Influenza virus antigenicity and broadly neutralizing epitopes

Gillian M. Air

Department of Biochemistry & Molecular Biology University of Oklahoma Health Sciences Center PO Box 26901, Oklahoma City, OK 73126-0901 Phone 405 271-2085

Gillian M. Air: Gillian-air@ouhsc.edu

Abstract

A vaccine formulation that would be effective against all strains of influenza virus has long been a goal of vaccine developers, but antibodies after infection or vaccination were seen to be strain specific and there was little evidence of cross-reactive antibodies that neutralized across subtypes. Recently a number of broadly neutralizing monoclonal antibodies have been characterized. This review describes the different classes of broadly neutralizing antibodies and discusses the potential of their therapeutic use or for design of immunogens that induce a high proportion of broadly neutralizing antibodies.

Introduction

Influenza vaccines have been used since the 1940s. They are safe but need to be multivalent to protect against the multiple circulating viruses, and the components need to be updated nearly every year in response to mutations of the virus. The holy grail for influenza vaccine would be a single formulation that cross-protects against all current and future strains. Recent discoveries of cross-reactive monoclonal antibodies have given hope that a universal influenza vaccine may be possible.

This review covers recent work (approximately 2009 to 2014) to characterize neutralizing antibodies against influenza with emphasis on those that show some level of cross-reactivity between different subtypes.

Early observations

Human influenza virus was first isolated in 1933. Memories of the devastating death toll of the 1918–1919 epidemic fuelled efforts to develop a vaccine, spurred even more by the advent of the Second World War. By 1936 it had been recognized that influenza viruses are antigenically diverse. Methods to inactivate the virus with formalin overcame the inherent safety concerns of live virus vaccines and the vaccine given to troops in World War II was trivalent, containing A/PR/8/34, A/Weiss/43, and B/Lee/40. This vaccine was shown to

provide protection against type A and B viruses until 1947, when it dramatically failed. The 1947 viruses were originally classified as "A prime" but eventually were grouped into the H1N1 subtype, despite the marked change in antigenic properties. By 1954 there were two fundamental questions on antigenic variation [1]. One was whether the virus mutates in response to environment (such as infection of a new host, or presence of antibodies), versus the ideas of G. K. Hirst and J. Y. Sugg that a pre-existing variant is selected out by environmental pressure. The second question was whether there are a limited number of variants of influenza virus that wax and wane in the human population (J. Salk, T. Francis), or whether the virus is continually changing (F. L. Horsfall, F. M. Burnet). A finite number of variants would imply that a vaccine containing all of them would be effective. Unfortunately this is not the case, and we now know that influenza evolves linearly by selection of escape mutants, usually by antibodies, from a small population of variants generated by random mutation from the preceding virus. This means that development of a universal influenza vaccine requires a strategy other than including all known strains.

Antigenic drift and shift, neutralizing antigens, current vaccine strategies

Influenza viruses are classified by serological cross-reactivity, or lack thereof. Types A, B and C do not cross-react by any serological test. Type A viruses all share cross-reactivity of internal proteins, nucleoprotein (NP) and matrix (M1), but the surface glycoproteins hemagglutinin (HA, or H) and neuraminidase (NA or N) are divided into serological subtypes H1 to H16 and N1 to N9 that do not cross-react with serum antibodies. Only H1, H2 and H3 with N1 or N2 circulate in the human population. Recent influenza sequences from bats proposed as H17, H18, N10 and N11 have functionally different glycoproteins and the viruses have not yet been isolated [2]. A new subtype entering the human population is described as antigenic shift, such as when H2N2 viruses replaced H1N1 in 1957 and H3N2 replaced H2N2 in 1968. Antigenic shift is facilitated by the large variety of influenza viruses in bird populations and by the segmented nature of the genome that allows reassortment of genes in a mixed infection. Following antigenic shift, the new virus undergoes progressive changes due to antibody selection, known as antigenic drift.

All the genes of influenza virus undergo some degree of variation, all occurring by the same basic mechanism. Influenza has an RNA genome that codes for its own RNA polymerase. RNA polymerases in general lack the editing feature of DNA polymerases, an exonuclease domain that removes a mismatched 3' nucleotide before elongation can continue. Without this exonuclease activity, the intrinsic error rate of RNA polymerases is relatively high. Most of the resulting variants are lost in the population, but a few may be fixed by chance ("random drift"). Some mutations are positively selected, for example, to escape from antibody neutralization or for more efficient replication or better interaction with a specific host protein, and these variants rapidly take over the population because they confer an advantage. Changes in proteins selected for improved function may change their antigenic properties. There is a marked distinction between antigenic selection (resistance to the immune system) and antigenic change that is a consequence of some other selective pressure.

Definitions of "neutralizing" and "epitope"

Protection against influenza is mediated by innate systems and by T cells and antibodies. The relative roles of these vary with the patient's genetic profile and history of influenza, but overall the most important contribution to protection is from neutralizing antibodies, and current vaccines are measured by their power to induce neutralizing antibodies. The classical definition of "neutralizing" is to block the ability of a virus to attach to a cell; i.e. to block the first step in viral infection. Such antibodies sterically interfere with the receptor-binding site on the hemagglutinin (HA) so it cannot bind to its sialic acid receptor on the cell surface. However, there are antibodies that interfere in infection at later stages. While these do not meet the classical definition, they effectively neutralize the infection and in laboratory studies are equally protective.

What are the targets of neutralizing antibodies that do not block attachment to sialic acid? While antibodies can be raised that block various viral activities (RNA-dependent RNA polymerase, assembly of nucleoprotein complexes or of the viral matrix protein), these antibodies are not neutralizing or protective because they cannot access their targets during the normal course of infection. Neutralization targets are those outside the viral membrane and so exposed on the virus; the surface proteins HA, neuraminidase (NA) and M2 ion channel. The HA's first function is to bind sialic acid receptors, but after internalization, in the low pH environment of the endosome, the HA undergoes a conformational change to enable a fusion activity that allows release of the viral genome. So antibodies that efficiently block either binding to receptor, conformational change or the fusion function will also be neutralizing.

Antibodies can only neutralize if they block a function. It follows that they bind to native proteins. Typically they bind to multiple segments of the polypeptide chain that may be dispersed in the primary sequence but come together in the three-dimensional structure. Therefore most neutralizing antibodies bind to a so-called "conformational" epitope, that is lost if the protein is denatured or even partially unfolded.

Mapping antibody epitopes

For this review, an epitope is defined as the amino acids on the antigen that make contact with antibody. Some of the interactions of antibody with antigen are more important than others. The critical contacts, with highest interaction energy, can be identified by selection of escape mutants, or by exhaustive mutagenesis, or by hydrogen exchange methods. These studies only give a partial view of the epitope, albeit the most energetically important view. Full descriptions of epitopes can be made from X-ray crystal structures of the antigen-antibody complex; this is currently the only available method that shows all the atomic interactions between antigen and antibody. An excellent discussion of broadly neutralizing epitopes characterized by X-ray crystallography is given by Lee and Wilson [3].

How polyclonal is human serum?

Much of our knowledge of neutralizing epitopes comes from studies with monoclonal antibodies (mAbs), that allow selection of escape mutants that can be attributed to a single

selecting antibody. A monoclonal antibody also allows complexes of antigen and antibody to be crystallized for structural analysis. The human response is, of course, polyclonal, raising questions about the applicability of monoclonal studies (usually mouse) to human protection. Mouse monoclonal antibodies were used to map broad antigenic regions on the HA of H3N2 and H1N1 viruses by using competition assays and cross-reactivities of the mAbs with escape mutants selected by other mAbs. Five sites were found on the H3 HA (sites A through E, [4,5]) and four on H1 HA (Sa, Sb, Ca, Cb, [6]). An antibody that recognizes a change in Site A, for example, did not recognize changes in sites B-E. The deduction is that changes in all antigenic sites would be needed for a virus to escape the human immune system and begin a new epidemic. For some antigenic drift strains of H3N2 viruses this was the case, but more commonly, especially more recently, only one or two changes are found between epidemic viruses. It has been noted for some time that human H3N2 viruses show a changing pattern of immunodominance and it appears that for any given virus, the human response does not cover all antigenic sites and vaccine failure can be due to one or two mutations in the HA [7].

Broadly neutralizing monoclonal antibodies

The N-terminal ectodomain of the M2 ion channel protein is quite highly conserved, and immunization with recombinant M2 generates mAbs with considerable cross-reactivity between subtypes. These antibodies are rare in infected humans but they might be protective if they could be induced by recombinant M2, or amplified in vitro and given therapeutically. Both approaches have been tested and shown to be effective in animal models [8–10]. There is one report of a Phase 2 clinical trial of antibody TCN-032 which showed some reduction in symptoms but no protection from infection in volunteers challenged with influenza virus [11]. On the whole, the M2 approach has not yet shown efficacy; the neutralizing power of antibodies directed against M2 may be inherently low compared to those against HA.

There are recent reports of antibodies that have broad reactivity against NA. One epitope is within a conserved linear sequence in the active site of all type A subtypes and type B NA, but protection is less than 50% [12,13]. Others target a conformational epitope shared by seasonal H1N1, pandemic H1N1 and H5N1 viruses and show prophylactic activity [14]. Antibodies against NA do not block infection but they do block release of virus and so ameliorate disease (reviewed in [15]). The most effective neutralizing antibodies are against the HA.

Initial attempts to raise antibodies against conserved regions of the HA used small peptides as immunogens. The resulting antibodies were cross-reactive, but usually bound only to denatured HA and so were not neutralizing, reviewed in [16]. A mouse mAb designated C179 was shown in 1993 to neutralize viruses of the H1 and H2 subtypes, and partial mapping showed that the epitope was located on the stem of the HA, later fully mapped by X-ray crystallography [17,18]. Sixteen years later two reports appeared describing broadly neutralizing antibodies directed against the stem region of the HA, fully characterized by X-ray crystallography of HA-antibody complexes [19,20]. Many such stem-binding mAbs were reported in the next few years. There have also been descriptions of broadly neutralizing antibodies recognizing the receptor binding region of the HA head [21–23].

It has now become clear that there are varying definitions of "broadly neutralizing" antibodies. In influenza studies the traditional term was "cross-reactive" and it meant binding to more than one subtype of influenza. "Broadly neutralizing" has been applied to mAbs that neutralize multiple subtypes, but is also used by some authors to describe neutralization of more than one strain within a subtype. So one investigator's "broadly neutralizing" mAb may bind only some clades of H5 HAs while another's may bind representatives of all 16 HA subtypes. Table 1 shows the well-characterized epitopes of antibodies that bind to several strains of influenza virus published up to December 2014. The entries are sorted from most cross-reactive (14 of 16 subtypes of influenza A and influenza B), through those that bind more than one subtype within the phylogenetically-defined HA Group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16) or Group 2 (H3, H4, H7, H10, H14, H15), to those that bind multiple viruses of a single subtype. It should be noted that testing may not have included all subtypes so the sorting is only approximate. However, it does show that the most cross-reactive antibodies (e.g. CR9114, F16, F10, CR6261) bind to the stem of the HA while receptor site binding antibodies are more selective (e.g. S139/1 and C05). Antibodies that bind within a single subtype all have epitopes on the HA globular head, perhaps reflecting higher neutralizing power on the head domain but limited by higher sequence variation due to antigenic drift and shift.

The binding sites for representative mAbs that bind to the stem and receptor binding domains are shown in Figure 1. Note that although the stem antibodies bind in the same general region, the binding sites vary up and down and around the stem and in the angle of the antibody Fab relative to the HA. The antibodies that bind to conserved elements of the receptor binding site similarly vary in placement of the Fab [24]. Antibodies that are not cross-reactive bind to variable loops that surround the receptor binding pocket [25] while those with intra-subunit cross-reactivity show binding to somewhat conserved epitopes on the globular HA head [26,27].

The structural studies have also shown that antibodies can bind across the canonical antigenic sites A – E of H3 and Sa – Sb of H1. This is the case with broadly neutralizing antibodies that bind around the receptor binding site (Table 1) but also with some of the original mouse mAbs made against Aichi/68 H3 that are more specific (see structures referenced in [25]).

Conclusions and Future Prospects

For many years investigators searched for broadly-neutralizing antibodies in sera and did not find significant evidence, suggesting they are rare. There are several reports that breadth of the response can be widened by changing the immunization route or by using adjuvant, but the protective effect in humans is not clear. The recent advances in mAb technology, particularly in making human mAbs have resulted in many examples of antibodies that are truly broadly neutralizing, and these can be enriched in serum of mice by immunization with "headless" HA constructs [28,29] or by using HAs with different head domains for prime and boost [30,31].

The broadly-neutralizing mAbs are also of potential use therapeutically. Antibodies CR8020 and CR6261 completed Phase 1 clinical trials but a small challenge study did not show efficacy and a phase 2 trial was halted before enrollment. Another mAb called VIS410 is currently in Phase 1 trial but its epitope has not been published, at least not under that name (www.clinicaltrials.gov).

At this time there is a lot of activity to develop broadly neutralizing antibody technology against the variable influenza viruses, but challenges remain. Vaccines could be further refined to induce anti-stem antibodies but the neutralizing power when fusion is blocked may be inherently lower than that of antibodies that block receptor binding, as seen in the lack of efficacy in the challenge study. However, mAb technology allows engineering to improve avidity of antibodies and that may be a path to successful therapeutic cross-reactive mAbs.

Acknowledgments

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Highlights

- A vaccine that induces broadly-neutralizing antibodies would not need annual updates.

- Neutralizing epitopes are almost always conformational and discontinuous, not linear.

- Recent crystal structures show the location of cross-neutralizing epitopes on HA.

- Many broadly-neutralizing antibodies bind the HA stem, others bind the receptor pocket.
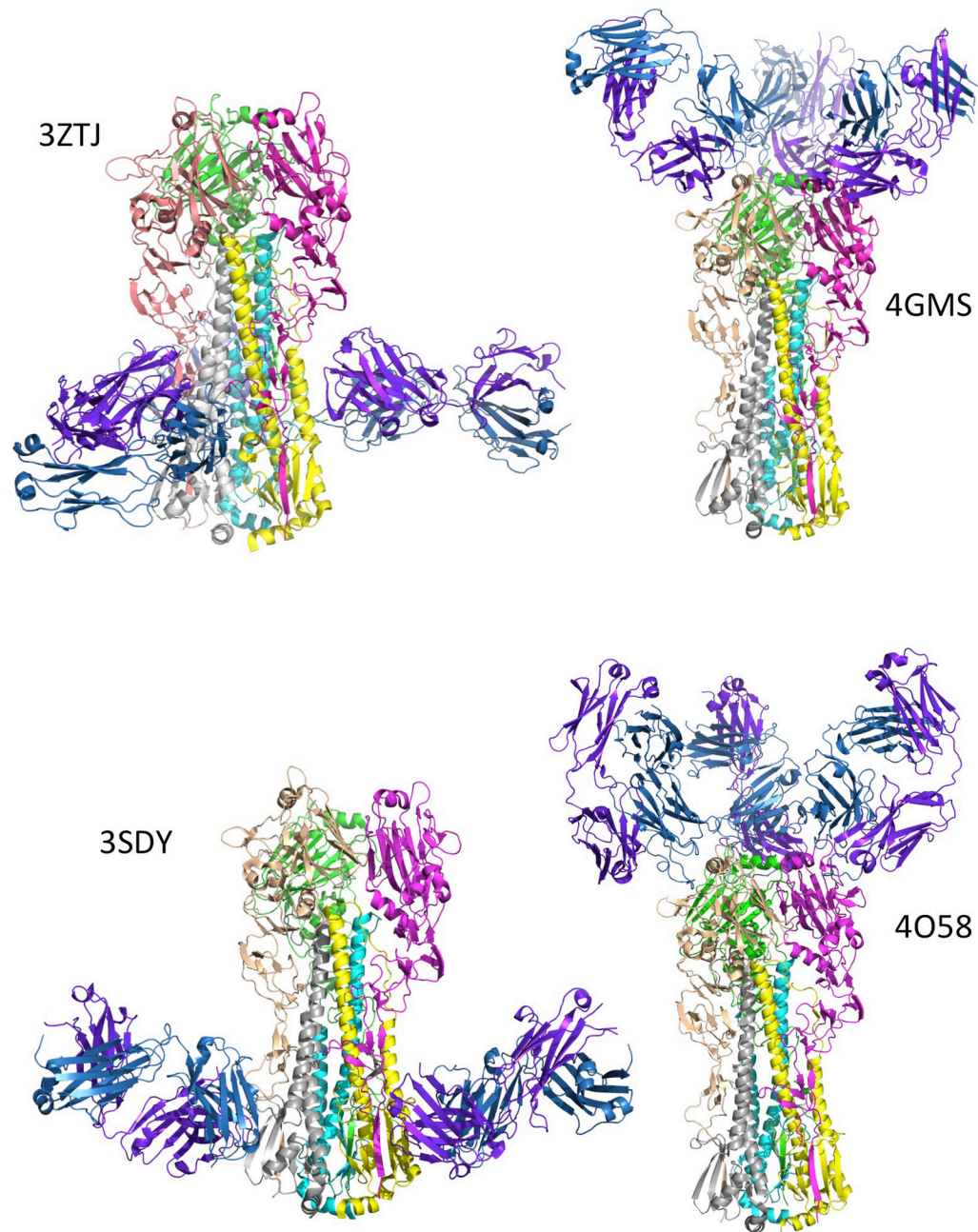
Figure 1.
Binding sites of representative broadly neutralizing antibodies on HA seen in X-ray crystal structures. The HA is shown as the trimer in approximately the same orientation in all panels. Monomer 1 colors are magenta (HA1) and yellow (HA2); monomer 2 are wheat (HA1) and gray (HA2); monomer 3 are green (HA1) and cyan (HA2). In all cases there are three antibody Fabs bound per HA trimer although the third is hidden from view in some cases. The antibody Heavy chain is colored purple and the Light chain is sky blue. The two stem-binding antibodies (F16 and CR8020, see Table 1) bind at different sites on the stem (PDB IDs 3ZTJ and 3SDY respectively). The antibodies that bind to the receptor binding

region (S139/1 and F045-092) bind at different angles (PDB IDs 4GMS and 4O58 respectively). The figures were made using PyMOL Molecular Graphics System, Schrödinger, LLC.

Table 1

Broadly neutralizing or cross-reactive epitopes on influenza virus HA

Antibody	Source	IEDB[1] ID	HA subtype	Contacts on HA1[2]	Contacts on HA2	Crystal structure (PDB[3] ID and viral HA)	Reference
CR9114	human V$_H$ 1-69	178112	14/16 A subtypes and B	H5: H44, Q46, D47, I48, S304, M305, P306	V364, D365, G366, W367, A382, K384, T387, Q388, I391, D392, V394, T395, V398, I402	4FQI A/Vietnam/1203/2004	[32]
CR9114	human V$_H$1-69	178113	14/16 A subtypes and B	H7: N53, T55, E56, T57, N307, L308, P309	I366, D367, G368, W369, A384, Y386, T389, Q390, I393, D394, I396, T397, L400, I404	4FQV A/Netherlands/219/2003(H7 N7)	[32]
CR9114	human V$_H$1-69	178114	14/16 A subtypes and B	H3: N54, T56, E57, L58, D307, K308, P309	I363, D364, G365, W366, A381, L383, T386, Q387, I390, D391, I393, N394, L397, I401	4FQY (Hong Kong/68 H3)	[32]
F16	human V$_H$3-30*18	164106	H1, H2, H5, H6, H8, H9, H3, H4, H7, H10	H1: H45, S46, S306, T333	V362, D363, G364, W365, L382, K385, T386, Q387, N388, I389, D390, T393, N397, I400, E401	3ZTN (Calif/04/2009 H1), 3ZTJ (Aichi/68 H3)	[33]
F10	human phage display	229014	H1, H2, H5, H6, H8, H9, H11	H24, H44, Q46, S304, M305	V364, D365, G366, W367, K384, T387, Q388, I391, T395, V398, N399, I402	3FKU Vietnam/1203/2004	[20]
CR6261	human V$_H$1-69	226309	Group 1 (H1, H2, H5, H6, H8, H9)	H25, H45, V46, N47, L496, S306, L307, P308, T333	D363, G364, W365, Q382, T385, Q386, I389, D390, T393, V396, N397, I400	3GBN (SouthCarolina/1918, H1), 3GBM (H5). Only H chain is bound	[19,34]
S139/1	mouse	176782	H1, H2, H3, H13, and H16 receptor site	H3: Y115, T148, G151, G152, S153, S154, W170, Y172, K173, S174, G175, S176, K206, E207, N210, L211, V213, L243		4GMS Victoria/3/75	[22]
C179	mouse	186645	H1, H2, H5, H6, H9	H2: H43, K45, I47, T302, L303, P304, T329	V358, D359, G360, W361, K378, T381, Q382, K383, F385, D386, V392, I396 I362, D363, G364, W365, Q382,	4HLZ Japan/305/1957	[17,18]
3.1	human V$_H$3-30	227882	H1, H2, H5, H6	H45, V47, N48, L49, N304, S305, S306, L307, P308, T333;	T385, Q386, I389, T393, V396, N397, I400;	4PY8 South Carolina/1/1918	[35]
C05	human	180352	H1, H2, H3, H9	Y114, T147, N149, G150, G151, S152, N153, S161, W169, T171, K172, Q205, E206, T208, S209, L210, L242		4FQR Aichi/1968 H3)	[36]

Antibody	Source	IEDB[1] ID	HA subtype	Contacts on HA1[2]	Contacts on HA2	Crystal structure (PDB[3] ID and viral HA)	Reference
1F2, 1F4, and 1E1	human	177664	H1 H3 H9		FIEGGWTGMVDGWYGYHH, part of fusion peptide		[37]
CR8020	human	167844	H3, H7, H10	E341;	E360, G361, I363, D364, R370, E375, T377, G378, Q379, A380, A381, L383, N491, E495	3SDY (Hong Kong/1/68 H3)	[38]
CR8043 (similar to CR8020)	human	196975	H3, H10, less H7	P37, E341, K342	E360, G361, I363, D364, R370, T377, Q379, L383	4NM8 HK/1/1968	[39]
F045-092 VH1-69	human	227712	H1, H2 H3	Y115, T148, N150, G151, G152, S153, S154, S162, W170, Y172, K173, S174, G175, N210, L211, L243		4O58 Victoria/3/75	[40] [23]
PN-SIA49	human V_H3-23	0	H1, H2, H5 Group 1	Mutations that reduce binding: H25A, N336A, P338A	M360A,D362A, G363A, W364A, T384A, V395A,N396A, E400A (sequence numbering refers to A/PR/8/34)		[41]
39.29	human	189321	H1, H3	H34, V36, N54, S70, K292, N294, P305, D307,	T334, V363, D364, G365, W366, Q379, A380, D382, L383, K384, T386, Q387, I390, D391, I393, N394, G395, L397, N398, I401, K403, T404, N405, R498	4KVN Perth/16/2009	[42]
1F1	human	179938	Various H1	K147, V149, S159, W167, K170, S173, H197, P200, T201, T203, D204, Q206, S207, L208, Q210, K236, D239, A241, G242		4GXU South Carolina/1/1918	[43]
2D1	human	164527	H1 1918 and 2009 (not seasonal H1)	S138, S139, P141, N142, K171, G172, S173, S174, Y175, P176, K177, S179, K180, S181, V183, N211, E260, T262		3LZF (S.Carolina/1918 H1) 3LZG (Cal/04/2009 H1) H+L binding	[27] [44]
5J8	human	191110	Seasonal and 2009 H1 except NewCal & Brisbane	K147, V149, T150, A151, G157, A158, K159, W167, A203, D204, Q206, S207, L208, K236, D239, Q240		4M5Z California/07/2009	[26]
CH65	human	159269	most H1 receptor site	G147, V148, S149, A150, W166, T168, G169, N171, G172, L173, N200, G202,		3SM5 (Solomon Is/3/06 H1)	[21]

Antibody	Source	IEDB[1] ID	HA subtype	Contacts on HA[2]	Contacts on HA2	Crystal structure (PDB[3] ID and viral HA)	Reference
CH67	human	180309	H1N1 1986–2007	G147, V148, S149, A150, W166, T168, N171, G172, L173, N200, G202, D203, R205, A206, L207, H209, K232, D238	D203, R205, A206, L207, K235, D238, R239, E240 D203, R205, A206, L207, K235, D238, R239, E240 D203, R205, A206, L207, K235, D238, R239, E240	4HKX Solomon Islands/3/2006	[45]
GC0587	mouse	226441	H1	T89, A90, S127, E129, R130, E132, P135, S138, K180, K186, K188, Y270, A273		4LVH Korea/1/2009 H1N1	[46]
GC0757	mouse	190190		E129, R130, F131, E132, P135, T137, S138, K180, S181, I183, Y270		4F15 Korea/1/2009 H1N1	[47]
2G1	human V_H1-69	181066	H2 human and swine	T138, Q139, T141, G144, G145, S146, R147, P155, T165, K166, K167, G168, S169, T203, L204		4HG4 Japan/305/1957	[24]
8F8	human V_H3-33	181071	H2 human and swine	T141, T143, G144, G145, S146, R147, A150, G153, N154, P155, W163, T165, E166, G168, T199, E200, T203, L204, Q236		4HF5 Japan/305/1957	[24]
8M2	human V_H1-69	181067	H2 human and swine	T141, T143, G144, G145, S146, R147, P155, W163, T165, K166, G168, S169, N196, D197, E198, T199, E200, R202, T203, L204, K232, G235, L236, G237, S238		4HFU Japan/305/1957	[24]
B-1, D-1	human	115630, 115652	H3 many strains	Within 189–197 NFDKLYIWG and 227–239 SSRISIYWTIVKP			[48]
F005-126	human	233323	H3 1968–2004	S107, K108, N187, D188, N189, P255, G256, S286, D287, A288, P289, P300, N301		3WHE Aichi/68	[49]

Antibody	Source	IEDB[1] ID	HA subtype	Contacts on HA1[2]	Contacts on HA2	Crystal structure (PDB[3] ID and viral HA)	Reference
100F4	human	0	H5 most clades	68, 112, 137, 143, 251, 254, and 255			[50]
65C6	human	164481	H5 all clades except 7.2	118, 121, 161, 164, and 167 (mature H5 numbering) P134, S137, K177, Y180, T183 (IEDB)			[51]
9F4	mouse	136074	H5 clades	271–281, centered on 272–275			[52,53]
AVFluIgG 01	human	170144	H5	I132, I133, P134, W138, S139, Y180, T183			[54]
H5M9	mouse	194677	most H5N1 clades	D59, D61, V63, K64, R69, E85, N88, P90, E91, H126, E128, E286, Y287, N289, N291		4MHH Vietnam/1203/2004	[55]
HA-7	mouse	186786	H5 several clades	88–90 NVP and 126–131 HFEKIQ		cryoEM	[56]
CR8059	human V_H1-18	178109	B strains	T52, K53, S54, H55, F56, K67, N74, T76, D77, H100, E101, G300, S301, L302, P303		4FQK B/Brisbane/60/2008	[32]
CR8071	human V_H1-18	178108	Most B strains	T52, K53, Y55, F56, K67, L73, N74, C75, T76, D77, H100, E101, V105, G299, S300, L301, P302, I304		4FQJ B/Florida/4/2006	[32]

[1] Immune Epitope Database www.iedb.org

[2] Numbering is from the initiating Met and is continuous through the signal peptide and across the HA1-HA2 junction. In most crystal structures the numbering is separate for HA1 and HA2, and it often follows homology to H3 HA.

[3] http://www.rcsb.org/pdb/