Table 4. Nasal HI and neutralizing antibody responses after two doses of the nasal vaccine

	Week	HI titers[a]	NT titers[a]
GMT (Mean geometric increase)	0	12.4 (1.00)	15.4 (1.00)
	6	38.8 (3.13)	90.5 (5.88)

[a]Nasal HI and neutralization titers were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HI and neutralization titers of < 1:20 were considered negative and were arbitrarily assigned a titer of 1:10.

Table 5. Serum and nasal HA-specific antibody responses after two doses of the nasal vaccine

	Isotype	Week	Serum	Nasal wash
GMT (Mean geometric increase)	IgG	0	83.7 (1.00)	80.0 (1.00)
		6	247.7 (2.96)	109.8 (1.37)
	IgA	0	14.6 (1.00)	122.0 (1.00)
		6	36.0 (2.47)	473.5 (3.88)

[a]Serum HA-specific antibody titers of <1:10 were considered negative and arbitrarily assigned a titer of 1:5. [b]Nasal HA-specific antibody responses were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HA-specific IgG and IgA titers of <1:160 were considered negative and arbitrarily assigned a titer of 1:80.

Table 6. Cross-reactive HI and neutralizing antibody responses to A/Sydney virus after two doses of the A/Victoria vaccine

Item	Antibody responses	Week	Serum[a]	Nasal wash[b]
GMT (Mean geometric increase)	HI antibody	0	21.2 (1.00)	13.6 (1.00)
		6	30.5 (1.44)	22.2 (1.63)
	Neutralizing antibody	0	160.0 (1.00)	23.0 (1.00)
		6	233.2 (1.46)	48.7 (2.12)

[a]Serum HI and neutralization titers of <1:10 were considered negative and arbitrarily assigned a titer of 1:5. [b]Nasal wash HI and neutralization titers were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HI and neutralization titers of <1:20 were considered negative and arbitrarily assigned a titer of 1:10.

with the degree of protection. It was shown that in the upper respiratory tract the majority of the protective immunity induced by influenza virus infection is mainly due to S-IgA antibodies.[2,3] In this study, the nasal inactivated vaccine induced high levels of HI, NT and HA-specific IgA antibody responses in nasal mucus (**Fig. 3**; **Table 4, and 5**). Althogh several reports show a good correlation between HA-specific antibody titers and HI or NT titers in serum,[41,42] HA-specific antibody responses seem to be more sensitive than microneutralization assay in this study (**Fig. S2A and D**). In addition, NT titers were well correlated with HI titers in both serum and nasal wash (**Fig. 2B and 3C**). Among three antibody measurements (HI, microneutralization and HA-specific antibody ELISA), ELISA may be the best choice for achieving sensitivity, but microneutralization has the advantage of representing not only antibody responses against HA like HI and ELISA, but also those against neuraminidase (NA), since it was already shown that NA-specific antibody can contribute partially to the virus neutralization.[43] Therefore, neutralizing antibody might be more useful for the evaluation of protective antibody response.[10] Together with the fact that at present there are no guidelines available for the evaluation of antibodies in nasal mucus, we chose to focus on the neutralizing antibodies. In the present study, HI, neutralizing and HA-specific antibody titers in nasal mucus, as well as in serum, showed synchronous changes in many of the subjects following nasal vaccination, although the degree of the respective responses and the type of dominant neutralizing antibody varied slightly from subject to subject (data not shown). In addition, antibody responses decreased with age; subjects aged > 60 y produced only low levels of HI and neutralizing antibodies (**Figs. 1, 2, and 3**). Thus, as reported previously, the magnitude of the antibody response in each subject appeared to change depending on parameters that affect the immune responses of the host, such as pre-vaccination antibody levels, sex and age.[34,44]

HI and neutralizing antibodies cross-reactive with the A/Sydney virus were detected in pre-vaccination serum and nasal wash, and were enhanced after two doses of the nasal A/Victoria vaccine, although the mean geometric increase in the cross-reactive antibody responses increased only about 1.5- to 2-fold (**Table 6**). These results suggest that cross-protection against infection with different strains of influenza virus could be enhanced by an intranasal inactivated whole virus vaccine. Since this field is relatively unexplored yet, it will be interesting to find to which extent this cross-protection can be enhanced by optimizing intranasal vaccination.

The present study first demonstrated that intranasal vaccination with an inactivated whole virus vaccine alone (45 μg HA per dose) could induce serum HI antibody responses which exceeded the EMA criteria for serum HI antibody titers as well as serum neutralizing antibody responses, and that high levels of nasal HI and neutralizing antibody responses were detected using concentrated nasal wash samples. Thus, at least among adults with some immunological memory induced by previous infection or vaccination, the intranasal inactivated whole virus vaccine could be a promising candidate for a needle-free mucosal vaccine. Antibody responses induced by intranasal vaccination in individuals who are naïve to influenza virus antigens remain to be determined. In addition, further studies which compare the HI and NT antibody responses between groups treated with intranasal and existing non-mucosal vaccines are needed to gain more insight in the possible benefits of intranasal vaccination. Some clinical trials have already shown that trivalent inactivated whole virus vaccines induce significantly higher vaccine-specific IgA antibody responses in intranasally immunized subjects than in intramuscularly-immunized elderly subjects, with no significant differences in serum HI antibody responses between the groups.[16] A drawback for analyses of nasal antibody responses is that there are currently no criteria to evaluate these type of responses. In this study it is shown that the measurement of nasal HI and NT titers can provide important data in addition to the serum

antibody titers. Improved knowledge on these different types of antibody responses in different types of samples will be useful to increase the understanding of immune responses to both vaccination and infection and will be of help for defining the criteria for evaluation of responses in nasal mucus. In order to optimize the accuracy of the data acquired from nasal mucus, the techniques for recovery of nasal antibodies from nasal wash samples might need further improvement.

In conclusion, high levels of serum HI antibody responses, which exceeded all criteria used by the EMA for the evaluation of vaccine efficacy, could be induced by intranasal administration of an inactivated whole A/Victoria virus vaccine containing 45 μg HA in healthy adults. The serum antibody responses were accompanied by high levels of HI, NT and HA-specific IgA antibodies in nasal mucus. The results show that the intranasal vaccination induces both high levels of serum and nasal antibody responses that may be involved in mounting effective protective responses, including cross-protection, against both upper and lower respiratory tract infection by various influenza viruses. The inactivated whole virus vaccine therefore appears to be a promising candidate for intranasal vaccination.

Materials and Methods

Subjects. The study subjects comprised 50 healthy volunteers aged between 22 and 69 y (average 36.5 ± 12.8 y); 14 (28%) were female and four subjects (8%) were over 60 y of age. None of the subjects had to be excluded due a history of allergy to eggs, past or current neurological conditions, or respiratory illness or fever at the time of vaccination. Based on the answers of the volunteers on questions regarding their history of influenza infection and vaccination during the past 5 y, almost all subjects were considered to have acquired at least some degree of immunity to influenza viruses. Written informed consent was obtained from each subject before the onset of the trial. The protocol and other relevant study documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Viruses and vaccines. Influenza viruses, A/Victoria/210/2009 (H3N2; A/Victoria) and A/Sydney/05/1997 (H3N2; A/Sydney) strains, were obtained from the National Institute of Infectious Diseases (Tokyo, Japan), propagated in the allantoic cavity of 10-d-old embryonated hen's eggs, and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated using a previously described method.[45,46] In brief, 10-fold serial dilutions of allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney cells (MDCK: ATCC No. CCL-34) in a 96-well culture plate and incubated for 3 d at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effect observed in the virus-containing wells was evaluated using a microscope and the $TCID_{50}$ was calculated using the Reed-Muench method.[47] An inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus, containing 45 μg HA per dose, was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Kagawa, Japan). The vaccine was prepared from the purified viruses, which were sedimented through a linear sucrose gradient and treated with formalin by the method of Davenport et al.[48]

Vaccinations and adverse effects. Intranasal vaccination with an inactivated whole virus vaccine (45 μg HA/dose) was performed twice, with a 3-week interval (week 0 and 3), by spraying 0.25 ml of vaccine into each nostril (0.5 ml total) using an atomizer (Keytron: Ichikawa, Chiba, Japan). The mean droplet diameter of the mist generated by the atomizer was 56.5 μm (range: 10 μm to 90 μm). Blood and nasal wash samples were taken from each of the subjects prior to vaccination and three weeks after each vaccination (week 0, 3, and 6).

A health check sheet was given to each subject to record any symptoms after the nasal vaccination as well as their answers to questions regarding their medical history. Medical examinations were held every 3 weeks after the primary and secondary vaccinations to assess their medical condition. Adverse effects were evaluated in terms of local reactions (discomfort, pain in the nose, sneezing, stuffiness and/or running nose, throat pain, or cough) along with systemic reactions, such as malaise, headache, fever and abdominal pain, by means of health check sheets and personal interviews.

Nasal wash specimens. About 80 ml of nasal wash was collected by washing the nasal cavity several times with a nose irrigation device (Hananoa: Kobayashi Pharmaceutical, Osaka, Japan) according to the manufacturer's instructions.[31] The collected nasal wash samples were filtered using bottle top filters (Nalgene Nunc International, Chiba, Japan) with membranes covered with a cotton mat to remove mucopolysaccharides and other debris. The pooled cleaned nasal wash samples were then concentrated to a final volume of approximately 1 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO 30.000: Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash samples were stored at −80°C until use. The protein concentration in the concentrated nasal wash was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization and haemagglutination inhibition assays. NT titers were examined using microneutralization assays as previously described with minor modifications.[41,46] Briefly, serum samples were treated with a receptor-destroying enzyme (RDE: Denka Seiken, Niigata, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C, and then diluted 1:10 before the assay. Nasal wash samples were adjusted to 1 mg/ml total protein, and the final samples contained about 1/10 of the total IgA found in nasal mucus (0.22 mg/ml; original concentration in nasal mucus is 2.20 mg/ml).[31] These standardized nasal wash samples were treated with RDE and heat-inactivated similar to the serum samples, and then diluted 1:20 for use in the assays.

2-fold serial dilutions of samples were mixed with an equal volume of diluent containing influenza virus equivalent to 100 $TCID_{50}$ and added to the wells of a 96-well plate containing a monolayer culture of MDCK cells. Four control wells containing virus or diluent alone were included on each plate. The plates were incubated for 3 or 4 d at 37°C in a 5% CO_2 humidified atmosphere. All wells were observed for the presence or absence of cytopathic effects and then fixed with 10% formalin phosphate

buffer for more than 5 min at room temperature and stained with Naphthol blue black. After washing and drying, cells were solubilized with 0.1 M NaOH and the absorbance (A) was read at 630 nm. The average A_{630} value was determined from virus-only controls (A_{virus}) and medium-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula $X = (A_{cell} - A_{virus})/2 + A_{virus}$, were considered positive for neutralization. The titers recorded were the reciprocal of the highest dilution, where A_{630} was > X.

HI titers were examined using a microtitration method as previously described.[49] Serum and nasal wash samples were prepared in the same manner as for the neutralization assay and treated with packed red blood cells to remove non-specific haemagglutination-inhibiting materials. The starting dilutions for the HI assay were 1:10 and 1:20 for the serum and standardized nasal wash samples, respectively.

Evaluation of serum HI titers. Serum HI titers were evaluated using the following three parameters: the ratio of the geometric mean titer (GMT) in post-vaccination to that in pre-vaccination (the mean geometric increase); the percentage of subjects showing an increase from a pre-vaccination titer of < 1:10 to a post-vaccination titer of ≥ 1:40 (the conversion rate) or showing a ≥ 4-fold increase from a pre-vaccination titer of ≥ 1:10 (significant increase in titer); and the percentage of subjects with a post-vaccination titer of ≥ 1:40 (the protection rate). Hereafter, the conversion rate or significant increase in titer is indicated as conversion rate. As shown in **Table 1**, the parameters for serum HI titers after vaccination are currently used as criteria to evaluate the vaccine efficacy by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA).[22-24]

Determination of HA-specific IgA and IgG antibody titers. The titers of IgA and IgG antibodies specific for the HA molecule of the A/Victoria virus (HA-specific IgA titer and HA-specific IgG titer, respectively) in the serum and standardized nasal wash samples were determined by ELISA. The ELISA assay was performed in microtiter plates (Costar, Cambridge, MA) using the following procedure. First, wells of microtiter plates were coated with HA molecules purified from the A/Victoria virus according to the procedure of Phelan et al.[50] Second, the HA molecules were incubated with 2-fold serial dilutions of serum or standardized nasal wash samples followed by detection with goat anti-human IgA (α-chain specific) or goat anti-human IgG (γ-chain specific) (BETHYL Laboratories, Montgomery, AL) antibodies conjugated to alkaline phosphatase. Third, the enzymatic reaction was started by adding 1 mg/ml of p-nitrophenyl-phosphate as the substrate. Color development was measured at 405 nm using a microplate reader (Model 680: Bio-Rad Laboratories, Hercules, CA). The antibody titer for a given sample was calculated as the reciprocal of the highest dilution of the test sample that gave an absorbance read at 405 nm (A_{405}) greater than a cut-off value equal to the mean A_{405} + 2SD of 11 2-fold serial dilutions (starting at 1:10 for serum and at 1:160 for nasal sample due to sample limitation) of the negative control samples (NT titer, < 10×2^0; HI titer, < 10×2^0; HA-specific antibody titer, < 10×2^0 for serum and < 10×2^4 for nasal wash) selected from the pre-vaccination serum and nasal wash samples of 50 subjects.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism statistical software package (Version 5.0c: Graph Pad Software Inc., CA USA). The threshold of statistical significance was set at 5% ($p < 0.05$).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/vaccines/article/25458

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Two Types of Antibodies Are Induced by Vaccination with A/California/2009pdm Virus: Binding near the Sialic Acid-Binding Pocket and Neutralizing Both H1N1 and H5N1 Viruses

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Abstract

Many people have a history of catching the flu several times during childhood but no additional flu in adulthood, even without vaccination. We analyzed the total repertoire of antibodies (Abs) against influenza A group 1 viruses induced in such a flu-resistant person after vaccination with 2009 H1N1 pandemic influenza virus. They were classified into two types, with no exceptions. The first type, the products of B cells newly induced through vaccination, binds near the sialic acid-binding pocket. The second type, the products of long-lived memory B cells established before vaccination, utilizes the *1-69* V_H gene, binds to the stem of HA, and neutralizes both H1N1 and H5N1 viruses with few exceptions. These observations indicate that the sialic acid-binding pocket and its surrounding region are immunogenically very potent and majority of the B cells whose growth is newly induced by vaccination produce Abs that recognize these regions. However, they play a role in protection against influenza virus infection for a short period since variant viruses that have acquired resistance to these Abs become dominant. On the other hand, although the stem of HA is immunogenically not potent, the second type of B cells eventually becomes dominant. Thus, a selection system should function in forming the repertoire of long-lived memory B cells and the stability of the epitope would greatly affect the fate of the memory cells. Acquisition of the ability to produce Abs that bind to the stable epitope could be a major factor of flu resistance.

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Introduction

Influenza is an infectious disease of the respiratory tract that affects millions of people every year. Since antibodies (Abs) play important roles in protection against influenza virus, preventive vaccination has been one of the most efficient measures of influenza control. Hemagglutinin (HA), which is the main target for virus-neutralizing Abs, mediates virus entry into cells at two steps [1]. First, HA binds to the cell receptor, sialic acid. After internalization of viruses by endocytosis, HA undergoes a drastic conformational change induced by low pH. Neutralizing Abs have one of the following activities: prevention of the binding reaction between HA and sialic acid [2] and prevention of low-pH-induced conformational change of HA [3]. The former binds to the site near the sialic acid-binding pocket on the globular head in HA1, and the latter binds to the stem region formed mainly by HA2. Since the dominant immune response is the first type and mutations can be easily introduced into the target sites without losing the receptor-binding activity, variant viruses that have acquired resistance to these Abs become dominant and cause annual epidemics. Historically, it was long believed that all effective neutralizing Abs are the first type; therefore, vaccine strains should be changed almost every year to remain effective. As long as the vaccine strain is a good match to the circulating virus, vaccination is effective for preventing virus infection.

The mode of response against influenza virus infection is very heterogeneous among the human population. For example, many people who have experienced several influenza infections in their childhood have not experienced additional influenza infections, even without vaccination. There has been no report clearly showing what kinds of ability such flu-resistant people obtained through repetitive influenza infections in their childhood. In 2009, a swine-origin H1N1 influenza virus (S-OIV) emerged and spread rapidly among the human population, resulting in its classification as the first pandemic of the 21st century [4]. It has been generally believed that humans lack immunity to the newly appearing influenza virus at the outbreak of a pandemic because they are naive to the virus. In the case of S-OIV, however, long-lived memory B cells that produce broadly neutralizing Abs not only against seasonal H1N1 viruses but also S-OIV are found in many elderly individuals [5–7].

To analyze the total repertoire of neutralizing Abs against influenza viruses in humans, we developed the following exper-

imental strategy in our previous study [8]. A large number of B lymphocytes are collected by apheresis from a donor, and a huge Ab library is constructed by using phage-display technology. The library is screened with formalin-treated virus particles. The clones that have both binding activity and neutralizing activity to the virus are isolated and their strain specificity is extensively characterized. Although only B lymphocytes that are circulating in peripheral blood are analyzed in our experimental system, the results appeared to represent the total repertoire of neutralizing Abs formed in the donor's body [8]. In the present study, we applied this strategy to the analyses of Abs that are present in the human body before and after vaccination with a newly appearing kind of virus. Since S-IOV newly emerged, the results should reflect only the effects of vaccination without the effects of a natural infection with living S-IOV.

Materials and Methods

Viruses

The following influenza viruses were used in this study. A/H1N1pdm: A/California/7/2009pdm (Cal09), A/Suita/1/2009pdm (Sui09); A/H1N1: A/New Caledonia/20/1999 (NC99), A/Solomon Islands/3/2006 (SI06), A/Brisbane/59/2007(Bri07). A/H3N2: A/Panama/2007/1999. A/H5N1: A/Indonesia/5/2005/PR8-IBCDC-RG2. Abbreviations for the strains are shown in parentheses.

Ethics statement

Ethical approval was granted by the Research Ethics Committee of Fujita Health University. Signed informed consent was obtained from the blood donor.

Construction of Ab library

Phage Ab libraries were constructed as described previously [8]. Briefly, mononuclear cells from a donor born in 1947 were collected by apheresis from the equivalent of 3 litters of blood before and after vaccination. The cells included 8.0×10^8 B lymphocytes (before vaccination) and 1.2×10^9 B lymphocytes (after vaccination). Large combinatorial Ab libraries were constructed by using the phage-display method as described previously [9]. The size of the libraries were: before vaccination, 1.6×10^9 clones for heavy (H) chain, 2.0×10^9 clones for light (L) chain and 1.4×10^{10} clones for Fab; after vaccination, 3.2×10^9 clones for H chain, 1.3×10^9 clones for L chain and 2.6×10^{10} clones for Fab.

Screening of the library

Phages bound to virus particles were selected by a panning method as described previously [10]. In brief, formalin-treated virus particles of Cal09 or Bri07 strains were used as antigens (Ags) in the screenings. After two and three pannings, *E. coli* (DH12S) cells were infected with the eluted phages and spread onto the LB plates containing 100 μg/ml ampicillin and 0.2% glucose. *E. coli* colonies harboring phagemid were picked up and grown in 2× YT medium containing 100 μg/ml ampicillin, 0.05% glucose and 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C overnight. During growth of *E. coli*, the Fab-cp3 form of Ab was secreted into the medium [11]. The culture supernatants containing Fab-cp3 molecules were subjected to enzyme-linked immunosorbent assay (ELISA) against H1N1 virus used as Ag in the screening and H3N2 virus. Clones that bound only to H1 were selected and subjected to further analyses.

ELISA

Formalin-treated virus particles were coated onto 96 well Maxisorp immunoplates (Nunc), and Fab-cp3 Ab in the supernatant of *E. coli* culture was added to each well. After incubation with rabbit anti-cp3 Ab (MBL), the wells were further incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L chain; MBL). Then, HRP substrate (OPD; Wako) was added to each well, and the color of the sample was developed. After stopping the peroxidase reaction by adding H_2SO_4, the absorbance of the sample at 492 nm was measured.

Sequence analysis

The nucleotide sequences of V_H fragments of isolated Ab clones were determined by using GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter) and a CEQ2000 DNA Analysis System (Beckman Coulter). The T7ETZ (5′-TAATACGACTCACTATAGGG-3′) was used as the V_H sequencing primer.

Virus neutralization test

For measurement of virus neutralizing activity, a focus reduction assay [12] was performed by using the single cycling (VN) or multiple cycling (M-VN) method. Two hundred or 500 μg/ml of Fab-PP Abs (P denotes a single Fc-binding domain of protein A) or two-fold serial dilutions of serum were mixed with an equal volume of 100 FFU of influenza virus and applied to MDCK cells in a 96-well plate. In the VN method, after incubation with the mixture, the cells were washed with serum-free MEM and cultured in MEM containing 0.4% BSA at 37°C for 15 h. In the M-VN method, after incubation, MEM containing 0.4% BSA, 5 μg/ml of acetylated trypsin, and 0.5% methyl cellulose of equal volume to the mixture was further added to the cells without removing the mixture and the cells were incubated at 37°C for 28 h. Then, the cells were fixed with ethanol and stained with PAP (peroxidase and anti-peroxidase) complex. The number of foci containing one and more cells (VN method) or four and more cells (M-VN method) was counted. The results were indicated as the focus reduction rate (%) for Fab-PP Ab or the reciprocal of the highest dilution of serum to show 50% focus reduction rate for serum.

Hemagglutination inhibition (HI) assay

The HI test was performed as described previously [12]. In brief, serial dilutions of 160 μg/ml of purified Fab-PP or donor's serum in PBS were prepared. Serial dilutions of Fab-PP or serum were preincubated with 4 HA units of virus per well. Guinea pig red blood cells (0.75%) in PBS were added to each well, and the plate was incubated at room temperature for 30–60 min. The results were shown as the lowest concentration (μg/ml) of Fab-PP Ab or the reciprocal of the highest dilution of serum to inhibit hemagglutination.

Competition ELISA

Competition ELISA was performed by using the Fab-PP form of Ab [13] or C179 [3] for detection of the binding activity to virus particles and the Fab-cp3 form of Ab as a competitor. Fab-cp3 molecules in the supernatant of *E. coli* culture were concentrated 20-fold before use. Formalin-inactivated virus particles were coated onto a 96 well Maxisorp immunoplate. A total of 50 μl of Fab-PP at an optimized concentration was mixed with 50 μl of 20-fold concentrated Fab-cp3 and the mixture was added to a virus-coated well. Then, peroxidase-conjugated rabbit anti-streptavidin Ab was added to each well as a secondary Ab. When C179

at the final concentration of 0.25 μg/ml was used for detection of the binding activity to virus strain, each well was incubated with peroxidase-conjugated goat anti-mouse IgG (H+L chain; MBL) as a secondary Ab. Then, HRP substrate (OPD; Wako) was added to each well, and the color of the sample was developed. After stopping the peroxidase reaction by adding H_2SO_4, the absorbance at 492 nm was measured.

Preparation of mAb specifically bound to 1-69Abs

Five kinds of Fab-PP form of 1-69Ab, F081-007, F083-103, F083-115, F083-305, and F083-311 were purified and injected into the foot-pad of Balb/c mice with Freund's complete adjuvant once and 11 days later without adjuvant once more. Three days later after the second injection, the lymphocytes were isolated from the inguinal lymph node and fused with the myeloma line P3-X63AG8.653. After cloning of hybridomas, the culture media were screened using the above 1-69Abs and various kinds of Abs that do not utilize *1-69* V_H gene by ELISA. The clones that bind to multiple kinds of 1-69Ab but do not bind to any of non 1-69Abs were selected. This part of the work was performed by MAB Institute Inc. (Sapporo, Japan).

Determination of the serum concentration of IgG that uses the V_H*1-69* germline gene

A human IgG ELISA Quantitation set (Bethyl Laboratories, Inc.) was used, with slight modification. K1-18 Ab for detection of IgG using V_H *1-69* germline gene and affinity-purified Human IgG Coating Antibody for standard curve were coated onto a 96-well Maxisorp immuonoplate. Serum or human reference serum (for standard curve) was added to the assigned well. After incubation with HRP-conjugated Human IgG Detection Antibody, TMB substrate was added to each well. The peroxidase reaction was stopped by adding H_2SO_4, and the absorbance of sample at 450 nm was measured. The concentration of IgG bound to K1-18 Ab was calculated from the standard curve of human reference serum.

Virus-neutralizing activity of serum in the presence of K1-18 Ab

The virus neutralization test described above was modified. In brief, serum treated with receptor destroying enzyme (RDE) was diluted at 1:10 or 1:20 in serum-free medium and mixed with an equal volume of 800 or 1,600 μg/ml of K1-18 Ab. After incubation, two-fold serial dilutions of the mixture were mixed with an equal volume of 100 FFU of influenza virus and applied to MDCK cells in a 96-well plate. After incubation, the cells were fixed with ethanol and stained with PAP (peroxidase and anti-peroxidase) complex. The reciprocal of the highest dilution of serum to show a 50% focus reduction rate was indicated as virus neutralizing activity.

Binding activity of K1-18 Ab to 1-69Abs

Ten μg/ml of Fab-PP form of 1-69Ab was coated onto 96 well Maxisorp immunoplates (Nunc), and K1-18 Ab was added to each well. After incubation with peroxidase-conjugated goat anti-mouse IgG (H+L chain, MBL), HRP substrate (OPD; Wako) was added to each well. After stopping the peroxidase reaction by adding H_2SO_4, the absorbance of the sample at 492 nm was measured.

Binding activity of Abs to virus particles under the presence of K1-18 Ab

Formalin-inactivated virus particles (Bri07) were coated onto a 96 well Maxisorp immunoplate. Fab-cp3 molecule that was diluted from 20-hold concentrated solution at an optimized concentration was mixed with an equal volume of 200 or 400 μg/ml of K1-18 Ab and incubated at 37°C for 1 h. The mixture was added to virus coated well and the wells were incubated at 37°C for 1 h. After incubation with rabbit anti-cp3 Ab (MBL), the wells were further incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L chain; MBL). Then, HRP substrate (OPD; Wako) was added to each well, and the color of the sample was developed. After stopping the peroxidase reaction by adding H_2SO_4, the absorbance of the sample at 492 nm was measured.

Nucleotide sequence accession number

The nucleotide sequences of the heavy and light chains of isolated Abs have been deposited in the DDBJ database (accession numbers AB826498 to AB826560 for the heavy chains and AB845357–AB845391 for the light chains).

Results

Two types of virus-neutralizing Abs with different characteristics

We examined the total repertoire of Abs induced by vaccination with S-IOV. The blood donor in this study was born in 1947 and was infected with influenza several times in his childhood (possibly by H1N1 and H2N2) and in 1968 (probably by H3N2). For 41 years afterwards, he did not contract influenza, and he was never vaccinated against influenza. The schedule of vaccination and blood collection is shown in Figure 1. The vaccination and blood collection were performed during the period from late October to mid-December 2009, and the examinee did not have an opportunity to be naturally infected with S-IOV. By using B lymphocytes collected before and after vaccination, two large Ab libraries were constructed and subjected to screenings by panning with pandemic H1N1 (Cal09) and seasonal H1N1 (Bri07) virus particles. After the second and the third rounds of panning with the viruses, 120 clones were isolated. The clones that bound to the H1N1 virus particle that had been used for screenings were further analyzed. The clones that bound to both H1N1 and H3N2 with equal strength were excluded, since it is likely that they were anti-NP Abs [8]. The HA-binding activity of respective clones was further confirmed by Western blot of virus proteins used as Ags in the screenings. Among the 240 clones isolated in respective screenings, the number of clones that were judged to be anti-HA Abs is as follows: screening 1 (after vaccination, with pandemic virus), 105 clones; screening 2 (before vaccination, with pandemic virus), 3 clones; screening 3 (after vaccination, with seasonal virus), 58 clones; and screening 4 (before vaccination, with seasonal virus), 16 clones. V_H nucleotide sequences of all these clones were determined. Comparison of the amino acid sequences revealed that these 182 clones were composed of 96 unique monoclonal Abs (mAbs). Based on sequence similarities of V_H fragments, the 96 clones were classified into 63 groups. The amino acid sequence of the V_H fragment and the nucleotide sequence of complementarity-determining region 3 (CDR3) of all the clones are available in Figures S1 and S2.

The HA binding activity, hemagglutination inhibition (HI) activity, and the virus-neutralizing activity [12] of representative clones of the 63 groups were examined. As shown in Figure 2, all of the representative clones were classified into two types with no exceptions. Clones classified as the first type bind only to the pandemic H1N1 and not to the seasonal H1N1. All of these clones show HI activity and were isolated only from the screening 1. Clones classified as the second type bind not only to the pandemic H1N1 but also to all of the seasonal H1N1s. While they do not

Figure 1. Schedule of vaccination and blood collection. In 2009, a donor born in 1947 was vaccinated with A/California/7/2009 pandemic vaccine strain two times (on November 2 and 16). Blood was collected from the donor two times before vaccination (on October 30 and November 2), once after 1st vaccination (on November 9) and 5 times after 2nd vaccination (on November 17, 23, 30, December 7, and 14). Large phage Ab libraries were prepared from blood collected on October 30 (before vaccination) and December 14 (after vaccination).
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show HI activity, most of them neutralize not only H1N1 viruses but also H5N1 virus. Judging from the low frequency of mutations, such as 0% to 5%, the majority of the first type of clones should be the products of B cells that have been newly induced through vaccination. On the other hand, all of the second type of clones (except for a few) should correspond to the products of long-lived memory B cells that were established before vaccination, judging from the high frequency of mutations, such as 10% to 15%. Furthermore, they all utilized the *1-69* V_H gene, with the exception of three clones.

Newly appearing Abs after vaccination

Since virtually all the first-type Abs showed HI activity, it is likely that their epitopes are located in the region surrounding the sialic acid-binding pocket. To systematically examine the relative position of the epitopes recognized by these clones, we adopted the competition method that we used in a previous study [14]. While respective mAbs are initially prepared as the Fab-cp3 form after the screening of libraries, they can be easily changed to the Fab-PP form (P denotes a single Fc-binding domain of protein A) in our vector construct [13]. If the epitope recognized by clone A is overlapped with that by clone B, the binding of the Fab-PP form of clone A to HA is largely disturbed by the presence of a large amount of Fab-cp3 form of clone B. Based on this principle, the competition study was performed by using 17 clones selected from the first-type Abs listed in Figure 2. As shown in Figure 3, 14 of the 17 clones competed well against one another for the binding to HA. In the case of three clones, F082-317, F082-254 and F082-022, the degree of disturbance for the binding was low. These observations are consistent with other data. Only F082-022 showed very low HI activity (titer 160 μg/ml). F082-254 binds to HA of not only pandemic virus but also seasonal virus and neutralizes both viruses. Furthermore, a high frequency of mutation, such as 18%, was observed in this clone. In the case of F082-317, the binding activity to HA is high but the neutralizing activity is relatively low.

Based on these observations, we concluded that the sialic acid-binding pocket and its surrounding region on HA are immunogenically very potent and that virtually all of the B cells whose growth is newly induced and expanded by vaccination produce Abs that recognize these regions. We also concluded that when formalin-treated virus particles that are not alive are used as vaccine, B cells producing Abs that are able to bind to HA but not able to neutralize virus are not induced at a substantial level. The second conclusion suggests that the non-neutralizing epitope on HA, even if it exists, is immunologically impotent.

Abs encoded by long-lived memory B cells

While the second type of clones is classified into 32 groups, they all utilized the *1-69* V_H gene, with the exception of three clones. Furthermore, the majority of these Abs were able to neutralize not only all H1N1 viruses but also the H5N1 virus. These observations suggest that the epitopes recognized by these clones should be located near the epitope recognized by C179, as shown by several groups [15,16]. We examined whether the binding of C179 to HA is disturbed by a large excess of the Fab-cp3 form of these clones. As shown in Figure 4, all of the clones including the three that did not utilize the *1-69* V_H gene disturbed the binding of C179 to HA. Thus, the second type of clones should bind to the membrane-proximal stem of HA in the same or similar way as other Abs using the *1-69* V_H gene (1-69Abs), such as CR6261 and F10, which have already been described by other groups [15,16]. Figure 4 also indicates that four clones, F081-268, F082-243, F082-237, and F083-373, showed weaker disturbance activity than the others. The data shown in Figure 2 indicate that F081-268 had strong HA-binding activity, but the neutralizing activity was weak. The other three clones showed relatively weak binding and neutralizing activities. Thus, while all of the epitopes recognized by the second type of Abs are not exactly the same as that recognized by C179, we did not find any clone that binds to an epitope totally different from that recognized by C179. This result suggests that this epitope has been shared and stably kept among group 1 viruses. Even if the immunogenicity of this epitope is weak, once humans acquire B cells that produce Abs that recognize this epitope, the B cells could remain in the body as memory cells for a long period and become major players against group 1 viruses. Interestingly, one clone (F083-115) that utilized the *1-69* V_H gene is able to neutralize even H3N2 virus.

Presence of Abs secreted into the serum

Two biological activities, HI activity and virus-neutralizing activity, against three viral strains, H1N1 pandemic virus, seasonal H1N1 virus and H5N1 virus, were measured in eight samples of sera collected from the examinee on different days, as shown in Figure 1. As indicated in Table 1, in the case of HI, the activity against seasonal H1N1 and H5N1 viruses was not detected in any

Gr.	Number: Unique clone	Number: Isolated clone	Number of isolated clone from each library: Cal09 Before	Cal09 After	Bri07 Before	Bri07 After	Representative clone	Germline gene	Identity (%)	Amino acid sequences for CDR3 region of clone	HI titer (μg/ml): Bri07	HI titer (μg/ml): Cal09	Binding activity: H1N1 NC99	H1N1 SI06	H1N1 Bri07	H1N1 Cal09	H3N2	PBS	Focus reduction activity (%): H1N1 Bri07 250	H1N1 Bri07 100	H1N1 Sui09 250	H1N1 Sui09 100	H5N1 250	H5N1 100	H3N2 250	H3N2 100
5	1	2		2			F082-124	1-18*01	94.3	DTTVTNEEINFYYGMDV	-	20	0.09	0.09	0.16	2.82	0.13	0.22								
4	5	42		42			F082-001	1-18*01	97.7	DTEVTNEEINFYYGMDV	-	10	0.09	0.08	0.15	3.33	0.13	0.23	23.8	-8.2	100	100				
7	1	1		1			F082-289	1-18*01	97.7	DTEVTSEEINFYYGMDV	-	10	0.11	0.09	0.20	2.99	0.15	0.28								
6	2	2		2			F082-355	1-18*01	98.9	DTTVTSEEINFYQGMDV	-	20	0.09	0.09	0.14	2.86	0.13	0.22								
8	6	6		6			F082-109	1-46*01	96.6	EFGANGEDIYFYHGMDV	-	10	0.15	0.08	0.14	3.10	0.23	0.20	26.2	6.6	86.5	54.5				
9	1	1		1			F082-022	2-5*09	98.9	SIGGYDGEGIFYNHYGMDV	-	160	0.09	0.08	0.14	2.05	0.12	0.19	45.9	26.2	17.4	15.7				
10	1	1		1			F082-380	3-7*01	96.6	DEWFGELGSSGMDV	-	20	0.14	0.11	0.30	3.19	0.40	0.47	18.9	11.5	100	94.9				
11	1	1		1			F082-290	3-9*01	95.5	DFAGEGHGSGSVDY	-	20	0.10	0.10	0.20	3.06	0.20	0.26	58.2	41.0	74.7	46.1				
12	1	1		1			F082-122	3-21*01	86.4	SATSYRDYLDRDFFYYALDV	-	10	0.21	0.13	0.38	3.39	0.16	0.26	60.7	68.0	69.7	49.4				
13	1	1		1			F082-262	3-21*01	86.4	SATSYRDYLDRDFYYYALDV	-	10	0.10	0.08	0.15	3.30	0.15	0.22								
14	1	2		2			F082-105	3-30*01	96.6	DHLNSEIVATITGFLDY	-	5	0.10	0.09	0.18	3.32	0.14	0.26	13.9	6.6	100	100				
15	1	1		1			F082-393	3-30*01	93.2	DKLNSEMVATITGFLDY	-	10	0.08	0.08	0.15	3.03	0.12	0.22								
16	1	1		1			F082-392	3-30*01	94.3	DKLNSEMVATITGFMDY	-	10	0.09	0.08	0.13	3.18	0.12	0.16								
20	1	1		1			F082-108	3-30*03	95.5	DKLNSDEVTTITGFLDY	-	2.5	0.13	0.13	0.24	3.30	0.15	0.32								
17	1	1		1			F082-102	3-30*01	90.9	DNLNSELVATITGFLDH	-	5	0.09	0.10	0.18	3.47	0.15	0.25								
18	1	1		1			F082-376	3-30*01	97.7	DNLNSDEVATISGFLDY	-	20	0.09	0.09	0.15	3.15	0.13	0.21								
19	1	1		1			F082-005	3-30*01	95.5	DYLNSEMVATITGFLDS	-	40	0.10	0.09	0.14	2.76	0.12	0.15								
21	2	7		7			F082-104	3-30*01	94.3	EPSNTEDIRGIEGVFDY	-	2.5	0.14	0.13	0.23	3.62	0.20	0.33	-0.8	18.9	93.3	84.8				
22	1	1		1			F082-254	3-30*02	81.8	DAYSSGDTYYYGLDV	-	10	0.91	0.55	0.48	1.84	0.12	0.16	100	100	100	100				
23	2	2		2			F082-003	3-43*02	95.5	DRGTGEQIAVVTALIDY	-	2.5	0.08	0.08	0.15	3.18	0.14	0.22	77.9	38.5	100	98.3				
24	1	2		2			F082-332	4-39*01	95.5	HGYGDYVGYFDY	-	20	0.11	0.10	0.24	3.07	0.16	0.35	33.6	1.6	100	100				
25	4	6		6			F082-208	4-59*01	96.6	VLRWLGEEDADAFDI	-	10	0.10	0.08	0.16	3.15	0.14	0.24	1.6	11.5	32.6	25.8				
26	1	1		1			F082-341	4-59*01	82.8	GFGMVGDTVDDLYNGMDV	-	10	0.11	0.09	0.20	3.29	0.16	0.31	21.3	16.4	100	100				
27	1	2		2			F082-351	4-59*01	97.7	VQRPYGDYAAGAFDI	-	10	0.10	0.10	0.20	3.38	0.16	0.27	36.1	-15.6	100	96.6				
28	1	1		1			F082-120	4-59*01	94.3	VQRPYGDYITGAFDI	-	10	0.11	0.11	0.14	2.69	0.12	0.18								
29	1	1		1			F082-352	5-51*01	97.7	RTWYYDGSGPDPSRDAFDI	-	10	0.10	0.10	0.20	3.24	0.14	0.25	18.9	6.6	100	100				
30	1	1		1			F082-316	7-4-1*02	100	DLGNGEDIAVQPGTIGVDY	-	5	0.10	0.08	0.16	3.29	0.14	0.23								
31	1	2		2			F082-020	7-4-1*02	100	DLGNGEDIAVQPGTTGVDY	-	5	0.10	0.10	0.19	3.55	0.59	0.30	48.4	32.0	98.3	98.3	6.6	10.5	-2.7	2.7
32	1	1		1			F082-359	7-4-1*02	97.7	DLGNGEDIVVQPATIGVDY	-	5	0.18	0.18	0.22	2.54	0.25	0.24								
33	3	3		3			F082-317	7-4-1*02	96.6	GTEVTTEEIYFYYGMDV	-	20	0.16	0.17	0.28	3.32	0.19	0.35	-0.8	16.4	71.3	36.0				
34	1	1		1			F082-248	7-4-1*02	96.6	GTEVTTEEINFYYGMDV	-	20	0.09	0.08	0.17	2.30	0.14	0.17								
46	1	1				1	F083-116	1-3*01	87.5	AEKWLADYFYYFGMDV	>160	>160	2.93	3.13	2.80	0.36	0.17	0.17	93.0	81.3	54.5	42.7	34.2	21.1	5.6	-11.9
66	1	1				1	F083-341	1-46*01	89.8	DREESLFAGAIYNYYYDMDV	>160	80	2.57	2.66	2.43	1.41	0.29	0.26	95.3	85.9	100	94.9	98.7	90.8	2.1	-18.4
45	1	1			1		F081-268	4-34*01	97.7	KGGAKLLYFDWLASAFDI	>80	>160	2.49	2.27	1.89	1.18	0.23	0.21	nd	41.4	nd	57.9	nd	63.2	nd	-19.7
1	2	5	1		4		F080-227	1-69*01	92.2	GPNYYENFFDY	>160	>160	3.15	3.16	3.10	2.92	0.14	0.15	98.0	98.0	100	100	52.9	67.6	-0.7	-0.7
2	4	12	1	1	1	9	F082-220	1-69*01	88.6	GPNYYESYFDY	>160	>160	3.37	3.37	3.15	2.72	0.16	0.24	100	100	97.7	95.3	97.1	90.6	34.2	3.4
36	2	2		1		1	F082-243	1-69*01	88.6	GPNYYENYFDF	>160	>160	0.96	1.21	1.05	0.80	0.13	0.11	94.1	84.2	85.9	97.7	52.9	50.6	-2.7	7.5
49	1	1				1	F083-115	1-69*01	85.2	GPNYYESYLDF	>160	>160	2.50	2.49	2.23	1.83	0.27	0.28	100	96.1	83.6	93.0	89.4	91.8	91.8	54.8
47	2	7				7	F083-308	1-69*01	89.8	GPNYFESYFDN	>160	>160	1.79	1.92	1.82	1.21	0.16	0.14	100	100	100	97.7	49.4	29.4	-6.8	1.4
48	2	2				2	F083-389	1-69*01	79.5	GPNYYETYLDN	>160	>160	2.72	2.80	2.83	2.49	0.14	0.15	100	98.0	100	-18.3	60.8	54.6	19.9	-0.7
3	2	2	1		1		F081-281	1-69*01	85.6	GPHYYESHLDY	>160	>160	2.86	2.86	2.98	2.67	0.18	0.24	100	98.0	100	100	85.9	81.8	-8.9	11.6
35	1	1		1			F082-117	1-69*01	80.7	GPHYYVSYFDS	>160	>160	3.35	3.37	3.26	3.11	0.15	0.12	100	100	100	100	95.9	94.1	-4.8	3.4
39	2	4		2		2	F082-237	1-69*01	86.4	GNTYYSSYFDQ	>160	>160	1.94	1.89	1.95	2.62	0.13	0.14	64.5	48.7	100	95.3	41.2	31.2	15.8	-2.7
54	1	3				3	F083-354	1-69*01	87.5	GSTYYSSYFDQ	>160	>160	3.05	2.92	3.01	2.54	0.16	0.18	100	96.1	94.2	100	80.7	66.4	-11.0	-0.7
37	2	6		1		5	F083-311	1-69*01	85.2	SGTYYYSYFDS	>160	>160	2.59	2.70	2.64	1.82	0.15	0.17	100	100	100	93.0	97.1	90.0	5.5	-8.9
41	2	4			3	1	F081-107	1-69*01	81.8	SGTYYVSYLDS	>160	>160	2.92	3.02	2.94	2.66	0.15	0.19	92.1	96.1	100	100	85.9	81.2	40.4	3.4
52	1	1				1	F083-328	1-69*01	80.7	SGTYYVSFFDY	>160	>160	2.66	2.56	2.43	2.02	0.22	0.23	100	100	100	100	93.2	86.9	5.5	9.6
40	3	3			2	1	F081-264	1-69*01	84.1	SGSYYPDYFQY	>160	>160	3.10	3.26	3.07	3.09	0.19	0.40	100	98.0	100	100	97.6	94.1	-6.8	-8.9
42	2	6			1	5	F083-249	1-69*01	89.8	SPTYYPGALDM	>160	>160	3.13	3.18	3.02	2.88	0.15	0.20	100	100	97.7	100	98.2	96.5	34.2	15.8
55	1	1				1	F083-305	1-69*01	86.4	APLIYNWYFDL	>160	>160	3.07	3.07	2.88	1.23	0.15	0.19	96.1	96.1	69.5	88.3	22.4	2.4	-6.8	-17.1
56	1	1				1	F083-318	1-69*01	83.0	APLIYNWYYDL	>160	>160	3.01	2.90	2.85	0.71	0.16	0.27	92.1	84.2	27.9	13.5	1.7	-9.5	7.5	7.5
57	1	2				2	F083-103	1-69*01	83.0	HPTYHYGSAMDY	>160	>160	2.43	2.61	2.39	1.82	0.16	0.19	92.1	84.2	83.6	60.2	61.2	30.0	13.7	11.6
58	1	2				2	F083-307	1-69*01	89.8	HPTYYFGSAMEY	>160	>160	3.30	3.32	3.04	2.37	0.19	0.28	100	100	64.8	76.6	31.8	28.8	11.6	-6.8
60	1	1				1	F083-373	1-69*01	83.0	HPTYYYGSPMDY	>160	>160	1.39	2.40	1.31	0.17	0.13	0.18	74.3	70.4	7.7	1.9	15.4	-3.3	-21.2	-8.9
59	1	1				1	F083-351	1-69*01	83.0	HPMYHYGSAMDY	>160	>160	3.21	3.20	3.09	2.62	0.21	0.30	100	100	85.6	88.5	5.4	-2.7	-6.8	-6.8
61	1	2				2	F083-302	1-69*01	80.7	HSGYHLIGYFDS	>160	>160	1.92	2.08	2.00	1.55	0.23	0.11	98.0	98.0	100	100	85.9	72.9	-11.0	3.4
62	2	2				2	F083-349	1-69*01	85.2	EEGYYYGSGPLDS	>160	>160	3.17	3.08	2.93	2.79	0.15	0.15	92.1	92.1	91.3	97.1	63.9	40.2	-8.9	13.7
63	1	1				1	F083-336	1-69*01	89.8	NSGYHISGFYLDY	>160	>160	1.81	1.91	1.89	1.56	0.15	0.13	100	100	100	100	100	98.8	-8.9	-11.0
38	2	4		1		3	F082-338	1-69*01	86.4	SLGYHTQYNGMDV	>160	>160	3.01	2.88	2.93	2.82	0.13	0.12	100	100	97.7	100	85.3	67.1	-0.7	-2.7
43	1	2			2		F081-007	1-69*01	85.2	HPTYHFDKSGYRFDS	>160	>160	2.84	2.82	2.72	2.33	0.14	0.13	100	100	100	100	98.8	97.6	13.7	11.6
64	1	1				1	F083-301	1-69*01	87.5	SRGYSFGYGTDYFDY	>160	>160	1.34	1.51	1.44	0.46	0.16	0.13	92.1	86.2	81.3	83.6	47.1	40.6	7.5	-2.7
65	1	1				1	F083-366	1-69*01	84.1	NYYGSGTYFNDAFDI	>160	>160	2.93	2.81	2.84	1.13	0.18	0.26	98.0	96.1	19.2	13.5	-3.9	-0.8	-6.8	-13.0
44	1	1			1		F081-008	1-69*02	92.3	YQSSDYYNSEYFQH	>160	>160	3.28	3.17	2.89	0.43	0.19	0.22	100	100	97.7	95.3	56.5	48.8	15.8	3.4

Figure 2. Activities of representative clones classified into 63 groups. The first three columns contain the numbers of clones isolated by the screenings. Germline genes were identified by comparing the amino acid sequence of V_H of the representative clone with the sequences of all of the germline V_H genes, and the identity (%) is indicated. The amino acid sequence of CDR3 is shown. The binding activity to four H1N1 (NC99, SI06, Bri07 and Cal09) and one H3N2 virus particles was examined by enzyme-linked immunosorbent assay (ELISA). Absorbance at 492 nm is shown as follows: ≥1.00 (red), 0.50–0.99 (orange), and 0.10–0.49 (yellow). The virus neutralizing activity of 100 or 250 μg/ml Fab-PP Ab against H1N1, H5N1, and H3N2 viruses was examined by the focus reduction test. The reduction rate is shown as a percentage as follows: ≥60% (dark blue), 40%–60% (blue), and 20%–40% (light blue). The HI activity of Fab-PP Ab was measured by using two H1N1 (Bri07 and Cal09) viruses. The lowest concentration (μg/ml) of Fab-PP Abs to inhibit hemagglutination is shown.
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of the sera. HI activity against H1N1 pandemic virus started to appear 2 weeks after the first vaccination. In the case of virus-neutralizing activity measured by standard focus reduction assay, the activity against H1N1 pandemic virus already started to increase 1 week after vaccination and reached a plateau 2 weeks after vaccination. Against seasonal H1N1 virus, virus-neutralizing activity was detected even before vaccination and increased 1 week after vaccination. Against H5N1 virus, virus-neutralizing activity

Fab-pp

Fab-cp3	F082-001	F082-332	F082-341	F082-020	F082-351	F082-122	F082-104	F082-208	F082-109	F082-352	F082-003	F082-105	F082-290	F082-380	F082-317	F082-254	F082-022
F082-001	107.0	87.7	96.0	100.1	99.6	96.5	96.7	96.1	97.2	96.0	98.7	99.0	81.3	96.5	80.0	96.7	53.5
F082-332	89.1	89.8	92.9	94.2	84.6	93.4	88.4	81.8	92.6	93.5	93.2	93.5	95.5	91.9	96.7	92.6	69.4
F082-341	90.7	90.1	99.0	97.7	95.6	94.7	94.3	102.1	96.2	97.6	99.7	100.3	92.6	96.1	99.9	95.6	72.7
F082-020	100.1	100.0	100.2	100.3	102.5	98.2	97.1	108.9	99.8	97.7	99.4	99.7	92.8	96.5	100.1	60.6	64.3
F082-351	91.5	103.1	101.2	104.8	99.9	96.1	100.7	107.8	101.6	99.2	102.5	102.2	95.9	100.4	100.7	94.9	68.1
F082-122	72.4	84.9	87.5	86.5	71.4	93.5	71.2	71.1	86.1	89.7	87.7	84.2	87.9	82.7	94.1	94.3	63.5
F082-104	100.7	92.2	97.8	97.6	99.2	89.9	96.7	98.7	72.6	97.7	97.9	97.2	95.2	96.6	98.6	85.6	72.5
F082-208	87.7	94.3	94.7	96.1	91.9	95.6	95.8	96.0	96.9	96.6	96.9	97.1	93.6	94.9	97.1	91.3	69.8
F082-109	92.4	98.4	94.3	97.1	93.2	94.3	94.2	103.3	96.5	96.6	97.0	97.3	86.9	96.1	99.3	92.7	74.4
F082-352	93.0	101.8	98.4	99.5	88.6	92.3	96.0	92.7	97.2	96.5	98.3	98.9	94.1	94.5	97.3	91.7	67.5
F082-003	98.2	81.1	96.5	89.6	90.3	90.6	79.9	89.6	88.4	92.4	85.6	86.6	71.8	89.3	97.4	95.0	32.5
F082-105	93.6	99.0	99.1	96.0	101.1	95.8	95.3	102.6	98.1	97.9	98.0	97.7	89.7	97.2	99.3	97.8	60.3
F082-290	90.1	96.0	76.3	79.2	97.9	97.2	100.2	98.7	100.6	78.1	99.3	101.7	95.4	97.4	87.2	86.0	88.8
F082-380	91.8	85.4	95.1	92.0	79.6	78.1	86.6	79.8	91.9	88.0	89.7	90.6	79.4	85.3	94.1	79.8	67.2
F082-317	76.6	87.2	74.9	82.1	71.3	45.3	69.6	84.0	80.9	82.2	77.7	84.0	82.8	83.9	90.4	87.6	40.7
F082-254	79.6	81.0	72.7	50.1	63.8	67.8	39.3	28.7	44.4	48.9	57.3	62.8	67.2	57.2	63.7	84.6	34.3
F082-022	79.2	86.7	72.0	62.6	64.7	81.7	52.5	57.9	79.8	82.0	71.6	79.7	77.2	73.7	91.2	89.2	31.8
F008-009	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F033-367	13.7	13.6	18.1	16.0	-14.9	5.4	-2.4	-17.7	9.7	1.6	5.5	4.4	-2.1	-2.3	-7.7	5.6	11.1

Figure 3. Competitive inhibition of binding to HA among the Abs newly induced by vaccination. The binding activity of Fab-PP Ab (indicated at upper side) to Cal09 virus particles was measured by ELISA under the presence of a 10-times greater concentration of Fab-cp3 Ab (indicated at the left side). F008-009 and F033-367 are not anti-HA Abs and were used as controls. The binding inhibition was calculated as follows: the absorbance value under the presence of F008-009cp3 was used as 100% binding, and the degree of reduction in the absorbance value under the presence of Fab-cp3 of Ab was measured and shown as percent inhibition. Percent inhibition is shown as follows: ≥70% (white), 50%–70% (blue), 0%–50% (grey). The experiment was performed at least three times in duplicate.
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was not detected by the standard focus-reduction assay [12] but was detected by a more sensitive method that is described in Materials and Methods.

To prove that 1-69Abs are really responsible for neutralizing H1N1 pandemic virus, seasonal H1N1 virus and H5N1 virus, we prepared mAb that can specifically bind to 1-69Abs, that is, anti-idiotypic Ab. We expected that the region including two amino acids, isoleucine at the 53rd residue and phenylalanine at the 54th residue, in the V_H domain should be immunogenic in mice since the presence of two hydrophobic residues in CDR2 is very unique and found only in 1-69Ab of humans [17]. Furthermore, we presumed that the anti-idiotypic Ab may inhibit the binding of 1-69Ab to HA since these two amino acids are directly involved in the Ab/HA interaction [15,16]. We successfully isolated mAb K1-18 that can bind to around 80% of 1-69Abs listed in Figure 2 and can inhibit the binding of a half of the 1-69Abs to HA as indicated in Figure 5A and B.

By using K1-18 as a probe, the amount of the IgG form of 1-69Ab in serum was measured. The results indicated that it was present at a concentration of 4.58 μg/ml in the serum collected on October 30 (before vaccination) and increased after vaccination to an concentration of 11.24 μg/ml in the serum collected on December 14. Virus-neutralizing activity against pandemic and seasonal H1N1 viruses was measured in the presence of K1-18. In the case of H1N1 pandemic virus, K1-18 definitely inhibited the neutralizing activity, as indicated in Table 2. Against seasonal H1N1 virus, the inhibition was clearly observed, although not perfectly. Thus, we concluded that 1-69Abs were really present in

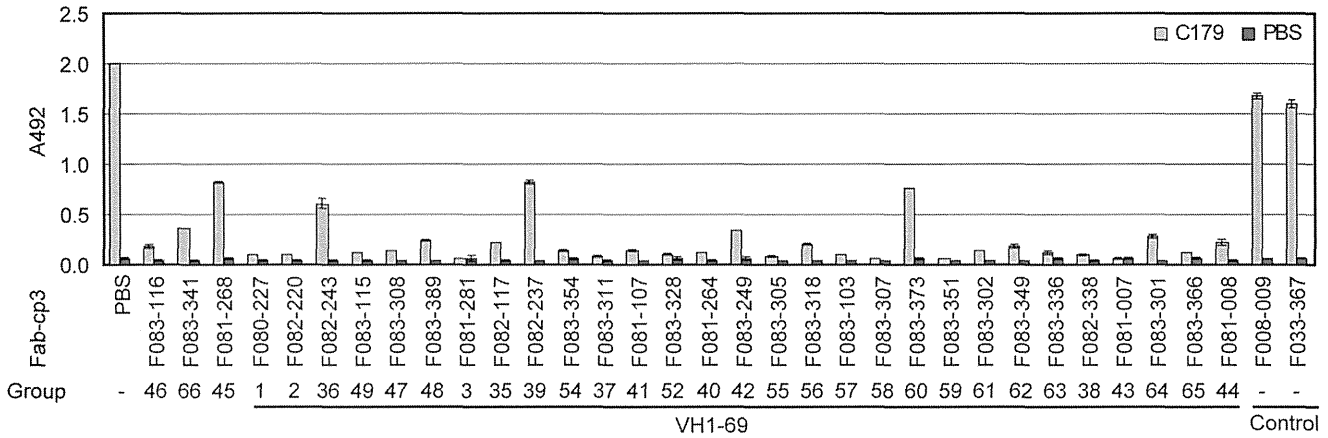

Figure 4. Inhibition of the binding of C179 to HA by type 2 Abs. Binding of C179 to Bri07 virus particles was examined under the presence of a 10-times greater concentration of various Fab-cp3 Abs by ELISA. F008-009 and F033-367 are not anti-HA Abs and were used as negative controls. The group number is indicated under the name of the clone. The experiment was performed two times in duplicate, and the error bars show standard deviation.
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the sera and functioned in the neutralization of H1N1 viruses. Furthermore, the data in Table 2 suggested that when two types of functionally different Abs co-exist, the first type prevents HA/receptor interaction and the second type prevents low-pH-induced conformational change of HA, and the virus-neutralizing activity synergistically increases.

Discussion

Historically, it has been believed that vaccines remain the most reliable method to control seasonal epidemics of influenza. However, the mode of response against influenza virus infection is very heterogeneous among the human population. Since we experience an outbreak of flu almost every year in Japan, most of the people may easily have opportunities to be infected by influenza viruses. Nevertheless, there are many people who appear to be resistant to the seasonal epidemics without vaccination. On the other hand, there are many people who are vaccinated almost every year but contract influenza quite often. In the present study we analyzed the repertoire of neutralizing Abs against H1N1 viruses in a flu-resistant person. We also tried to analyze the effects of vaccination with formalin-treated virus particles on the Ab repertoire under the conditions without effects of natural infection with live viruses. This experiment can be performed only at the outbreak of a pandemic. Since people are naive to the pandemic virus, we can perfectly exclude the possibility that the examinee has been naturally infected with the virus used as vaccine.

We obtained very simple results. Only two types of Abs were isolated with no exceptions. The first type is the products of B cells newly induced through vaccination and the second type is the products of long-lived memory B cells established before vaccination. While there should be more clones that we have overlooked in this screening, it is unlikely that we have specifically overlooked clones with characteristics very different from those listed in Figure 2. For example, Abs that bind to HA of seasonal H1N1 virus but not to HA of pandemic virus were not isolated in the screening 4 (before vaccination, with seasonal virus), and all of the 16 clones were the second type of Abs. Since the library used in this screening was constructed from B cells before vaccination,

Table 1. HI and virus neutralizing activity of serum against H1N1 and H5N1 virus strains.

	A/Suita/1/2009H1N1pdm		A/Brisbane/59/2007(H1N1)			A/Indonesia/5/2005(H5N1)		
Date of blood collection	**HI[a]**	**VN50[b]**	**HI**	**VN50**	**M-VN50[c]**	**HI**	**VN50**	**M-VN50**
2009.10.30[d]	<40	<40	<40	40	160	<40	<40	ND[e]
2009.11.02[d]	<20	40	<20	20	160	<20	<40	ND
2009.11.09	<20	320	<20	80	160	<20	<40	ND
2009.11.17	80	1280	<20	80	320	<20	<40	40
2009.11.23	40	1280	<20	80	320	<20	<40	40
2009.11.30	40	1280	<20	80	320	<20	<40	40
2009.12.07	40	1280	<20	80	320	<20	<40	40
2009.12.14	40	2560	<40	80	320	<40	<40	ND

[a]HI activity.
[b]Virus neutralizing activity detected by VN method.
[c]Virus neutralizing activity detected by M-VN method.
[d]Serums before vaccination.
[e]Not determined.
doi:10.1371/journal.pone.0087305.t001

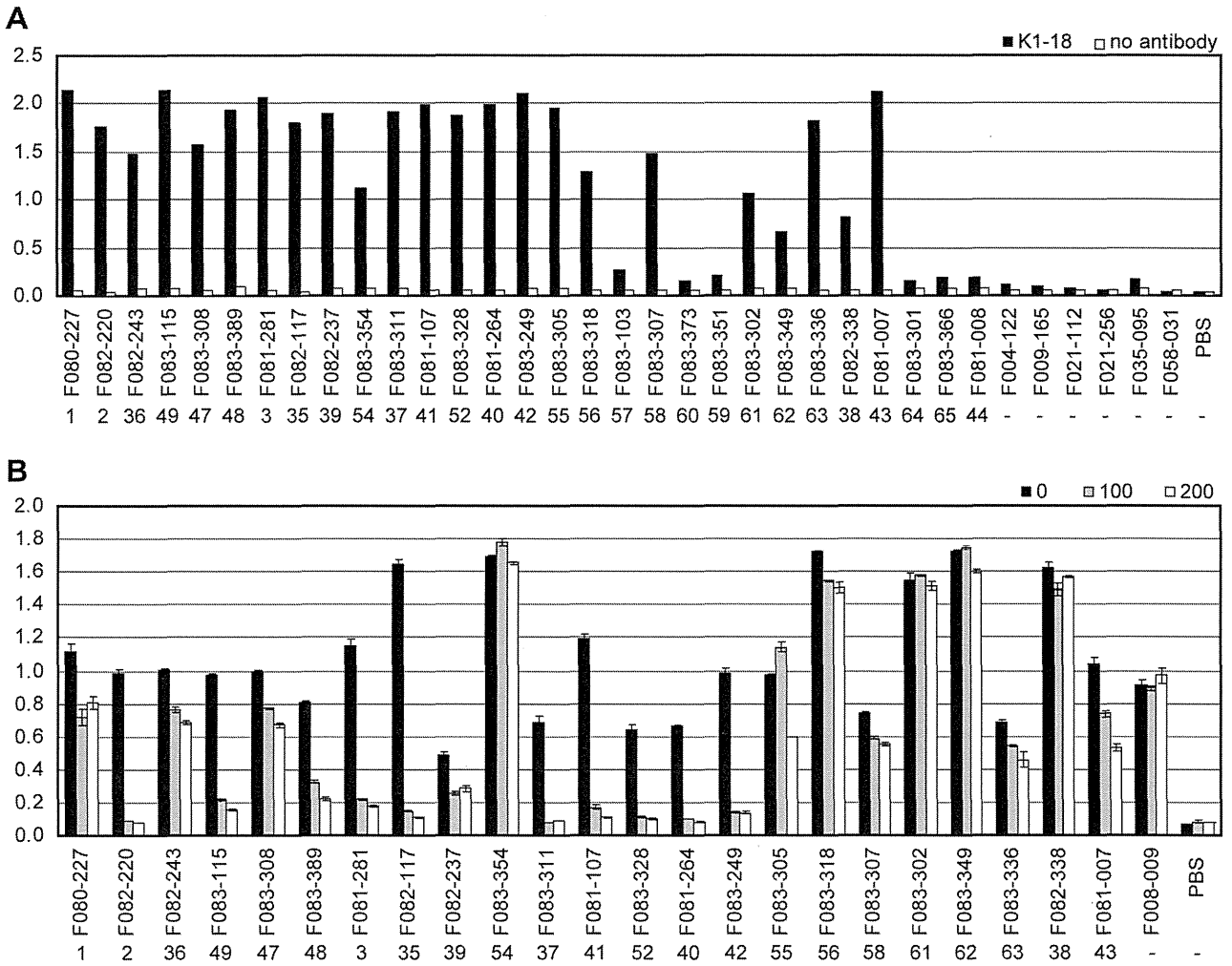

Figure 5. Characterization of K1-18 Ab. (A) Binding activity of K1-18 Ab to 1-69Abs. Binding activity of K1-18 Ab to 1-69Abs was examined by ELISA. F004-122, F009-165, F021-112, F021-256, F035-095, and F058-031 are not 1-69Abs. The group number is indicated under the name of the clone. (B) Binding activity of 1-69Abs to virus particles under the presence of K1-18 Ab. Binding activity of 1-69Abs to Bri07 virus particles was examined under the presence of 100 or 200 μg/ml K1-18 Ab by ELISA. F008-009 is not anti-HA Ab and was used as a negative control. The group number is indicated under the name of the clone. The experiment was performed in duplicate, and the error bars show standard deviation.
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expansion of the first type of B cells through vaccination did not influence the characteristics of clones isolated from the screening 4. Thus, the number of B cells that produce Abs that bind to the globular head of HA of seasonal H1N1 virus should have been at a low level, even if they existed. This conclusion is further supported by the following observation. In the serum before vaccination, the

Table 2. Virus neutralizing activity of serum in the presence of K1-18 Ab.

	A/Suita/1/2009H1N1pdm			A/Brisbane/59/2007(H1N1)		
	Concentration of K1-18 (μg/mL)			Concentration of K1-18 (μg/mL)		
Date of blood collection	800	400	0	800	400	0
2009.11.02[a]	<40	<40	40	<20	<20	20
2009.11.09	80	320	320	<40	<40	40
2009.11.17	640	640	1280	40	40	40
2009.11.23	320	640	2560	40	40	40
2009.12.07	320	640	2560	40	40	80
2009.12.14	320	320	2560	<40	40	80

[a]Serum before vaccination.
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virus-neutralizing activity against seasonal H1N1 virus examined by focus reduction assay was at a detectable level, but the HI activity was not detected (Table 1). Since the surrounding regions of the sialic acid-binding pocket are immunogenically very potent, B cells producing Abs that bind to the epitopes could be preferentially generated. Therefore, these results should have reflected the selection mechanism of long-lived memory cells.

Our results also indicate that majority of the memory cells present in the donor's body produce Abs with similar characteristics, that is, they utilize the *1-69* V_H gene, bind to the stem of HA, and neutralize both H1N1 and H5N1 viruses. They are major players in protection against H1N1 virus infection in the donor's body. We analyzed a person who had experienced influenza disease in their youth but never suffered from this disease afterwards. Since he has never been vaccinated against influenza, the Ab repertoire formed in his body was generated only by the effects of infection with live viruses. This observation is consistent with the following hypothesis. In our previous study [8], we analyzed the repertoire of neutralizing Abs against H3N2 viruses in a donor born in 1960. Many anti-HA Abs were isolated, and they were divided into three major groups with three distinct strain specificity: 1968–1973, 1977–1993 and 1997–2003. While five sites, such as A, B, C, D, and E, located on the globular head of HA have been identified as neutralizing epitopes [18,19], most of the clones that neutralize the 1977–1993 strains bound to site C [20]. Between 1977 and 1993, many mutations were introduced into sites A and B, but no mutations were introduced into site C. In order to explain these observations, we proposed the following hypothesis [20]. After a set of B cells producing Abs that can neutralize the viruses are generated by infection and/or vaccination, they will take various courses under further stimulation with the Ags. Some B cells disappear but others remain as memory cells. Furthermore, there should be long-lived memory cells and short-lived memory cells. Humans who experience an outbreak of flu almost every year have opportunities to be infected by novel influenza viruses that have drifted away from previous viruses. Some memory cells produce Abs that are able to neutralize the novel viruses, but others produce Abs that cannot neutralize them. They would be selected through the presence or absence of stimulation with the Ags. According to this hypothesis, the stability of the epitope would greatly affect the fate of memory B cells. As already shown by other groups [15,16], the epitope recognized by 1-69Abs is extremely stable among group 1 viruses. Thus, the cells producing 1-69Abs were selected as long-lived memory cells.

We analyzed only one person in this study. Therefore, the observations described in this paper should not be generalized at present. However, all humans should have potential for generating 1-69Abs that can broadly neutralize group 1 viruses, since only the *1-69* V_H gene is required for producing a broadly neutralizing Ab without participation of the V_L domain in forming the Ag-binding site [15,16], and furthermore, the requirements of the CDR3 sequence in V_H for binding to the HA stem appeared to be limited [15,16]. Thus, we propose that the resistance of people against influenza infections is explained by acquisition of the ability to produce Abs that bind to the stable epitope and that many people show the resistance against influenza since humans are able to easily generate 1-69Abs that neutralize all of group 1 viruses. The present study gave the example only for resistance against group 1 viruses. If this is the case, humans should also have abilities to generate Abs that can neutralize all H3 viruses. However, there have been few papers reporting isolation of broadly neutralizing Abs against H3 viruses from humans. CR8020 reported by Ekiert *et al.* [21] showed broad neutralizing activity against group 2 viruses including H3, and it binds to the HA stem distinct from the epitope recognized by 1-69Abs. F16 reported by Corti *et al.* [22] neutralized both group 1 and group 2 viruses, and it binds to a conserved epitope in the F subdomain of HA. In our previous studies [8,14,20], we analyzed B lymphocytes collected from three donors born in 1944, 1960, and 1974 and revealed the repertoire of neutralizing Abs against H3 viruses. As summarized above, the Abs produced in two donors born in 1960 and 1944 were divided into three major groups with three distinct strain specificities: 1968–1973, 1977–1993 and 1997–2003. On the other hand, the Abs in the donor born in 1974 were composed of only four different types. Two of them showed narrow strain specificity: most strongly bound to the 1973 strain, and bound to the 1997 to 2003 strains. The other two types showed extremely broad strain specificity: bound to all of H3 strains, and bound strongly to all of H3 strains, moreover, weakly to group 1 viruses. These observations could be interpreted as follows: since he obtained B cells producing Abs that neutralize all of H3 viruses, the B cells producing Abs that specifically neutralize the viruses present between 1980 and 1995 disappeared.

How to select the B cells that produce Abs binding to the stable epitope as long-lived memory B cells would be explained by the following observations. HI activity against H1N1 pandemic virus started to appear in the serum 2 weeks after the vaccination. On the other hand, in the case of virus-neutralizing activity measured by focus reduction assay, the activity against H1N1 pandemic virus already started to increase 1 week after vaccination and reached a plateau 2 weeks after vaccination. When viruses infect a person, memory B cells that produce Abs with the ability to neutralize the infected viruses are stimulated to grow earlier than the birth of B cells newly induced by infection with the viruses.

While further experiments are required to examine whether Abs produced in the type 2 clones are strong enough for preventing infection with future pandemics caused by highly pathogenic avian influenza (HPAI) H5N1 virus, we may expect that the presence of type 2 clones as memory cells could be helpful for preventing expansion of the pandemic viruses in their bodies. Thus, we propose that the strategy for protection against the H5N1 pandemic should be designed according to the immunological profile of each individual. Presence of anti-HA Abs utilizing the *1-69* V_H gene could be a useful indicator for presence of broadly neutralizing Abs and anti-idiotypic Abs against 1-69Abs could be the reagent.

Supporting Information

Figure S1 Amino Acid sequences of VH fragment of representative clone in each group.
(TIF)

Figure S2 Nucleotide sequences for CDR3 region of representative clone each group.
(TIF)

Author Contributions

Conceived and designed the experiments: NO RKK YI YO YK. Performed the experiments: NO RKK YI. Analyzed the data: NO RKK YK. Wrote the paper: NO YK.

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Receptor mimicry by antibody F045-092 facilitates universal binding to the H3 subtype of influenza virus

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Influenza viruses present a significant health challenge each year, as in the H3N2 epidemic of 2012-2013. Here we describe an antibody, F045-092, that possesses broadly neutralizing activity against the entire H3 subtype and accommodates the natural variation and additional glycosylation in all strains tested from 1963 to 2011. Crystal structures of F045-092 in complex with HAs from 1975 and 2011 H3N2 viruses reveal the structural basis for its neutralization breadth through insertion of its 23-residue HCDR3 into the receptor-binding site that involves striking receptor mimicry. F045-092 extends its recognition to divergent subtypes, including H1, H2 and H13, using the enhanced avidity of its IgG to overcome lower-affinity Fab binding, as observed with other antibodies that target the receptor-binding site. This unprecedented level of antibody cross-reactivity against the H3 subtype can potentially inform on development of a pan-H3 vaccine or small-molecule therapeutics.

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Influenza virus infects millions annually and causes severe illness and economic distress. The constant threat of a novel pandemic influenza virus looms large as with the emergence in 2009 of the H1N1 swine flu that antigenically closely resembled the 1918 H1N1 Spanish flu, which resulted in death of ~50 million people worldwide. Fortunately, the novel 2009 H1N1 virus was much less severe and subsequently became the circulating seasonal H1 strain. The H3N2 virus in 2012–2013 was more virulent than usual and caused epidemics in the northeastern United States[1]. The unpredictable epidemic and pandemic potential of these viruses underscores the need for broad-spectrum therapy. To combat flu infections, small molecules are available that target the neuraminidase surface glycoprotein on influenza virus, but the effectiveness of these drugs is diminishing[2,3] and underscores the need for new classes of drugs. Vaccine therapy remains the best means as a preventative countermeasure from flu infections. However, vaccine strains must be reformulated almost every year to stay ahead of the ever-changing virus and may not provide protection if they are an inadequate match to the dominant circulating virus.

Much of the challenge in raising a broadly neutralizing antibody response against influenza by a universal flu vaccine arises from the diversity and hypervariability of the haemagglutinin (HA) surface glycoprotein. There are currently 18 identified HA subtypes (H1–H18) of influenza A viruses, where aquatic birds act as the primary reservoir. Among these, only the H1 and H3 subtypes currently circulate in humans although sporadic cases of H5, H7, including the most recent H7N9 outbreak[4,5], and H9 have caused deaths and severe illness. Thus, emerging viruses are carefully monitored for any propensity to acquire human-to-human transmission that would increase the potential to cause a human pandemic. Despite extensive sequence diversity across HA subtypes, viral attachment to terminal sialic acid glycans on host cells is a conserved function of the HA that initiates viral infection[6,7], although the newly discovered bat H17 and H18 HAs appear to be exceptions[8–10]. The HA interaction with sialosides is very weak (K_d ~ mM) and the virus is thus reliant on multivalency to achieve high enough affinity for productive receptor binding and subsequent receptor-mediated endocytosis.

HA is synthesized as a single polypeptide precursor (HA0) that assembles into trimers, which mature after proteolytic cleavage by host proteases between the HA1 and HA2 subunits to produce a pH-dependent metastable intermediate. In structural and functional terms, HA can be divided into a membrane-proximal 'stem', which constitutes the core fusion machinery that is triggered by low pH in the endosomes[11], and a membrane-distal 'head', which contains the receptor-binding site (three per HA trimer). The receptor-binding site is a broad and shallow pocket located at the apex of the HA head and its outer ridges are formed primarily by four loops named after their locations in the HA sequence: the 130 loop, 150 loop, 190 helix and 220 loop.

Blocking HA–receptor interactions can prevent infection by influenza virus. However, despite the functional conservation of receptor binding, the design of entry inhibitors to the HA still remains a significant challenge due to the shallowness of the receptor-binding pocket and sequence divergence within and across HA subtypes. Nevertheless, a number of HA receptor-binding-site-targeted antibodies have been structurally characterized that have broader-spectrum activity against a single subtype[12–15] or across multiple subtypes[16,17], thus suggesting that the receptor-binding pocket is indeed a site of vulnerability on HA[18]. The breadth of recognition of these antibodies is surprising as it was popularly believed until recently that the HA head could only elicit strain-specific antibodies. All of these antibodies that target the receptor-binding site insert a single complementarity determining region (CDR) loop (HCDR2 or HCDR3) into the receptor-binding site to prevent viral-host recognition; however, due to the larger footprints of antibodies in comparison to the sialic acid receptor, they invariably contact variable residues on the outer edges of the receptor-binding pocket and, hence, have limited breadth compared to stem-directed antibodies.

Here, we describe crystal structures of antibody F045–092 bound to divergent HAs from the 1975 and 2011 H3 subtype. The antibody was selected from a phage-displayed library, derived from human B lymphocytes, and was reported to have activity against a panel of H3 strains from 1968 to 2004 and possesses neutralizing activity against all H3N2 viruses that were tested within this time span as well as certain H1N1, H2N2 and H5N1 viruses[19]. The antibody contacts a minimal epitope in the HA receptor-binding site primarily by insertion of its 23-residue HCDR3 and achieves exceptional receptor mimicry. Full coverage of an entire subtype by interaction with the receptor-binding site is of great significance and the structural information from the complexes may potentially advance the design of novel small-molecule inhibitors as well as inform on the formulation of immunogens as vaccine candidates against H3 viruses.

Results

Binding affinity of F045–092 against HAs. To further profile its breadth, binding of F045–092 Fab and IgG was measured against a panel of HAs that encompass representative strains from nearly all HA subtypes by bio-layer interferometry (Table 1 and Supplementary Table 1). F045–092 has heterosubtypic activity and binds HAs from the H1, H2, H3 and H13 subtypes. Most notably, F045–092 Fab binds all 13 representative HAs from strains from the H3 subtype that span five decades from 1963 to 2011, including the 2012–2013 H3 vaccine strain A/Victoria/361/2011 (Vic2011/H3). Although the HAs of some strains are strongly bound by the Fab (K_d ~ 5–50 nM), others have only moderate to weak binding (K_d ~ 50 nM to 5 μM), some of which are outside the threshold thought to be necessary for effective neutralization (K_d < 250 nM)[17]. However, the bivalent F045–092 IgG shows increased affinity through avidity (K_d < 20 nM) that significantly improves F045–092 binding to all strains compared with the monovalent Fab (Table 1). These IgG-binding data are consistent with the reported neutralization data[19]. Avidity has also been observed, and now appears to be a common feature, for other receptor-binding-site-targeted antibodies[16,17] as the close packing and high density of HA trimers on viruses[20] likely promotes and facilitates bivalent binding by IgG. Moreover, binding of F045–092 was tested against strains beyond the years that were tested for neutralization (1968–2004)[19]. Despite further antigenic variation, the more recent strains (2007–2011) are bound with high affinity by IgG, indicating that F045–092 may truly be a pan-H3 antibody.

Crystal structures of the F045–092–HA complexes. To decipher the structural basis for this pan-H3 recognition by F045–092, crystal structures of the F045–092 Fab in complex with the HAs from the A/Victoria/3/1975 (Vic1975/H3) and A/Victoria/361/2011 (Vic2011/H3) viruses were determined at 2.75 Å and 6.50 Å resolution (Table 2). In addition, crystal structures of the F045–092 Fab and the unliganded Vic2011/H3 HA were determined at 1.50 Å and 1.75 Å resolution (Table 2), respectively, and served as high-resolution starting models for refinement of the Fab–HA complexes. In the crystal asymmetric unit, one copy of the Fab is bound to one HA protomer of the HA trimer in the F045–092–Vic1975/H3 complex. The HA protomer and the Fab variable domain are well ordered, as judged from the electron density,

Table 1 | Binding breadth of F045-092 Fab and IgG to influenza HA subtypes and strains.

Subtype	Strain	Binding		Neutralization*
		F045-092 Fab	F045-092 IgG	F045-092 IgG
H1N1	A/Beijing/262/1995	+	+ + +	NT
H1N1	A/New Caledonia/20/1999	+	+ + +	+ + +
H2N2	A/Adachi/2/1957	+	+ + +	NT
H2N2	A/Okuda/1957	NT	NT	+ + +
H3N8	A/duck/Ukraine/1/1963	+	+ + +	NT
H3N2	A/Aichi/2/1968	NT	NT	+ + + +
H3N2	A/Hong Kong/1/1968	+ + +	+ + + +	NT
H3N2	A/Victoria/3/1975	+ + +	+ + + +	NT
H3N2	A/Bangkok/1/1979	+ +	+ + + +	NT
H3N2	A/Leningrad/360/1986	+ + +	+ + + +	NT
H3N2	A/Beijing/353/1989	+ +	+ + + +	NT
H3N2	A/Kitakyushu/159/1993	NT	NT	+ + + +
H3N2	A/Shangdong/9/1993	+ + + +	+ + + +	NT
H3N2	A/Panama/2007/1999	+ + +	+ + + +	NT
H3N2	A/Moscow/10/1999	+ + + +	+ + + +	NT
H3N2	A/Wyoming/3/2003	+	+ + +	NT
H3N2	A/Brisbane/10/2007	+ +	+ + + +	NT
H3N2	A/Perth/16/2009	+ +	+ + + +	NT
H3N2	A/Victoria/361/2011	+ +	+ + + +	NT
H13N6	A/gull/Maryland/704/1977	+	+ + +	NT

Dissociation constants: NT, not tested; + K_d = 500–5,000 nM; + + K_d = 50–500 nM; + + + K_d = 5–50 nM or IC_{50} < 5 μg ml^{-1}; + + + + $K_d \leq 5$ nM or IC_{50} < 0.05 μg ml^{-1}.
*Virus-neutralizing activities adapted from Ohshima *et al.*[19]

whereas the Fab constant domain appears to be disordered, as frequently found in other Fab–HA complexes[14,17,21–23] due to flexibility of the elbow region connecting the Fab variable and constant domains[24]. The asymmetric unit of the F045–092–Vic2011/H3 complex contains two HA trimers bound by six copies of the Fab. Despite the moderate resolution, the HA and Fab molecules can be clearly observed in the electron density maps (Supplementary Fig. 1). All six Fab variable domains similarly recognize HA, but have a slight difference in approach angle on average (~14° rotation) in comparison to the F045–092–Vic1975/H3 complex; at this stage, these values are still qualitative given that we are comparing different resolution structures. No major conformational changes were observed between the apo and bound Fab in either complex. There is also good structural agreement between the HAs from the Vic1975/H3 and Vic2011/H3 complexes (Cα root mean squared deviation (r.m.s.d.) of ~0.4 Å) despite their divergence over 36 years, which amounted to 62 amino-acid mutations in the HA ectodomain.

The F045–092 Fab binds near the apex of HA to a single protomer of the HA trimer (Fig. 1) and contacts portions of antigenic sites A, B and D[25]. The interactions are mediated only through the heavy chain using all three of its CDR loops, whereas the light chain is positioned ~14 Å away from the HA main chain and only comes within ~10 Å of a conserved glycan at Asn165 from a neighbouring HA protomer in the trimer (Fig. 1c). Indeed, the heavy chain CDRs form a ridge and protrude much further from the Fab core than the CDRs from the light chain (Supplementary Fig. 2). F045–092 reaches into the receptor-binding site through insertion of its long 23-residue HCDR3, which notably contains an intrachain disulphide separated by only two amino acids (Fig. 2a). This unusual disulphide in HCDR3 has only been seen in only a few other antibody structures[26–28]. As the HCDR3 and sialoglycan receptor bind to the same specificity pockets in the receptor-binding site (Fig. 2b), the F045–092 antibody is able to block viral–host interactions. Only ~970 Å^2 of total molecular surface is buried in the F045–092–Vic1975/H3 interaction, divided evenly between HA and the Fab, and is the smallest footprint for any HA–antibody complex to date (Supplementary Table 2).

The *VH1-69* germline is well known for producing heterosubtypic HA antibodies, which use a hydrophobic HCDR2 loop to target the stem of group 1 HAs[21,26]. However, other *VH1-69* germline-encoded antibodies have been shown to insert their HCDR2 into the receptor-binding site[14]. F045–092 is also a *VH1-69* germline-encoded antibody but primarily uses its HCDR3, which comprises 58% of the total surface area contributed by the Fab. The hydrophobic residues at the tip of HCDR2 (somatic Phe53 and germline Phe54) that are the hallmark of the *VH1-69* family surprisingly only make minimal contacts with the outer ridges of the 150 loop and the 190 helix of HA.

Sequence analysis of the F045–092 epitope. To delineate the F045–092 binding determinants, the sequence of the HA epitope was analysed. F045–092 contacts a minimal epitope in and around the conserved receptor-binding site (Fig. 3a). Although F045–092 does indeed contact absolutely conserved residues involved in receptor recognition, the antibody also contacts some variable residues outside the receptor-binding site (Fig. 3b). The F045–092 epitope on the HA is quite variable across all human H3 strains and, at first glance, broad-spectrum recognition to a site with this relatively poor sequence conservation, apart from a handful of key residues that bind sialic acid, would not have been predicted as compared with the near absolute conservation of the HA stem-targeted epitopes[21,22,26,29–32] (Fig. 3c and Supplementary Table 3). Yet, F045–092 can tolerate natural variation in H3 strains that span from 1963 to 2011 (Table 1). Furthermore, the current 2013–2014 H3 vaccine strain (A/Texas/50/2012) is antigenically similar to Vic2011/H3 and has an identical F045–092 epitope.

Among the four loops that form the receptor-binding pocket, only the 150 loop does not contact the sialoglycan receptor and, therefore, it can accommodate high sequence variability as it is not functionally constrained for receptor recognition. Notwithstanding, the 150 loop contributes 40% of the total HA buried surface area in the F045–092–HA complex. However, the antibody appears to make non-specific interactions with the side chains of variable residues of HA such as with residues 156 and

Table 2 | Data collection and refinement statistics.

	F045-092-Vic1975/H3 complex	F045-092-Vic2011/H3 complex	F045-092 Fab	Vic2011/H3 HA
Data collection				
Space group	P321	C2	$P2_12_12_1$	H32
Cell dimensions				
a, *b*, *c* (Å)	99.1, 99.1 336.7	318.1, 187.2, 353.6	69.5, 77.3 81.5	100.7, 100.7, 383.3
α, β, γ (°)	90, 90, 120	90, 90.5, 90	90, 90, 90	90, 90, 120
Resolution (Å)	50-2.75 (2.92-2.75)*	50-6.50 (6.89-6.50)	50-1.50 (1.60-1.50)	50-1.75 (1.85-1.75)
R_{merge}	0.10 (0.71)	0.07 (0.50)	0.04 (0.51)	0.07 (0.89)
$I/\sigma I$	13.3 (2.3)	10.8 (2.1)	22.7 (2.3)	21.2 (2.4)
Completeness (%)	99.5 (97.7)	92.8 (91.7)	96.8 (83.4)	99.8 (99.0)
Redundancy	6.1 (6.1)	2.0 (2.0)	5.6 (4.4)	8.9 (8.6)
Refinement				
Resolution (Å)	49.0-2.75	49.0-6.50	43.6-1.50	46.8-1.75
No. of reflections	50,811	38,102	67,979	75,930
R_{work}/R_{free}	17.7/22.4	20.1/24.8	17.5/20.7	16.5/18.5
No. of atoms				
Protein	7,078	42,816	3,436	3,929
Glycan/ligand/ion	236	1,489	5	178
Water	76	0	457	426
B-factors				
Protein	57	145	26	34
Glycan/ligand/ion	86	168	73	69
Water	37	-	34	37
r.m.s. deviations				
Bond lengths (Å)	0.010	0.006	0.010	0.009
Bond angles (°)	1.25	1.22	1.26	1.18
PDB code	4O58	4O5I	4O5L	4O5N

r.m.s., root mean square.
*Values in parentheses are for the highest-resolution shell.

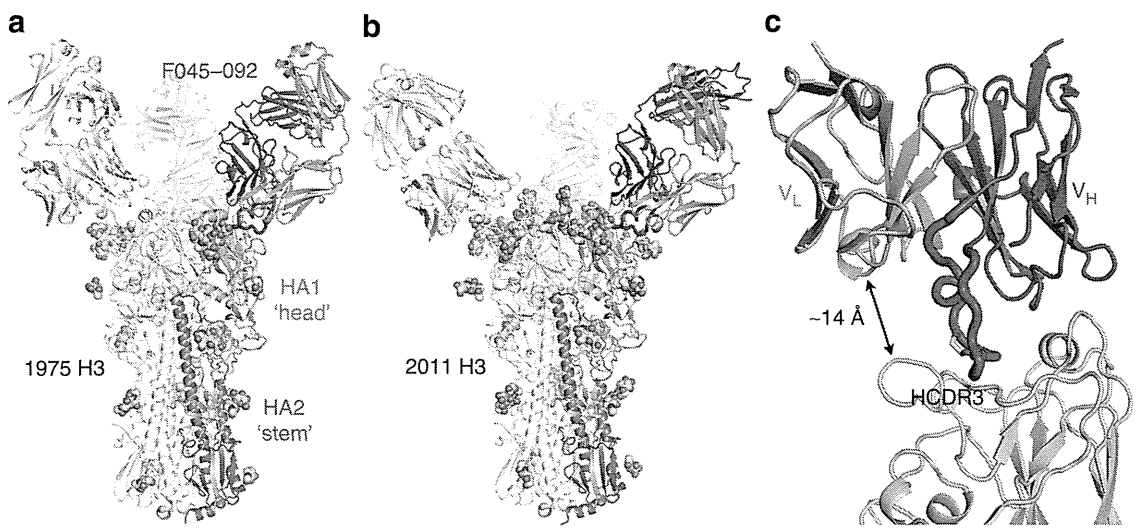

Figure 1 | Antibody F045-092 binds the influenza HA receptor-binding site. Crystal structures of F045-092 Fab in complex with (**a**) Vic1975/H3 HA and (**b**) Vic2011/H3 HA. One Fab-HA protomer is coloured with HA1 in light blue, HA2 in green, the Fab heavy chain in purple and the Fab light chain in pink. Glycans are shown as spheres with carbon in yellow, oxygen in red and nitrogen in blue. (**c**) F045-092 binds HA using only its heavy chain and inserts its HCDR3 into the HA receptor-binding site. The HCDR3 contains a disulphide bridge, which is shown in yellow sticks.

158 that have a sequence conservation of 69% and 46% across all human H3 viruses, respectively (Supplementary Fig. 3). Interactions with these and other variable residues appear to be permissive and do not create destabilizing clashes with F045–092, as the antibody is able to tolerate natural variation across decades of antigenic drift (Table 1).

In addition to the sequence variability in and around the receptor-binding site, insertions and deletions at or near the receptor-binding site loops can alter the conformations of the loops to evade immune recognition. In particular, the conformation of the 130 loop is important for F045–092 recognition as the antibody contacts this loop primarily through main-chain interactions and,

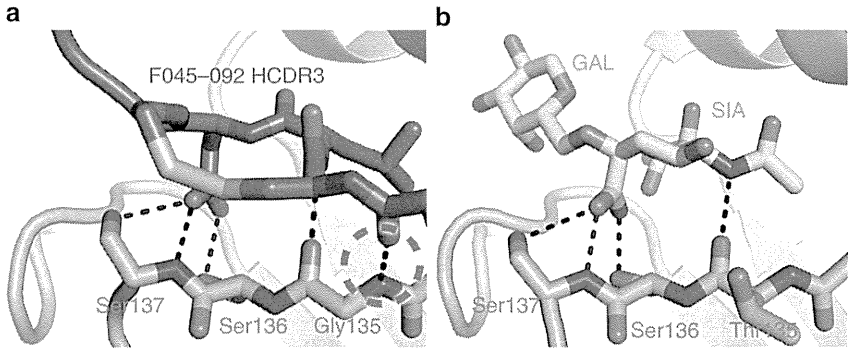

Figure 2 | Receptor-binding site recognition by F045-092 to Vic1975/H3 in comparison to the α2,6 sialoglycan receptor. (**a**) F045-092 inserts its HCDR3 into the HA receptor-binding site. The carboxylate side chain of F045-092 Asp100e overlaps with the (**b**) carboxylate moiety of the sialic acid of the α2,6 sialoglycan receptor (PDB 2YP4) and uses identical hydrogen-bonding interactions, which are shown as black dashed lines. An additional hydrogen bond between the main chains of F045-092 and HA is circled in red dashed lines. The Leu100d side chain of F045-092 overlaps with the acetamide moiety of sialic acid.

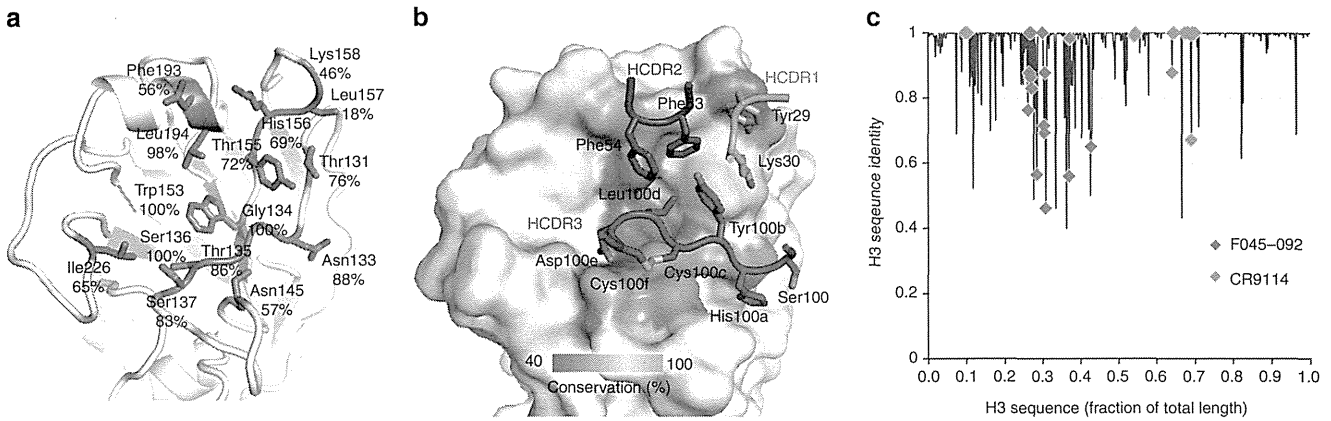

Figure 3 | Sequence conservation of the F045-092 epitope across human H3 viruses. (**a**) The F045-092 contact residues on HA are depicted as sticks. The per cent conservation for the most common residue found in the H3 subtype is labelled, which is not always identical to the residue at that position in the Vic1975/H3 sequence. (**b**) Interaction of F045-092 with Vic1975/H3 illustrating the sequence conservation of neutralizing epitope on the HA surface. For identification of the HA residues and positions that are colour coded on the surface, see (**a**). The F045-092 contact residues are labelled, coloured by HCDR, and shown as sticks. (**c**) Per cent identity of the most common residue among human H3 isolates plotted against the linear sequence of HA. Residues contacted by F045-092 or CR9114 are indicated by red or green diamonds, respectively. Although F045-092 contacts more hypervariable residues than CR9114 (but in different regions- head versus stem), it can still broadly neutralize the H3 subtype.

thus, is not restricted by the natural variation of the residues in this loop in the H3 subtype. For instance, the 133a insertion (between residues 133 and 134) observed in ~78% of human H1 strains (observed in the 1918 and 2009 pandemic viruses) and in ~78% of human H5 strains produces a bulge in the 130 loop that protrudes from the receptor-binding site and would clash with F045–092 and other antibodies[12,16,17]. Notably, the H1 and H2 isolates recognized by F045–092 do not contain the 133a insertion. Also, a conserved two amino-acid insertion is present in the 150 loop of all H7 strains that likely will sterically prevent F045–092 binding. Although these idiosyncratic structural features of the receptor-binding loops limit the recognition of these particular strains and subtypes by F045–092 (Supplementary Table 1), no insertions or deletions exist for any H3 strains, suggesting that sequence variation alone dictates immune evasion in the H3 subtype.

HA glycan evasion. In addition to continual antigenic drift, influenza HA can evade immune detection by masking neutralizing sites with glycans[33–37]. Within the H3 subtype, five conserved glycans exist at HA positions 22, 38, 165 and 285 on the HA1 subunit as well as 154 on HA2. Over time, H3 strains have incorporated and generally have retained several additional, potential N-linked glycosylation sites (PNGs) on the HA head (Fig. 4a). For example, Vic1975/H3 has seven PNGs per HA protomer, whereas Vic2011/H3 has these same seven and five additional PNGs in the HA head. This finding suggests that addition of glycans to H3 HAs is accretive, similar to the continual selection of variants in the HA amino-acid sequence by antigenic drift[38]. The added PNGs do not appear to occlude receptor binding but decorate the regions surrounding the receptor-binding site. Nevertheless, F045–092 evades these glycans to contact the HA protein surface. Most notably, a PNG in the 130 loop at Asn133 appeared in H3 viruses in 1996 and has been retained in successive viruses. As seen in the Vic2011/H3 complex, the glycan at this site is directed away from the receptor-binding site and does not interfere with F045–092 binding as the antibody slides in between the glycan and the HA 140 loop (Fig. 4b). Accordingly, the A/Panama/2007/1999 (H3N2) and A/Moscow/10/1999 (H3N2) strains, which have a PNG at Asn133, are both bound with very high affinity by the F045–092 Fab (K_d < 20 nM). In addition, a PNG at Asn144 began to emerge in 1996, but no corresponding density for carbohydrate was observed in the Vic2011/H3 complex; however, the Asn side

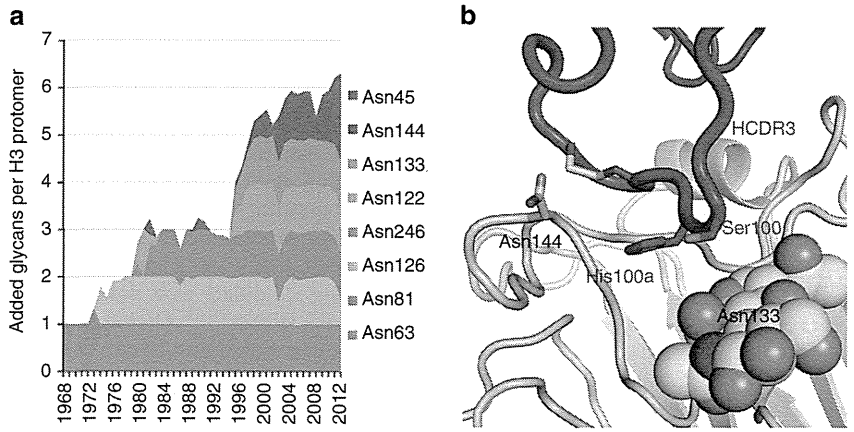

Figure 4 | Emergence of predicted N-linked glycans (PNGs) across human H3 viruses and evasion by F045-092. (**a**) Stacked graph showing the normalized percentage of strains that contain an additional PNG on HA1 at each indicated position. Seven PNGs have been incorporated in the HA since 1968 in addition to the five absolutely conserved PNGs in H3 strains (HA1 Asn22, Asn38, Asn165, Asn285; HA2 Asn154), except for the PNG at Asn81 (1968-1977). (**b**) F045-092 evades the glycan at Asn133 proximal to the receptor-binding site in the Vic2011/H3 complex. The glycan is shown as spheres, with carbon in yellow, oxygen in red and nitrogen in blue. No density corresponding to the PNG at Asn144 (shown as sticks) was observed.

chain is directed away from the combining site (Fig. 4b) and suggests that potential glycosylation at this site would not obstruct F045–092 recognition, which is substantiated by the binding data.

F045–092 receptor mimicry. The F045–092 HCDR3 adopts a protruding, blunt-ended conformation, which inserts into the receptor-binding site. The inserted residues include a disulphide loop consisting of residues Cys100c, Leu100d, Asp100e and Cys100f, which form a type I-like β turn. Remarkably, the side chains of the two central residues of the disulphide loop overlap with and chemically resemble sialic acid of the sialoglycan receptor (Fig. 2). In particular, the isobutyl side chain of Leu100d aligns with the acetamide of sialic acid and similarly enters the hydrophobic pocket formed by the nearly universally conserved HA residues Trp153 and Leu194. Moreover, the carboxylate side chain of Asp100e closely aligns with the carboxylate of sialic acid and uses the same network of hydrogen bonds, including Ser136, which was predicted to contribute to antibody recognition[19]. Furthermore, the F045–092 HCDR3 curls over the 130 loop and creates an additional hydrogen bond between the Fab main chain at Tyr100b and HA1 Gly135 (Fig. 2).

The importance of the HCDR3 disulphide loop was investigated by alanine scanning mutagenesis. Individually mutating either Leu100d or Asp100e to alanine or eliminating the disulphide loop abolishes binding by F045–092 Fab to nearly every H3 strain tested (Supplementary Table 4). These results suggest the identities of these four residues are the key determinants for F045–092 recognition of the H3 HA.

Alanine mutagenesis of His100a, which is positioned in the groove between the 130 and 140 loops, does not have any major effect on the affinity of F045–092 to HA (Supplementary Table 4). Its side chain is directed away from the HA and evades the glycan at Asn133 in the Vic2011/H3 complex (Fig. 4b). The residues that form and surround the vicinity of this groove, which is adjacent to the receptor-binding pocket, are highly conserved among H3 isolates (Supplementary Fig. 4). This cleft may then be a potential secondary hotspot for the expansion of receptor-binding site directed small molecules or engineered proteins.

Discussion

Influenza H3N2 viruses have circulated in humans ever since the 1968 Hong Kong pandemic. In the span of nearly five decades, the H3 HAs have diverged by antigenic drift so that the current H3 strain has only ~80% sequence identity to the 1968 H3 strain, with the majority of that variation focused in the HA head. In addition, glycans are periodically incorporated and retained as an immune evasion strategy employed by H3 viruses to shield antigenic sites[33,34]. The extent of the additional masking of the HA surface by glycans from 1968 to present can be clearly visualized in the Vic2011/H3 HA structure (Supplementary Fig. 5). The crystal structures of heterosubtypic, pan-H3 antibody F045–092 in complex with the HAs from 1975 and 2011 H3N2 viruses reveal how the antibody can broadly neutralize the H3 subtype through insertion of a long HCDR3 loop into the HA receptor-binding site. Although F045–092 does not make specific contacts with the HA glycans around the receptor-binding site but rather avoids them, its mode of binding to HA resembles to some extent the exceptionally long HCDR3 loops of many anti-HIV antibodies, especially those that use their HCDR3 loops to reach into recessed sites or penetrate through the glycan shield of HIV-1 Env to contact the protein surface below[39–42].

The principal contacts of F045–092 to the HA are via HCDR3, which contains a disulphide loop. The antibody binds a minimal HA epitope that is located in and around the receptor-binding site and has the smallest footprint on HA to date (Supplementary Table 2). The HCDR3 Asp100e and Leu100d, which are between the Cys residues (Cys100c, Cys100f) that form the disulphide loop, mimic the hydrogen-bonding network of the carboxylate and the hydrophobic acetamide moiety of the endogenous sialoglycan receptor, respectively, and are critical for antibody recognition to the HA. Furthermore, an additional hydrogen bond is formed between the main chains of F045–092 and the HA 130 loop, which represent an interaction that sialoglycan receptors do not utilize.

Yet, due to the larger footprint of the antibody on HA (~490 Å^2) compared with sialic acid (~190 Å^2), it is inevitable that F045–092 will contact some non-conserved residues outside the receptor-binding site. However, F045–092 is able to overcome the antigenic variation of the H3 subtype, as well as the addition and accumulation of glycans, surrounding the receptor-binding site and binds some HA strains with a K_d ~nM as compared with a K_d ~mM for the sialoglycan receptor, a difference of six orders of magnitude. Studies[43] indicate that H3 strains with increased glycosylation have significantly decreased affinity for receptor,