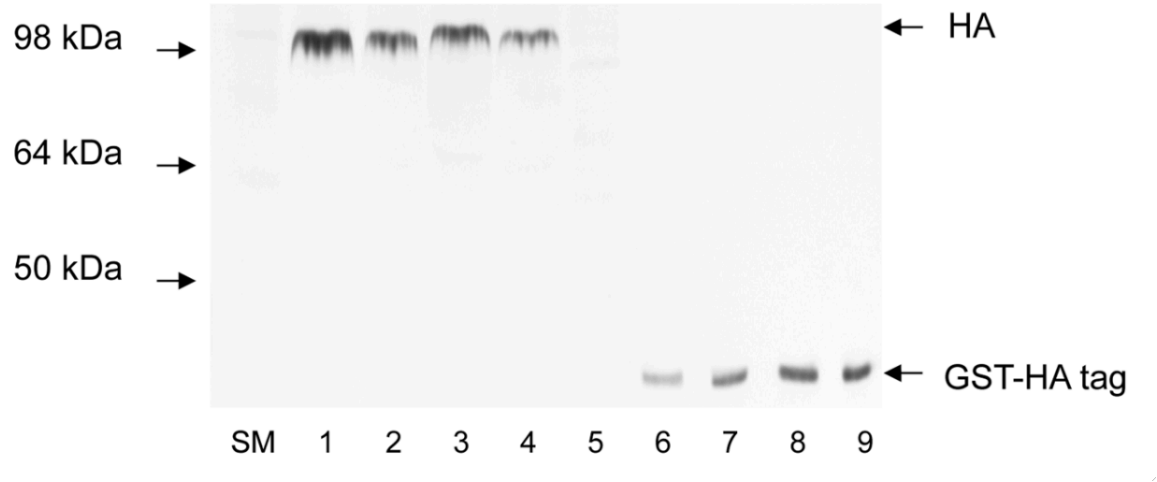

Figure 19. Quantitation of expressed HA by immunoblotting assay using anti-HA-tag antibody.
Different concentrations of standard protein GST-HA tag were visualized in the same membrane as HAs. 1-2, 309 HA (10µl, 7µl); 3-4, mutant HA HL156-157KS (10µl, 7µl); 5, mock infected; 6-9. GST-HA tag 4ng, 6ng, 8ng, 10ng, respectively. The bands were scanned and quantitated using ImageQuant software (Molecular Dynamics). Amounts of HA were determined from a standard curve of GST-HA tag fusion protein. Standard curves were built for each sample of HA.

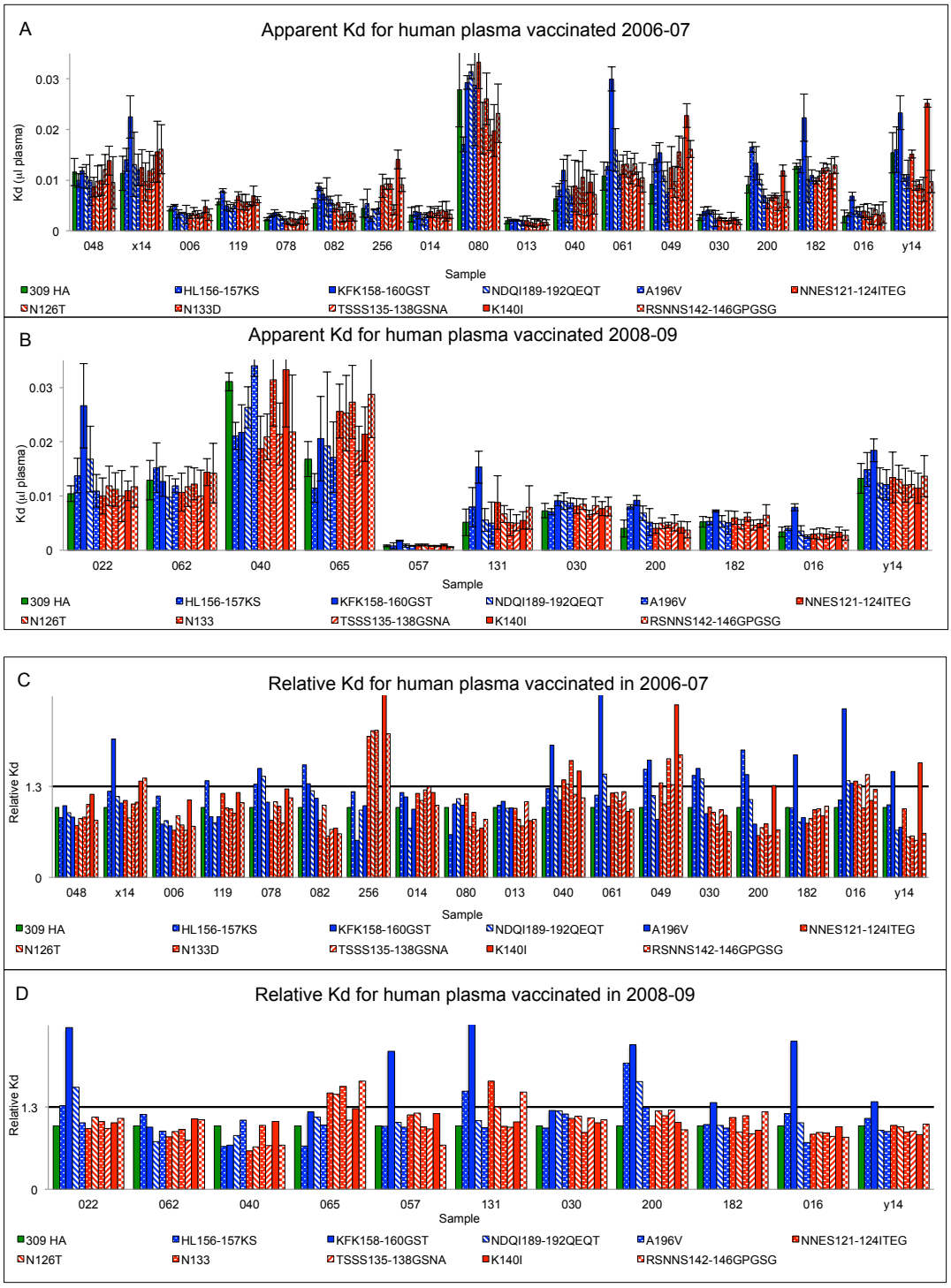

Figure 20. Analysis of human plasma samples vaccinated in season 2006-07 (H3 component A/Wisconsin/67/05) and season 2008-09 (H3 component A/Uruguay/716/07). A, B. Overall affinity (Kd ± St. dev.) of antibodies in human plasma against wt 309 HA and mutants. Overall affinity labeled as dissociation

constant (Kd) of binding of antibodies in plasma from subjects to wild type and mutants in antigenic site A or B; higher Kd is lower affinity. Kds were measured as described in Materials and Methods and the units are µl plasma in the standard assay. Results are plotted as mean Kd ± standard deviation over 3 experiments. **C, D.** Relative Kd of antibodies in human plasma against mutants relative to binding of wild type A/OK/309/06 HA. Kds of binding of plasma antibodies to mutants HA are calculated by normalizing Kd of wild type 309 HA to 1.0.

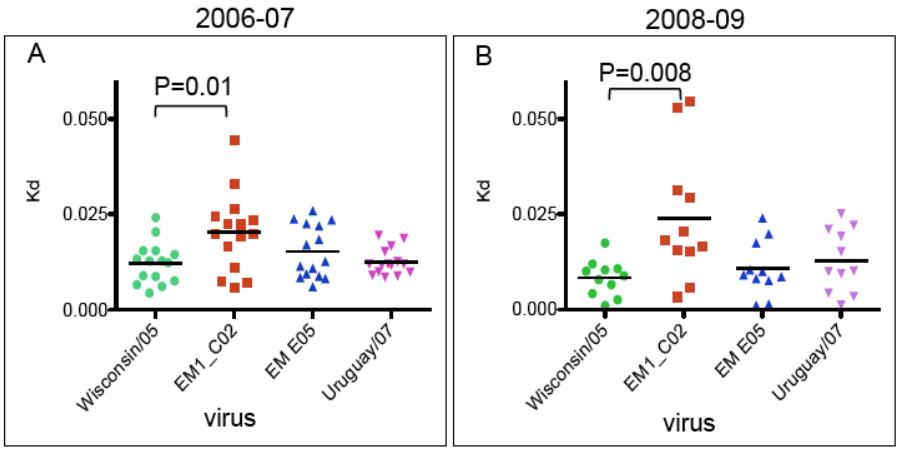

Figure 21. Overall dissociation constants (Kd) of antibodies in human plasma after vaccination.
Kd of antibodies in season 2006-2007 (A) and season 2008-2009 (B). Plasma samples were tested against H3 vaccine viruses A/Wisconsin/67/05 (2006-2007 vaccine) and A/Uruguay/716/07 (2008-2009) and against escape mutant viruses EM 1_C02, EM E05. The median Kd is represented by a horizontal bar, and the p values are from the Student's T test.

Supporting Information

Table S1. Overall affinity (Kd) of binding of human antibodies to 309 HA and mutants after vaccination against A/Wisconsin/67/05 in the 2006-07 trivalent vaccine.

Table S2. Overall affinity (Kd) of binding of human antibodies to 309 HA and mutants after vaccination against A/Uruguay/716/07 in the 2008-09 trivalent vaccine.

TABLES

Table 1. Mutations made in antigenic site A and B of A/Oklahoma/309/2006 HA. The amino acids were changed to those of Aichi/68

Mutations made	Change	Site	Charge change	Other
NNES121-124ITEG	N121I	A	0	
	N122T	A	0	Remove glycosylation site
	S124G	A	0	Remove side chain
N126T	N126T	A	0	Remove glycosylation site
N133D	N133D	A	1-	Remove glycosylation site
TSSS135-138GSNA	T135G	A	0	Remove side chain
	S137N	A	0	
	S138A	A	0	
K140I	K140I	A	1-	Same position as E05 escape mutant
RSNNS142-146GPGSG	R142G	A	1-	Remove side chain
	S143P	A	0	
	N144S	A	0	Remove glycosylation site
	S146G	A	0	Remove side chai
HL156-157KS	H156K	B	1-	Remove stacking with F159
	L157S	B	0	
KFK158-160GST	K158G	B	1-	Remove side chain
	F159S	B	0	Same as1_C02 EM
	K160I	B	1-	
NDQI189-192QEQT	N186Q	B	0	
	D187E	B	0	
	I190T	B	0	
A196V	A196V	B	0	

Table 2. Binding of human monoclonal Abs 1_C02 and E05 to 309 HA and mutants in antigenic site A and B expressed in insect cells.

HA	Antigenic site	Kd, nM ± SD	
		1_C02	**E05**
309 wt	Wild type	1.0 ± 0.2	2.0 ± 0.1
NNES121-124ITEG	A	2.1 ± 0.3	1.2 ± 0.1
N126T		1.8 ± 0.3	1.1 ± 0.1
N133D		0.8 ± 0.2	0.6 ± 0.3
TSSS135-138GSNA		1.0 ± 0.3	0.6 ± 0.2
K140I		1.0 ± 0.4	>1000
RSNNS142-146GPGSG		1.3 ± 0.4	>1000
HL156-157KS	B	>1000	0.3 ± 0.1
KFK158-160GST		6.0 ± 3	0.8 ± 0.2
NDQI186-190QEQT		1.5 ± 0.3	1.0 ± 0.3
A196V		1.1 ± 0.2	1.9 ± 0.1

Table 3. Dissociation constants (Kd) for binding of human monoclonal Abs 1_C02 and E05 to H3 viruses.

	Kd (nM) ± SD	
	1_C02	**E05**
A/Beijing/89	No binding	No binding
A/Panama/99	No binding	No binding
A/Wyoming/03	>1000	No binding
A/California/04	No binding	97.8 ± 26.3
A/Wisconsin/05	2.1 ± 0.3	6.6 ± 0.5
A/Oklahoma/06	1.0 ± 0.2	2.0 ± 0.3
A/Uruguay/07	No binding	No binding
A/Perth/09	No binding	No binding
EM 1_C02 (F159S)	No binding	6.0 ± 0.5
EM E05 (K140E/T)	1.9 ± 0.1	No binding

Table 4. Decreased binding to site A and B mutants by human plasma after vaccination in 2006-07 or 2008-09.

Mutant	Vaccinated 2006-7 (18 subjects)		Vaccinated 2008-9 (11 subjects)	
	Number[1]	Percent	Number	Percent
Site B				
HL156-157KS	6	33	3	27
KFK158-160GST	11	61	7	64
NDQ189-192QEQT	5	28	2	18
A196V	1	0	0	0
Total subjects binding site B	12	66	7	64
Site A				
NNES121-124ITEG	4	22	2	18
N126T	3	17	2	18
N133D	3	17	1	9
TSSS133-138GSNA	2	11	0	0
K140I	6	33	0	0
RSNNS142-146GPGSG	3	17	2	18
Total subjects binding site A	7	39	2	18
Total A, B or both	13	72	8	73
Not A or B	5	28	3	27

[1] Number of subjects who showed decreased avidity (Kd increased by ≥30%) to the mutant HA.

Table 5. Differences in HA1 sequence between Wisconsin/05 and Uruguay/07 PR8 re-assortant vaccine strains. Changes in parentheses are not present in other isolates from the same season, so may not be antigenically significant. The percent variation analyses in the footnotes were done using the Influenza Research Database (178).

Amino acids	**50**	122	**140**	**142**	**156**	186	**188**	**194**	**196**	223
Site	**C**	-	**A**	**A**	**B**	-	**B**	**B**	**B**	-
California/7/04	**G**	(N)	K	R	H	G	N	L	T	V
Wisconsin/67/05 X-161b	**G**	(D)[2]	K	R	(Q)[3]	(V)[4]	D	L	A	(I)[5]
Oklahoma/309/06	**E**	N	K	R	H	G	D	L	A	V
Brisbane/10/07	**E**	N	**I**	R	H	G	D	P[6]	A	V
Uruguay/716/07 X-175	**E**	N	**I**	(G)[7]	H	G	D	(P)[5]	A	V

[2] All Wisconsin/67/05 sequence have D but 99.7% of 2005/6 isolates have N
[3] Q in PR8 reassortants not isolates from primary chick kidney cells or egg passages
[4] V in PR8 reassortants and some other Wisconsin/67/05 entries; 98.8% of 2005-06 isolates have G
[5] in all Wisconsine/67/05 entries but 97% of 2005/06 isolates have V
[6] P in egg-passaged viruses but not many others
[7] G only in reassortant X-175

CHAPTER III

MANUSCRIPT TO BE SUBMITTED

Escape mutant viruses of A/Perth/16/09 (H3N2) selected by postvaccination human plasma contain mutations in antigenic site B but not in antigenic site A

Lyubov I. Popova, Ann H. West, John T. West, Gillian M. Air

Abstract

In the 44 years since it appeared in humans in 1968, influenza subtype H3 hemagglutinin (HA) has accumulated over 20% amino acid sequence changes in HA1 that are believed to be predominantly due to selection for escape from antibodies. Five antigenic sites were mapped on the structure of HA from the 1968 and 1971 viral isolates based on escape mutant virus analysis and competition assays using monoclonal antibodies (mAbs). The goal of our research is to determine whether if neutralizing antibodies in post-vaccination human sera are directed to a dominant antigenic site. Sera from seven of nine individuals vaccinated in the 2010–2011 season (H3 component A/Perth/16/09) showed reduced binding to mutants in antigenic site B. We selected escape mutant viruses of A/Perth/16/09 using post-vaccination human plasmas from two individuals. Both escape variants contained two mutations (H156Q and A186V) in antigenic site B of HA. Our results suggest that antigenic site B of A/Perth/16/09 is immunodominant and the next H3 drift strain may need mutations in antigenic site B to escape from antibodies induced by vaccination.

INTRODUCTION

Influenza viruses are responsible for annual epidemics of respiratory disease and in temperate zones these epidemics occur each winter. Vaccination to induce neutralizing antibodies is the main strategy used to protect human health against influenza (56).

To escape from host defense, influenza viruses change their antigenic character by accumulating mutations in the viral surface glycoprotein hemagglutinin (HA), the major target of influenza neutralizing antibodies in a process named antigenic drift. Annual epidemics due to strains that have undergone antigenic drift result from amino acid changes in antigenically important regions of the HA. These regions are named antigenic sites of HA (66). Five antigenic sites (A-E) were partially mapped on the crystal structure of H3 HA based on escape mutations and antigenic drift variants (172, 210). Gerhard and Webster defined an antigenic site as a group of overlapping epitopes recognized by mAbs that compete with each other but do not compete with mAbs recognizing another antigenic site. On the HA crystal structure the antigenic sites are localized in loop areas surrounding the receptor-binding site of the surface of H3 HA (219) such that a bound antibody sterically inhibits interaction with receptor. While an antigenic site is a region of a molecule that is immunogenic, an epitope is defined as the amino acids (and sometimes sugars) that make contact with the antibody (48, 107). Variants that escape from neutralizing antibodies are known as escape mutant (EM) viruses. Most mutant HAs of influenza viruses selected by mAbs contain single substitutions that greatly reduce the binding of the mAb (35, 105, 172, 211).

The antibody epitopes on native proteins are conformational as studied in biochemical and structural analyses of protein-Fab complexes (10, 41, 124). The only method to fully map the epitope is the determination of the 3-D structure of a monoclonal antibody in complex with its antigen. A functional method to identify the most important residues of an antibody epitope is the isolation of escape mutants and the identification of amino acid changes that eliminate or reduce the binding of the antibody. The structural data establish that epitopes in native proteins consist of about 15-22 contact residues. Energetic calculations suggest that a small subset of 5-6 residues contribute most of the binding energy. The isolation of escape mutant viruses experimentally identifies those amino acids that interact with the greatest binding energy. Elimination of this critical contact will greatly reduce or even destroy the binding of antibody to antigen. To estimate the binding energy of amino acids with highest energy interaction in the antibody-antigen complex a large scale free energy perturbation the (FEP) method was used (232) to model the effect of single mutation on the antibody-antigen binding affinity of the H3N2 HA and Fab. They calculated that a single mutation T131I in H3N2 HA may cause a 5.2 ± 0.9 kcal/mol decrease in the HA/Fab binding affinity, mechanistically explaining how alteration of a single residue contact in escape mutant virus eliminates the binding to Ab.

The presence of five independent antigenic sites (A-E) on HA would appear to mean that mutations in all five sites would be needed for a virus to escape polyclonal antibodies in human serum, but this is not what is observed (ref Medimmune paper), so the host immune recognition to any particular influenza

virus might be limited to specific sites. If a hierarchy in immune response to these five sites existed, it would suggest immunodominance of particular antigenic sites over others. If we can define the hierarchy of immunodominance we may be able to predict the direction of antigenic drift and be prepared to make a vaccine ahead of a new variant emerging.

Escape mutant viruses are likely to mutate in critical contacts in a dominant antigenic site, allowing us to estimate the direction of polyclonal antibody selection of HA variants and thus allowing some predictions of antigenic drift. We isolated escape mutant viruses of A/Perth/16/09 selected with post-vaccination human plasmas and the fractionated IgG fractions from two individuals. Four independently selected escape mutants contain the same two mutations (H156Q and A186V) in antigenic site B of HA and there is no mutation in antigenic site A, C, D or E of HA that could contribute to the decreased binding of the plasma antibodies to the escape mutant virus. Our results suggest that antigenic site B of A/Perth/16/09 was immunodominant in both subjects.

MATERIALS AND METHODS

Viruses and cells

The viruses used in this study were vaccine strains reassorted with PR8, A/California/07/2004 (CDC, #2005712034), A/Wisconsin/67/2005-X161b, A/Uruguay/716/2007 X-175, and A/Perth/16/2009 (IRR, #FR-370), obtained from the CDC, and A/Oklahoma/309/2006, a Wisconsin-like H3N2 isolate, referred to as

"309". Viruses were grown in embryonated chicken eggs or in Madin-Darby canine kidney (MDCK) cells in DMEM: Ham's F12 medium (1:1) with ITS+ (BD Biosciences) and trypsin added, as previously described (109). *Spodoptera frugiperda* Sf9 insect cells (Invitrogen, Cat. No 11496-015) were used as the host for baculoviruses expressing wild type and mutant HAs. Sf9 insect cells were grown in BD BaculoGoldTM TNM-FH Insect medium (BD Biosciences).

Virus growth in the presence of polyclonal antibodies and Igs

Allantoic fluid of Perth vaccine strain was incubated 30 min to infect MDCK cells at serial 10-fold dilutions in the absence or presence of plasma from vaccinated subjects (5–50 μl) in each infection medium. After three days incubation at 37°C, the extent of cytopathic effect (CPE) was estimated by eye and the NA activity was determined to monitor growth of mutant viruses.

Isolation of viral RNA, reverse transcription and PCR amplification (RT-PCR)

The procedures for isolation of total viral RNA and RT-PCR to synthesize HA1 cDNA fragments for sequencing were as described in Gulati et al. (77).

RESULTS and DISCUSSION

Reactivity of wild type and mutant HAs with polyclonal antibodies in human plasma after vaccination

The sequence changes between A/Oklahoma/309/2006, the 309 mutants we previously made (ref) and A/Perth/16/2010 are shown in **Table 6**. From these changes we would anticipate that the sera of subjects vaccinated with Perth would not discriminate mutations at position 138 or 140 of OK/309/06, since these were mutated in Perth. Similarly, there might not be discrimination of mutants at 158 or 189 since these are also mutated in Perth, although to different amino acids than in our engineered mutations. Antibodies in human plasma that were induced through vaccination in 2010–2011 (H3N2 component A/Perth/16/09), were evaluated for their capacity to bind wild type 309 and mutant recombinant HA proteins (**Fig. 22**). As we predicted, we found that eight of nine subjects (89%) vaccinated with the Perth had no significant recognition of Site A mutants due to strain changes at 138 and 140 relative to wt 309 HA. The Student's T test showed that a Kd increase >30% as a significant reduction of binding of antibodies in human plasmas. No subject discriminated the mutant with a change at N189Q in accord with the Perth strain mutation N189K. However, 7 of 9 subjects discriminated the mutant that containing K158G, even though the vaccine strain has K158N. Five of these seven subjects also recognized the mutant 156-157, positions that did not change between 309 and Perth.

Antibodies in human plasma samples after vaccination with the 2010–2011 trivalent subunit vaccine (H3 component A/Perth/16/09) showed decreased binding to mutants in antigenic site B in five of nine plasmas (**Fig. 22**).

Selection of escape mutants with human polyclonal Abs

We selected escape mutant viruses by growing A/Perth/16/07 virus in the presence of serum of human subject 009. We had information that this subject had never been vaccinated against influenza until 2009 when the pandemic H1N1 (H1N1pdm) vaccine was administered, followed in 2010 with the trivalent vaccine containing H3N2 component A/Perth/16/09. Polyclonal antibodies in plasma 009 show high affinity binding to A/Perth/16/09 and decreased binding to mutants HL156-157KS and KFK158-160GST. When 009 plasma was included in the infection medium we did not observe viral growth in the first or second passage but cytopathic effect (CPE) was seen by P3. The sequence of HA1 RT-PCR products of P2 revealed nucleotide changes that resulted in two amino acid mutations at residues H156 and A186 of A/Perth/16/09. To test if there are two changes in HA1 or a mixed population of viruses with single amino acid changes in HA1 we repeated selection of viruses at limiting dilution for three to eight passages from P1. In passages P3 to P9 of the EM virus we observed the same sequence changes. We concluded that an escape mutant virus EM 009 selected by polyclonal antibodies of subject 009 contained two substitutions at H156S and G186V in antigenic site B of HA1.

To test if selected the virus with these two mutations is resistant to polyclonal antibodies in other human plasmas we chose subject 085 plasma that also showed a high affinity of binding to HA (**Figure 22**) to select EM 085. This escape mutant showed the same amino acid substitutions at positions H156 and

G186 in antigenic site B, although this escape mutant also had a third change at 183 which is outside the antigenic regions.

Immunoglobulin fractionation and selection of EM with eluted fractions of human plasma

To confirm that mutations H156 and G186 were selected by antibodies and not by other factors in human serum that might select mutants in receptor binding, we fractionated human plasmas 009 and 085 using a minicolumn of Protein A-Sepharose beads. We collected the flow-through fraction and then eluted the immunoglobulins. The flow through and immunoglobulin fractions were tested for the absence or presence of immunoglobulins by gel electrophoresis and ELISA. We then applied the same selection procedure, growing virus in the presence of flow through or eluted Ig fraction. The results are summarized in **Table 7**. The enriched antibody fractions selected the mutations at 156 and 186, while the non-Ig fraction of subject 085 selected the mutation at 183, suggesting that this is selection for growth (receptor affinity) rather than an immune selection. The flow through from subject 009 did not select ant mutations.

Our results suggest that antibodies in human plasma selected the mutations at residue position H156 and G186 in both escape mutants.

We measured binding of polyclonal antibodies of nine human plasma samples to Perth/09 and EM 085 viruses by native ELISA. Our results show that the affinity of binding of EM 085 to serum antibodies is significantly decreased compared to the binding of A/Perth/09 in eight of nine tested plasma (**Figure 24,**

Figure 25). This data correlate with the results of testing human plasma antibodies against site-directed HA mutants (**Figure 22**).

Conclusions: These results indicate that a majority of neutralizing antibodies in plasmas of two subjects 009 and 085 are directed to antigenic site B and not to other antigenic sites in A/Perth/16/09.

We selected EM viruses using polyclonal antibodies contained in plasma of two A/Perth/16/09 vaccinees. Independently selected EM viruses contain the same amino acid changes at positions H156 and V186 of HA1. EM viruses show resistance against neutralizing antibodies directed to antigenic site B.

Table 6. Sequence changes in 309 mutants, Perth/16/2009, Perth escape mutants and Victoria/361/2011

Site	C	C	E	A	A	A	B	B	D	B	B	B	D	D	D	C
	45	48	62	138	140	144	156	158	173	186	189	198	212	214	223	312
OK/309 virus	S	T	E	S	K	N	H	K	K	G	N	A	T	I	V	N
309 HA mutant				A	I		K	G			Q					
A/Perth virus	S	T	K	A	I		H	N		G	K	A	T	S	V	N
Perth EM virus						K	Q		Q	V						
Victoria virus							H	N		G	K	S	A	I	I	S
A/Victoria egg grown virus	N	I	E	A	I	N	Q(R)		Q	V						

Table 7. Differences in HA1 sequence in escape mutants of A/Perth/16/09 selected with polyclonal antibodies in postvaccination human plasma samples 009 and 085.

Sample used for escape mutant selection	Mutation in HA of A/Perth/09		
	H156Q	G186V	Other
Total 009 plasma	Yes	Yes	No
Protein A Flow through of 009 plasma	No	No	No
Eluted fraction of Abs from 009 plasma	Yes	Yes	No
Total 085 plasma	Yes	Yes	H183L
Flow through of 085 plasma	No	No	H183L
Eluted fraction of Abs from 085	Yes	Yes	T30M

Table 8. Growth detection of escape mutant viruses measured by RT-PCR, NA activity or CPE.

	Perth with plasma 009			Perth with plasma 085			A/Perth/09, no plasma		
	RT-PCR	NA activity	CPE	RT-PCR	NA activity	CPE	RT-PCR	NA activity	CPE
P1	+	+	-	+	+	-	+++	+++	+++
P2	++	++	-	++	++	-	+++	+++	+++
P3	+++	+++	+++	+++	+++	+++	+++	+++	+++

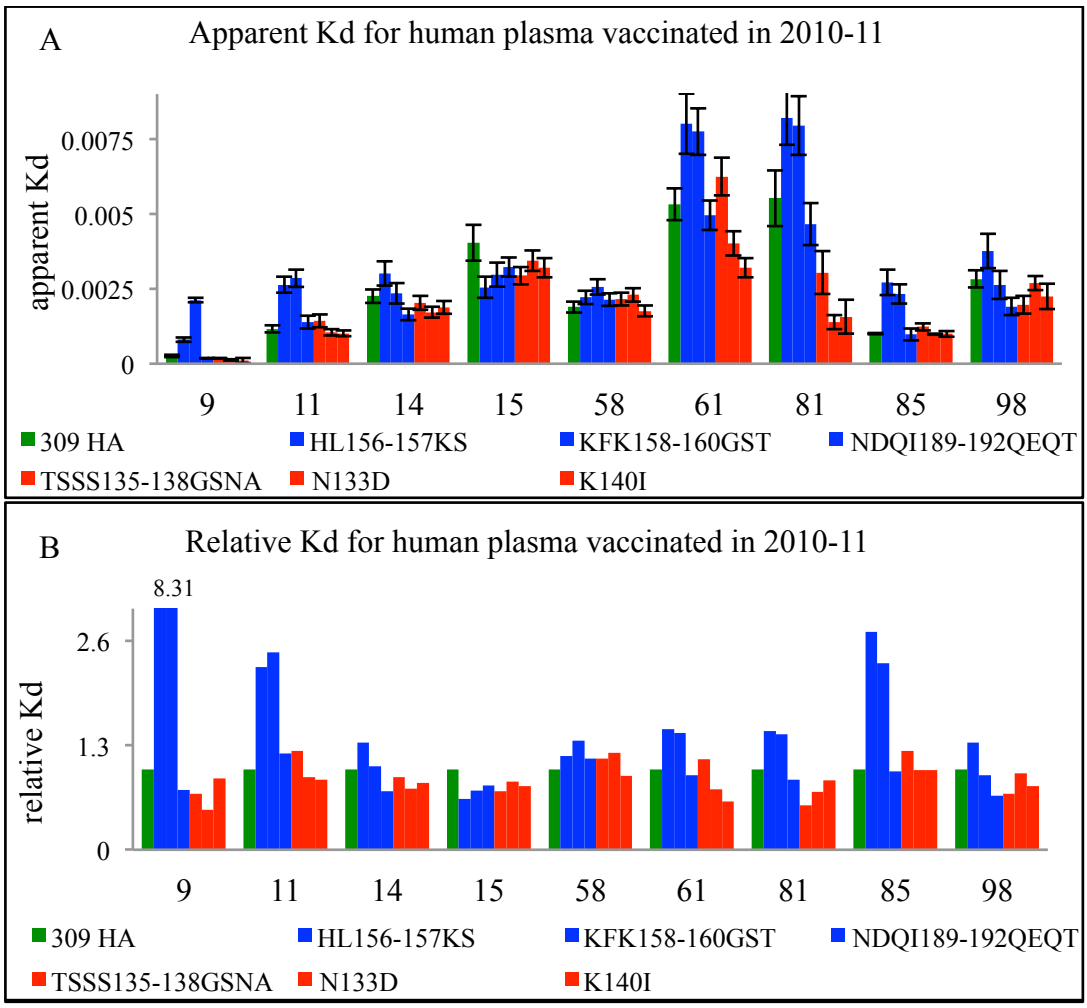

Figure 22. Analysis of human plasma samples vaccinated in season 2010–2011 (H3 component A/Perth/16/09). Overall affinity (Kd) of antibodies in human plasma against wt 309 HA and mutants. **A**. Overall affinity (Kd ± St. dev.) of antibodies in human plasma against wt 309 HA and mutants; higher Kd is lower affinity. Kds were measured as described in Materials and Methods and the units are µl plasma in the standard assay. Results are plotted as mean Kd ± standard deviation over 3 experiments. **B.** Relative Kd of antibodies in human plasma compared to to binding of wild type A/OK/309/06 HA. Kds of binding of plasma antibodies to mutants HA are calculated by normalizing Kd of wild type 309 HA to 1.0.

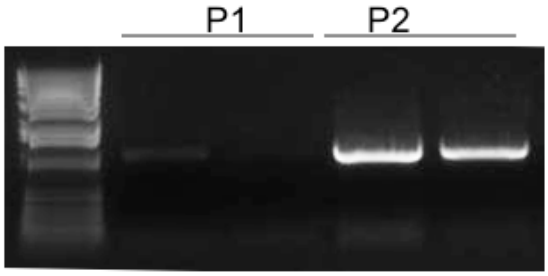

Figure 23. The HA1 fragments of RT-PCR in 1% agarose gel stained with EtBr. Two samples from each passage were used for cDNA synthesis of P1 and P2 HA1 for escape mutant virus selection. In P1, there was a low NA activity and no CPE. In P2, NA activity was high and PCR products were detected, but there was no CPE (**Table 8**).

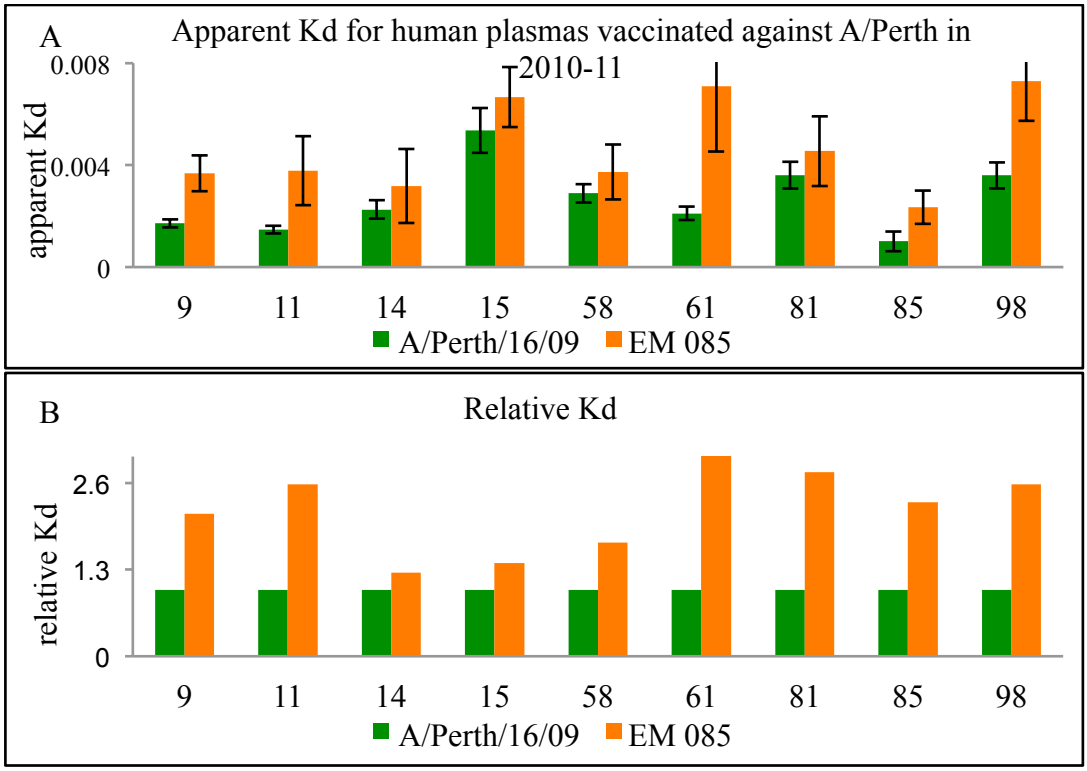

Fig. 24. Analysis of binding of A/Perth/16/09 and EM 085 to polyclonal antibodies. A. Overall affinity (Kd ± St. dev.) of antibodies in human plasma against Perth/09 and selected EM 085 was tested by native ELISA. **B.** Relative Kd of antibodies in human plasma are calculated by normalizing Kd of A/Perth/09 to 1.0.

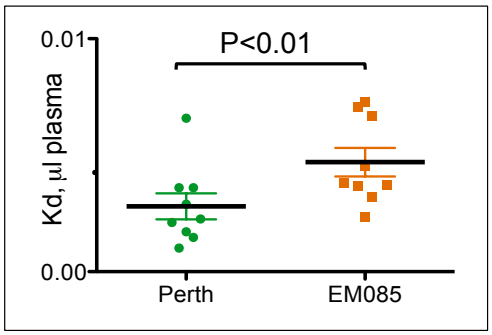

Fig. 25. Overall dissociation constants (Kd) of antibodies in human plasma after vaccination. EM085 virus containing the mutations H156Q, V186G in antigenic site B of HA1 decreases the binding to antibodies in human plasma vaccinated against A/Perth/16/09. Kds of antibodies were tested against A/Perth/09 and selected EM 085 viruses. The median Kd is represented by a horizontal bar, and the p values are from the Student's T test.

CHAPTER IV

DISCUSSION

Influenza viruses undergo a progressive and largely unidirectional antigenic drift to escape from neutralizing antibodies produced in response to infection and, most recently, mass vaccination. Vaccination is the primary means to protect human health against influenza virus. The production of flu vaccine is expensive and almost eight months are required to produce the needed large scale of each component in the yearly updated vaccine. Flu vaccines are reasonably effective but it takes time to develop neutralizing antibodies. For vaccine effectiveness, the components of the vaccine have to match the currently circulating epidemic virus and the vaccines have to be reformulated every year because of antigenic drift. Several approaches have been proposed to simplify the vaccine production and decrease its cost. In the mid 80s many researches were concentrated on designing vaccines from short synthetic polypeptides. However, there is still no licensed vaccine designed from short synthetic polypeptides. Another approach has been DNA vaccination. DNA vaccination is a technique for protection of an organism against flu virus by injecting it with genetically engineered DNA to produce an immunological response. Such vaccines remain experimental. Currently, vaccines under development are based on whole influenza protein (M2, NA) or specific part of HA2 but not short polypeptides or genes encoding proteins.

Human vaccination with influenza viruses began in the 1940s to protect troops in the Second World War. Currently, massive vaccination of humans against

flu is a normal procedure at the beginning of winter season. In a 2010 survey of healthcare workers, 63.5% reported that they received flu vaccine during the 2010-2011 winter season.

Influenza virus developed the mechanism to avoid immune system pressure. Neutralization resistance has continued to accumulate amino acid changes in antigenic sites of HA since the H3N2 subtype of influenza virus appeared in humans in 1968, causing antigenic drift. In the early years after the emergence of H3N2, multiple sequence changes were observed in viruses in humans from one epidemic isolate to the next. During the first 10 years of H3N2 virus circulation (1968–1979), 33 amino acid changes accumulated in HA1 (10.1%), but the vaccine was changed only three times. In recent years the vaccine has been changed more frequently even though there are fewer changes in circulating viruses. For example, the only consistent differences between Wisconsin/2005-like viruses and Brisbane/Uruguay/2007-like viruses are G50E in site E and K140I in site A. These two mutations were sufficient to necessitate changing the H3N2 component of vaccines to be changed for the 2008–2009 flu season. It is not known what rate of accumulation of mutations in HA was before widespread vaccination. Does vaccination increase the rate of accumulation of mutations in HA thereby accelerating the antigenic drift or not? However, it is known that that since the beginning of vaccination against Hepatitis B in 1988 mutation at G145R is accumulated in the “a” immunodominant determinant sequence causing false–negative response to diagnostic immunoassays. This mutation might be a consequence of vaccination.

Neutralizing antibodies are directed to another surface glycoprotein, NA. The importance of neuraminidase in antigenic drift of influenza virus is yet to be investigated. Analysis of amino acid sequences of NA in human isolates shows that the rate of change is similar for NA and HA. Antibodies against NA are less abundant than HA presumably because there is less antigen on the virus but antibodies against NA block infections. Currently, it is required that influenza vaccines contain 15 μg of each HA. The amount of NA is not required to be measured and is inconsistent and usually low (54). Due to accumulation of mutations in NA the inclusion of NA in the vaccine might increase the protection of vaccination against influenza virus.

Five antigenic sites have been mapped on the surface of the receptor-binding domain of subtype H3 HA, but at any given time, the host immune response may be limited to only the most immunodominant antigenic sites. We have studied recognition of sites A and B in human plasma after 2006 and 2008 vaccinations by mapping the binding of two human monoclonal antibodies 1_C02 and E05.

The map of antigenic sites is available for HA of A/Aichi/2/68 which is only about 80% identical to HA in recent circulating viruses. Since 1968, mutations have accumulated in more than 20% amino acids of HA1 including additions of N-linked carbohydrate sites. Mutations in HA as well as extra glycans might have an effect on antigenic sites. We tested the binding of two human monoclonal antibodies 1_C02 and E05 with high affinity of binding in single nM range specific to HA of A/Wisconsin/67/05.

For E05 we found that mutations K140I and RSNNS142-146GPGSG in antigenic site A eliminate the binding of E05, but other mutations in antigenic site A, as well as mutations in antigenic site B, have no effect. The E05 epitope therefore appears to be centered around amino acids 140–146. Two escape mutants selected with E05 were isolated; both had changes at aa140 (K140T and K140E), confirming the data of the biochemical analysis defining the E05 epitope. In circulating viruses, A/Brisbane/07 and A/Uruguay/07, the HA contains mutation K140I in antigenic site A. We tested the binding of escape mutant K140E and binding of A/Brisbane/07 and A/Uruguay/07 to E05. There is no binding of E05 to EM E05 as well as viruses contained the mutation K140I. This result suggested that K140 amino acid residue is critical to the "energetic" epitope of E05. Abolishing of binding affinity to mutant RSNNS142-146GPGSG suggest that the epitope of E05 includes the amino acid residues in this region. There are two possibilities of binding disruption of E05 to mutant RSNNS142-146GPGSG: conformational change and addition of sugar chains. Both might affect binding E05 to K140. However, we found the changing of glycosylation sites N133D and N122T do not affect the binding of E05. The explanation for this observation might be that the epitope of E05 does not cover the whole A loop, but includes the amino acids around K140.

For 1_C02 we found that ELISA analysis of mutants in site B shows that mutation HL156-157KS dramatically decreases the binding of 1_C02. The adjacent mutation KFK158-160GST, which includes F159S, results in only a six-fold

decrease in affinity. 1_C02 binds to mutant NDQI186-190QEQT and A196V as well as to wild type.

Antibody 1_C02 binds to Wisconsin/67/05 and Oklahoma/309/06 viruses, but not to California/07/04 or Uruguay/716/07. To understand the effect of mutation HL156-157KS, we used SWISS-MODEL to model the structure of Oklahoma/309/06 HA and its mutants onto the crystal structure of H3 HA of A/California/07/04, which was determined recently in our laboratory. In California/07/04, H156 is stacked against F159 and also makes contact with T196. F159 is present in Wisconsin/67/05 and Oklahoma/309/06, but the change T196A may alter the interactions in this region. In the energy-minimized model of A/Oklahoma/309/06, F159 is in the same orientation as in California/07/04, but the orientation of the side chain of F159 is changed in the HL156-157KS mutant. We propose that reorientation of F159 in B-loop in the structure of HA disrupts the binding of mAb 1_C02.

An escape mutant virus selected by 1_C02 (EM1_C02) contains a single substitution, F159S, in antigenic site B. ELISA data of binding 1_C02 to EM 1_C02 show there is no binding. But mutant KFK158-160GST, which includes F159S, results in only a six-fold decrease in affinity. We explained it by the increased flexibility of GST158-160 compared to KFK, which allows antibody 1_C02 to bind. However, it cannot bind when only F159 is changed, as in the escape mutant. The role of flexibility in binding of protein to antibody is not completely understood. The initial step of antibody recognition is followed by formation of a stable complex. After formation antigen-antibody complex the

structural rearrangements have been observed in the structures. We hypothesized that the additional contacts in mutant GST158-160 might be created contributing to the binding energy.

From these results we conclude that antigenic sites A and B originally mapped on the X-31 structure, at least approximately, apply to modern H3 HAs. Antigenic sites A and B in recent H3 influenza viruses are immunogenic. A single mutation in an antigenic site can eliminate the binding of neutralizing antibodies to HA, even when those antibodies are in human serum.

The epitopes are conformational. The only way to obtain the complete map of a conformational epitope is to solve the 3D structure of the antigen-antibody complex, but so far we have not been able to grow crystals of the HA-Fab complexes of 1_C02 and E05 hmAbs. To better understand effect of mutations on the antibody epitope we analyzed the model structures for EM1_CO2, EM E05 and for mutants HL156-157KS, KFK158-160GST. We created two model structures based on HA of A/Aichi/02/68 and recent viral HA of A/California/04. Both models of mutants show that mutations make conformational changes in the B loop. However, we observe that these models show different changes suggesting that we do need the real structure of complex HA with 1_C02 and E05 to determine the epitope of antibodies.

It was proposed that epitopes are sequential or conformational. Sequential epitopes are built from single continuous length of the polypeptide chain. Conformational epitopes contain several discrete amino acid sequences separated in primary structure. In both cases the epitopes are structural. The epitope of E05 is

likely sequential covering the part of loop A. The epitope of 1_C02 is likely conformational. The epitope of 1_C02 is within antigenic site B. Antigenic site B includes two parts: the B loop and the "190" helix on the top of the HA structure. The sequence of the B loop in the HA polypeptide chain is separated from the 190 helix. But the top of the B loop and the 190 helix come together to create antigenic site B. We did not observe that mutations in 190 helix affect the binding of 1_C02. We found that mutants NDQ189-192QEQT, A196V localized in 190 helix and loop after helix do not affect the binding of 1_C02. That means the epitope of 1_C02 include the amino acids in B loop, but not in 190 helix. However, in some publications (89) antigenic site B is divided into two parts B1 and B2. B1 is organized by B loop, B2 is organized by 190 helix. From our data of analysis of the epitope of hmAb 1_C02 we observe that B loop of antigenic site B is recognized but second component 190 helix of antigenic site B is not.

We tested two neutralizing monoclonal antibodies directed to HA of A/Wisconsin/67/05 isolated from patient vaccinated against A/California/04 and A/Wisconsin/67/05.

The presence of five independent antigenic sites on HA would appear to mean that at least five amino acid sequence changes needed for a new antigenic variant to emerge. The host immune response may be limited to only the most immunodominant antigenic sites of HA.

Early studies of antigenic sites on HA of A/Memphis/1/71 suggested that antigenic site A was immunodominant. To study the immunodominance of antigenic sites in recent viruses we tested the reactivity of polyclonal antibodies in

human plasma samples after the seasonal 2006 vaccination (H3N2 2006–2007 component A/Wisconsin/67/05) and/or after vaccination in 2008 (H3N2 2008–2009 component A/Uruguay/716/2007) to wild type HA and mutants in antigenic sites A and B.

To test the antibodies in human plasma after vaccination, we used the same mutants in antigenic sites A and antigenic sits B that we used to test the epitopes of human monoclonal antibodies 1_C02 and E05. Our results with monoclonal antibodies show that mutations at amino acid residue involved in antibody epitope abolish or decrease the binding. We anticipated that if antibodies in human plasma are dominantly expressed against epitopes in antigenic site A or B, we would observe reduced binding of sera to mutants in this antigenic site. We tested 18 human plasma samples collected six weeks after vaccination in fall 2006 (H3 component A/Wisconsin/67/05) and 11 samples collected after vaccination in fall 2008 (H3 component A/Uruguay/716/07). We measured binding of postvaccination plasma samples to the A/Wisconsin/67/05 virus and escape mutants EM 1_C02 and EM E05 derived from it using the native ELISA technique, capturing native viruses by binding to sialylated glycans on turkey erythrocyte ghosts used to coat the wells. Our data indicate that antibodies in most human plasma (66% of vaccinated in 2006–2008 and 64% of vaccinated in 2008–2009) are directed to antigenic site B of these viruses (see Table 4).

However, the ELISA test of polyclonal antibodies in postvaccination human plasma samples in the 2006–2007 season shows that in 39% of tested samples the antibodies are directed to antigenic site A. Thirty-three percent of analyzed samples

show that mutation K140I in antigenic site A decreased the binding to polyclonal antibodies suggesting that at least two seasons earlier neutralizing antibodies were directed to antigenic site A around K140. This mutation in HA1 was found in A/Brisbane /07. In two winter seasons, 2006–2007 and 2007–2008, A/Wisconsin/67/05 was the H3 component of the triple vaccine. In season 2008–2009, Brisbane–like virus A/Uruguay/716/07 was chosen as the H3 trivalent vaccine component due to mutations G50E and K140I in HA. We analyzed the binding of human monoclonal antibody E05 isolated from a patient vaccinated two seasons earlier with A/Uruguay/716/07. All these data indicate that through analysis of the directionality of polyclonal antibodies, we may predict what amino residue might be mutated in the next circulated virus.

From analysis of polyclonal antibodies in postvaccination human plasma samples, we concluded that in recent circulating viruses in the years 2006–2009, antigenic site B is immunodominant over antigenic site A. Most human subjects vaccinated in the years 2006–2007 (H3N2 component A/Wisconsin/67/05) and 2008–2009 (H3N2 component A/Uruguay/716/07) make antibodies against antigenic site B. From this we can propose that people vaccinated against A/Wisconsin/67/05 would be protected against A/Uruguay/716/07.

Mutations in antigenic site B are likely to drive the next antigenic drift. If the immunodominance of antigenic site B drives antigenic drift in circulating viruses, HA of A/Perth/16/09, compared to A/Uruguay/716/07 would accumulate mutations in antigenic site B. Indeed, HA of A/Perth/09 contains K158N, N189K in site B. Our biochemical data of analysis of postvaccination human plasma samples

in seasons 2006-2007 and 2008-2009 mainly decrease the binding to mutant KFK158-160GST (61% and 64% in both seasons consequently). EM 1_C02 isolated with purified monoclonal antibody contains mutation in F159. But 1_C02 is a single example of set of polyclonal antibodies from vaccinated patient. Mutation HL156-157KS decreases the binding of polyclonal antibodies (33% and 27%) in smaller ratio. These results suggest that it is likely that epitopes of majority of polyclonal antibodies after vaccination are centered around KFK 158-160 and HL 156-157 and mutations K158N, N189K in A/Perth/09 in antigenic site B might be as a results of escaping A/Uruguay/716/07 from neutralizing antibodies.

Other changes in the Perth vaccine strain in site A (N144K), site D (I214S), site E (E62K), and site B (P194L) are not found in other strains co-circulating with Perth/16/09 or subsequent isolates and so seem not to be antigenically important. However, a panel of mAbs generated by phage display from a single donor is described by Oshima et al. It was shown that monoclonal antibodies made by phage display bound to a wide variety of epitopes. These results show that there are antibodies directed to the minor antigenic sites that might select mutations in sites C, D, and E. We assume that changes in the Perth vaccine strain in site A (N144K), site D (I214S), site E (E62K), and site B (P194L) are result of antibodies which exist but they are in the minority.

To confirm that antigenic site B is immunodominant and that a majority of antibodies are directed to antigenic site B, we isolated escape mutant viruses with polyclonal antibodies. In this study we tested polyclonal antibodies in human sera after vaccination against A/Perth/16/09 in season 2010-2011 to mutant HA in

antigenic site A and antigenic site B. We tested antibodies in nine human plasma samples after vaccination with the 2010–2011 trivalent subunit vaccine (H3 component A/Perth/16/09). Our results show that seven of nine plasmas decreased binding to mutants in antigenic site B, and only one plasma shows decreased binding to mutants in site A and site B. Thus, immunodominance of antigenic site B is observed in A/Perth/16/09 as in A/Wisconsin/67/05 and A/Uruguay/716/07.

In this set of analyzed human plasmas we had human plasma sample 009 which show the highest affinity of binding to A/Pert/16/09, and polyclonal antibodies decreased the binding to mutants HL156-157KS and KFK158-160GST in antigenic site B as previously. The affinity of binding to wild type HA and mutant KFK158-160 ~10 times, and ~5 times for HL156-157. This sample shows the highest affinity of binding in whole set of plasmas tested in our study. Additional information is this person was vaccinated only once in season earlier in 2009 against pandemic H1N1 and he took first vaccination with triple vaccine containing H3N2 component A/Perth/09 in 2010-2011 season. We choose sample 009 for selection of the EM virus. We hypothesized if antigenic site is a group of overlapping epitopes to escape from neutralizing antibodies virus is likely to mutate in a dominant antigenic site allowing some predictions of antigenic drift. Viruses selected in the second passage, P2, and subsequent passages P3-P9 contained two substitutions H156S and A186V in antigenic site B of HA1. However, isolated escape mutant virus EM 009 is virus specifically escaped against polyclonal antibodies in sample 009. We used another human sample, 085, with a high affinity for binding to HA but less than for 009. Selected EM virus with the polyclonal

antibodies of human sample 085 shows the amino acid substitutions at the same positions 156 and 186 in antigenic site B, surprisingly! Escape mutant viruses selected in independent experiments contain amino acid substitutions at the same positions, 156 and 186, in antigenic site B but not other sites A, C, D or E in the HA1.

Isolated escape mutant viruses with polyclonal antibodies are the evidence that neutralizing antibodies in these two samples are directed to antigenic site B.

Due to predominance of antibodies against site B in the population in 2009-2011, predictive of the site B change K158N was later seen in the Perth16/09 and Victoria/361/11 epidemic strains.

A/Victoria/361/11 is a H3N2 component of the trivalent vaccine against influenza virus for winter season 2012-2013. Isolation of escape mutant viruses with polyclonal antibodies serum assume that the next drift of virus is due to amino acid change at H156 amino acid residue. Sequence analysis of HA of A/Victoria/361/11-like viruses shows that mutation at 156 amino acid residue mutated in egg-grown virus (**Table 7**). Thus, our result of EM 009 and EM085 predicted the change in currently circulated viruses.

What is the effect of mutation H156 on HA antigenicity? Two residues at 155 and 156 positions caused antigenic drift from A/Panama/2007/99 to A/Fujian/411/02, resulting in severe influenza epidemics in 2003 (89). After substitution of mutations at H155T and Q156H A/Panama/2007/02 became antigenically match to A/Wyoming/03/03, a A/Fujian/411/02-like virus and vaccine

strain for 2004-2005 season suggesting that H155T and Q156H caused antigenic drift.

Embryonated chicken eggs are currently the host in which we cultivate the virus for large scale supply and for vaccine production. The cultivation of influenza virus in embryonated chicken eggs frequently result in the selection of an HA differ antigenically from original viral strain containing amino acid substitutions (40, 91, 119, 144). Some variants of A/Victoria/361/11 virus adapted to growth in embryonated chicken eggs have a mutation H156Q. We predict that this virus will have altered antigenicity and variant H156Q cannot be used for vaccine production. There are big questions: What causes the changing of immunodominance from one antigenic site to another? Does immunodominance of particular antigenic sites changes as the antigen accumulates sequence changes?

The mechanism of this phenomenon is not known. There are several factors that might affect the immunodominance: accessability, hydrophilicity, and mobility.

Antigenic sites A and B are localized on the top of the HA molecule, furthest from the viral membrane and so they are accessible. Computer modeling of the immodominance of sites A and B are based on the accumulation of charged amino acids in antigenic loop structures, suggesting that these areas are very hydrophilic. Another factor, mobility, came from studies of protein-protein interaction. This factor was promising for the production of vaccines based on synthetic short polypeptides. But, to date, there is no licensed vaccine created by short peptides. From studies of synthetic peptides binding to antibodies specific to

the native structure of proteins, it was concluded that short peptides must be in motion to bind to the epitope of the antibody. It is likely that structural motion of short polypeptides is favorable.

Westhof et al. (212) compared the mobility of segments of polypeptide chains of tobacco mosaic virus (TMV) proteins with known antigenic sites. This analysis found that six of the seven sites showed high mobility. However, another study found that antibodies against native proteins bind better to polypeptides with low mobility (9). Thus, the idea that mobile sites of a protein are more immunogenic than sites with low mobility is not supported by the evidence.

The analysis of antibody epitopes in crystal structures shows that the epitopes of neutralizing antibodies are absolutely conformational and therefore restricted to native proteins. Recent data from the structural analysis of the "a" determinant in HBV show that the structure is stabilized by a backbone of conserved disulfide-bonded cysteine residues. mutation of the cysteine residues causes the reduced antigenicity. This is n example that in native proteins, low mobility in immunodominant site is might be critical and increasing the mobility causes the loss of immunogenicity. Structural analysis of antigenic site A and B of HA in recent viruses is yet to be studied.

Our biochemical study suggests that antigenic site B is immunodominant in viruses at least since 2005. Mutations accumulated in antigenic site B in viruses A/Wisconsin/05, A/Uruguay/07 and A/Perth/09 did not cause the changing of immunodominance of site B in these viruses.

CONCLUSIONS

Specific aim 1

1. Antigenic site A and antigenic site B are immunogenic in modern H3 HA.

   The epitopes of two human monoclonal antibodies 1_C02 and E05 specific to H3 HA of A/Wisconsin/67/05 were analyzed.

a. Antibody 1_C02 binds within antigenic site B centered at aaF159 in the B-loop. An isolated escape mutant virus contains mutation F159S in HA.

b. Antibody E05 binds within antigenic site A of HA. It is centered at aa140 in the A-loop. Mutation K140I in antigenic site A affects binding to hmAb E05. Two escape mutant viruses contain a mutation K140 in HA.

Specific aim 2

1. Antigenic site B is immunodominant over antigenic site A in recent H3N2 viruses.

   Polyclonal human plasma antibodies from subjects vaccinated in the 2006–2007 (H3N2 component A/Wisconsin/67/05) and 2008–009 seasons (H3N2 component A/Uruguay/716/07) mostly bind to antigenic site B.

Specific aim 3

1. This is first study of immunodominance of antigenic sites of HA analyzed with postvaccination human plasmas.
2. Isolated in four independent experiments escape mutant viruses on A/Perth/16/09 contained two amino acid changes H156Q, V186A in HA1 provide support that antigenic site B is immunodominant obtained by analysis of postvaccination human plasma samples.

SUMMARY

The achievement of this study is that the immunodominance of antigenic sites in recent viruses are tested in postvaccination human plasma samples. We concluded that antigenic site B has been immunodominant at least since 2005. Our results suggest that accumulation of mutations in immunodominant site B in A/Wisconsin/67/05, A/Uruguay/716/07, A/Perth/16/09 did not affect immunodominance of site B.

Our study gives the opportunity the vaccine design ahead of influenza epidemics.

ABSTRACT/ORAL PRESENTATIONS

1. **Lyubov Popova,** Gillian Air. Effect of mutations in antigenic site B of hemagglutinin of influenza virus A/Oklahoma/309/2006. 97th Annual Conference of OAS, Southern Nazarene University, Bethany, Oklahoma, October 31, 2008;

2. **Lyubov Popova**, JingQi Feng, Ann H. West, Gillian Air. Effect of mutations in antigenic sites A and B in hemagglutinin of virus A/Oklahoma/309/2006 on binding with human monoclonal antibodies 1_C02 and E05 against influenza HA. GREAT Symposium, OUHSC, March 31-April 2, 2009 (Graduate Student Award for Scientific Achievement);

3. **Lyubov Popova**, Ann H. West, Gillian M. Air. What antigenic sites are recognized by antibodies in human plasma vaccinated against influenza virus? GREAT Symposium, OUHSC, March 29 – April 1, 2010

4. **Lyubov Popova**, JingQi Feng, Ann H. West, Gillian M. Air. Effect of mutations in antigenic sites A and B in hemagglutinin of virus A/Oklahoma/309/2006 on binding with monoclonal antibodies against influenza HA. Annual meeting of the American Society for Virology, Canada, Vancouver, July 11-15, 2009;

5. **Lyubov Popova**, Patrick C. Wilson, Kenneth Smith, Ann H. West, Gillian M. Air. Evidence for immunodominance of antigenic sites on hemagglutinin of recent H3N2 influenza viruses. Gordon Research Conference "Antibody Biology & Engineering", March 7-12, 2010 (Travel Award of the Department of Biochemistry and Molecular Biology);

6. **Lyubov Popova**, Ann H. West, Gillian M. Air. Effect of mutations on immunodominance of antigenic sites of hemagglutinin of influenza viruses H3N2. 29th Annual meeting of the American Society for Virology, July 17-21, 2010 (Travel Award of the American Society for Virology);

7. **Lyubov Popova**, Lalitha Venkatramani, Ann H. West, Gillian M. Air. Effect of mutations in antigenic sites A and B of hemagglutinin of H3N2 influenza virus A/Oklahoma/309/06 on binding to neutralizing antibodies. GREAT Symposium, OUHSC, March 29-April1, 2010, in GREAT Symposium, OUHSC, March 28-31, 2011;

8. **Lyubov Popova**, Ann H. West, Gillian M. Air. Immunodominance of antigenic sites of hemagglutinin of recent H3N2 influenza viruses. 30th Annual meeting of the American Society for Virology, July 16-20, 2011 (Travel Award of the American Society for Virology);

9. G. M. Air, **L. Popova**, L. Venkatramani, K. Smith, P.C. Wilson, L.M. Tomas, B.H.S. Mooers, J.A. James, L.F. Thompson. Neutralizing antibody epitopes on the hemagglutinin of recent H3N2 viruses. 4th ESWI Influenza Conference in Malta, September 11-14.

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Table S1. Overall affinity (Kd) of binding of human antibodies to 309 HA and mutants after vaccination against A/Wisconsin/67/05 in the 2006-07 trivalent vaccine.

sample	Kd, 10^{-3} µl plasma ± SD										
	309 HA	HL156-157KS	KFK158-160GST	NDQI189-192QEQT	A196V	NNES121-124ITEG	N126T	N133	TSSS135-138GSNA	K140I	RSNNS142-146GPGSG
048	11.6±0.2	9.9±0.1	11.9±0.6	10.7±0.2	10.0±4.0	8.6±2.9	9.8±5.1	10.0±3.9	12.0±1.9	13.8±3.0	9.4±2.8
x14	11.3±0.3	14.0±0.2	22.4±0.4	13.1±0.6	12.0±3.0	12.4±2.6	9.6±4.8	11.9±3.7	12.2±3.5	15.6±3.5	16.2±6
006	4.3±0.4	5.0±0.2	3.3±0.8	3.5±0.1	3.1±1.8	2.9±0.4	3.8±1.1	3.2±0.4	2.9±0.4	4.8±0.7	3.1±1.1
119	5.7±0.5	7.8±0.3	4.9±0.8	4.4±0.6	4.9±0.6	6.8±0.4	5.6±0.6	5.6±0.5	5.2±0.8	6.9±1.5	6.1±1.8
078	2.2±0.3	3.0±0.3	3.5±0.5	3.3±0.3	2.4±0.3	1.8±0.4	2.4±1.2	2.3±0.4	1.7±0.5	2.8±1.2	2.6±0.4
082	5.4±0.9	8.7±0.7	7.2±1.1	6.7±3.8	6.1±0.7	4.4±1.8	5.5±1.4	3.2±0.4	3.7±0.7	3.8±1.4	3.3±1.4
256	4.4±1.7	5.4±2.8	2.3±0.5	4.2±0.1	4.5±1.1	8.8±0.9	9.2±1.3	9.3±4.4	4.1±2.2	14.0±1.0	9.0±1.8
014	3.1±1.4	3.8±0.9	3.6±1.3	2.2±1.0	3.0±0.5	3.7±1.6	3.4±0.8	3.9±1.6	4.0±1.0	3.8±0.9	3.2±1.9
080	27.8±7.4	17.0±1.5	29.2±1.4	31.3±11.9	28.7±6.8	33.2±3.0	20.2±5.7	26.0±9.3	18.8±5.2	19.7±5.1	23.2±5.1
013	2.0±0.6	2.1±0.1	2.2±0.1	1.9±0.2	2.0±0.8	1.9±0.3	1.7±0.5	1.5±0.5	2.1±0.9	1.6±0.7	1.7±0.7
040	6.3±2.4	8.0±1.3	11.9±2.1	8.2±2.6	7.0±1.7	8.8±3.5	8.4±3.9	10.5±4.0	7.5±2.5	9.6±3.1	7.2±2.1
061	10.8±2.8	12.7±0.7	29.9±2.3	15.9±4.2	11.0±2.7	13.0±2.0	13.1±2.8	11.8±2.0	13.2±1.9	10.1±2.5	10.5±1.3
049	9.2±3.8	14.2±2.6	15.4±1.9	10.7±1.1	7.6±1.7	12.4±3.0	9.6±1.6	15.6±5.0	12.2±4.1	22.7±3.1	16.2±2.3
030	2.6±0.6	3.8±0.8	4.0±0.7	3.7±0.4	2.4±1.0	2.6±1.1	2.4±0.4	2.0±0.5	2.5±0.6	2.3±0.4	1.7±0.3
200	9.0±1.6	16.5±0.9	13.3±3.2	10.1±1.0	6.9±1.3	5.4±1.4	6.5±1.5	6.9±0.9	5.4±1.0	11.9±0.4	6.1±1.0
182	12.7±0.6	12.7±1.2	22.3±4.6	10.2±4.5	10.9±1.1	9.8±0.6	10.7±1.6	12.3±1.1	12.5±0.6	11.2±1.0	13.0±3.0
016	2.8±1.2	3.1±0.3	6.8±0.8	3.9±0.6	3.8±0.9	3.9±0.9	3.7±1.2	2.8±1.1	4.1±1.3	3.1±1.5	3.5±1.1
y14	15.3±3.9	16.0±4.5	23.3±3.3	10.4±6.5	11.0±2.8	15.1±2.3	8.8±2.1	9.1±3.7	8.1±0.7	25.2±0.9	9.7±0.8

Table S2. Overall affinity (Kd) of binding of human antibodies to 309 HA and mutants after vaccination against A/Uruguay/716/07 in the 2008-09 trivalent vaccine.

sample	Kd, 10^{-3} µl plasma ± SD										
	309 HA	HL156-157KS	KFK158-160GST	NDQI189-192QEQT	A196V	NNES121-124ITEG	N126T	N133	TSSS135-138GSNA	K140I	RSNNS142-146GPGSG
022	10.4±1.4	13.7±3.2	26.6±7.0	16.8±6.0	10.9±2.9	10.0±3.3	11.8±3.6	11.2±3.0	10.0±2.7	10.9±1.8	11.7±3.1
062	12.9±3.6	15.2±4.5	12.6±2.7	9.7±3.9	11.9±1.3	10.7±3.5	11.7±3.6	12.2±2.9	10.0±2.7	14.4±1.8	14.2±3.7
040	31.0±1.6	21.0±2.4	21.7±5.0	26.3±3.8	34.0±5.9	18.7±4.2	20.8±1.9	31.3±8.4	21.3±5.7	33.2±10.5	21.7±1.1
065	16.8±3.2	11.4±2.6	20.5±7.8	19.2±1.3	17.1±6.5	25.6±4.9	25.2±6.9	27.3±6.7	18.3±4.5	21.4±5.0	28.7±8.0
057	0.8±0.1	0.8±0.01	1.7±0.5	0.8±0.3	0.7±0.09	0.9±0.1	0.9±0.01	0.8±0.1	0.7±0.1	0.9±0.1	0.5±0.01
131	5.1±2.4	7.9±3.0	15.3±2.9	5.6±2.1	5.0±3.8	8.8±4.0	6.7±1.5	5.1±2.4	5.0±1.4	5.4±1.5	7.8±3.9
030	7.3±1.3	7.0±0.5	9.1±0.9	9.0±1.5	8.6±0.8	8.1±1.3	8.4±0.9	6.5±0.8	8.2±1.5	7.6±1.3	8.0±1.7
200	4.0±1.5	8.0±0.3	9.1±0.9	6.8±1.3	5.2±2.5	4.0±0.9	4.9±1.0	4.6±0.4	5.0±1.4	4.2±1.0	3.7±1.5
182	5.2±0.9	5.3±0.7	7.2±0.2	5.3±1.1	5.1±2.1	5.9±1.2	4.7±0.8	6.1±0.7	4.6±0.9	4.9±0.7	6.4±1.9
016	3.3±0.9	4.0±0.4	7.9±0.6	3.5±0.9	2.5±0.3	2.9±0.8	3.0±1.0	3.0±0.9	2.8±0.5	3.3±0.9	2.7±0.9
y14	13.2±2.7	14.8±3.1	18.3±2.1	12.4±2.8	12.0±2.8	13.3±4.6	13.1±2.5	12.0±3.1	12.1±2.4	11.4±2.7	13.6±3.7