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Heterosubtypic Antibodies to Influenza A Virus Have Limited Activity against Cell-Bound Virus but Are Not Impaired by Strain-Specific Serum Antibodies

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ABSTRACT

The majority of influenza virus-specific antibodies elicited by vaccination or natural infection are effective only against the eliciting or closely related viruses. Rare stem-specific heterosubtypic monoclonal antibodies (hMAbs) can neutralize multiple strains and subtypes by preventing hemagglutinin (HA)-mediated fusion of the viral membrane with the endosomal membrane. The epitopes recognized by these hMAbs are therefore considered promising targets for the development of pan-influenza virus vaccines. Here, we report the isolation of a novel human HA stem-reactive monoclonal antibody, hMAb 1.12, with exceptionally broad neutralizing activity encompassing viruses from 15 distinct HA subtypes. Using MAb 1.12 and two other monoclonal antibodies, we demonstrate that neutralization by hMAbs is virtually irreversible but becomes severely impaired following virus attachment to cells. In contrast, no interference by human anti-influenza virus serum antibodies was found, indicating that apically binding antibodies do not impair access to the membrane-proximal heterosubtypic epitopes. Our findings therefore encourage development of new vaccine concepts aiming at the induction of stem-specific heterosubtypic antibodies, as we provide support for their effectiveness in individuals previously exposed to influenza virus.

IMPORTANCE

The influenza A virus hemagglutinin (HA) can easily accommodate changes in its antigenic structures to escape preexisting immunity. This variability restricts the breadth and long-term efficacy of influenza vaccines. Only a few heterosubtypic antibodies (hMAbs), i.e., antibodies that can neutralize more than one subtype of influenza A virus, have been identified. The molecular interactions between these heterosubtypic antibodies and hemagglutinin are well characterized, yet little is known about the functional properties of these antibodies. Using a new, extraordinarily broad hMAb, we show that virus neutralization by hMAbs is virtually irreversible and that efficient neutralization is possible only if stem-specific hMAbs bind to HA before the virus attaches to the cell surface. No interference between strain-specific human serum immunoglobulin and hMAbs was found, indicating that preexisting humoral immunity to influenza virus does not limit the efficacy of stem-reactive heterosubtypic antibodies. This knowledge supports the development of a pan-influenza virus vaccine.

Hemagglutinin (HA), the major surface antigen of influenza A virus, exists in 18 subtypes and is responsible for virus entry into the host cell. Influenza virus vaccines are usually effective against seasonal influenza (1–3), but currently available vaccines elicit antibodies of limited breadth that neutralize only the inoculated and closely related seasonal strains. This strain-specific (or homotypic) nature of the antibody response implies that seasonal vaccines have to be regularly reformulated to reflect antigenic changes acquired by drifting. Furthermore, vaccines have to precisely match the antigenic outfit of the strains predicted to be predominantly circulating and may be ineffective if the prediction fails. Although rather rare, several human heterosubtypic monoclonal antibodies (hMAbs) have been described (4–16) and used to define highly conserved epitopes in the receptor-binding site and in the stem of the influenza virus HA. However, development of a universal influenza virus vaccine against these epitopes has so far been approached unsuccessfully using various strategies (17–22).

To date, it is also not clear whether the membrane-proximal locations of the conserved epitopes bound by broadly neutralizing hMAbs restrict the efficacy of heterosubtypic antibodies if virions are cell associated or if they are saturated with strain-specific, membrane-distally binding serum antibodies. These are likely to represent common conditions under which naturally occurring or elicited heterosubtypic antibodies will encounter the virus in humans.

MATERIALS AND METHODS

Characterization of donor RI13. Donor RI13, a 30-year-old Caucasian male, was identified in a different study as an individual with an average heterosubtypic antibody response (23). RI13 had been vaccinated six times against influenza A virus prior to blood donation, and cells were harvested prior to the arrival of the swine origin H1N1 virus in 2009.

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Isolation and characterization of MAb 1.12. A phage library was prepared as previously described (24). In brief, frozen peripheral blood mononuclear cells (PBMCs) from donor RI13 were used to purify B cells using anti-CD22-coated magnetically activated cell sorting (MACS) beads (~1.6×10^6 B cells were isolated). Following total RNA extraction (RNeasy Mini; Qiagen), reverse transcription into cDNA was performed using oligo(dT) primer (Promega) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturers' recommendations. Rearranged variable gene segment families were amplified individually and modified for phage surface expression in 3 subsequent PCRs. The resulting full-length Fab fragments were cloned into the pComb3X phage display vector and used to rescue a phage library with a total of 1.5×10^9 transformants, giving rise to a 3.3×10^{11} phage particles/ml library titer.

This phage display library was enriched for phages binding to biotinylated recombinant trimeric hemagglutinin immobilized on streptavidin-coated magnetic beads (the construction and biochemical characterization of these antigens will be reported elsewhere; the beads were purchased from Promega). Approximately 2.5×10^{12} phage were combined with bead-immobilized hemagglutinin (see below; the final concentration of protein was 50 to 100 nM, as determined for HA dimer) in the first round of selection. A total of 4 rounds of selection were performed with a 50 to 100 nM HA concentration (as determined for HA trimer in rounds 2 to 4) and increasing wash stringency (washing with Tris-buffered saline containing 0.05% Tween 20 [TBST]). Phage clones obtained from the 3rd and 4th rounds were screened for binding to various HAs in enzyme-linked immunosorbent assays (ELISA), and positive clones were sequenced.

A total of 48 clones from the 3rd and 4th rounds were analyzed for binding to recombinantly expressed H2 [A/Japan/305/1957(H2N2)], H3 [A/Moscow/10/1999(H3N2)], and H7 [A/fowl plague/Bratislava/1979 (H7N7)] hemagglutinins in ELISA. Remarkably, 43 out of 48 clones showed cross-reactivity to all tested HAs. Sequence analysis revealed that all the selected clones have almost the same heavy chain (HC) derived from the VH1-69 germ line gene joined to the same complementarity-determining region 3 (HCDR3), which contains a stretch of five adjacent tyrosine residues. In contrast, high variability and the use of multiple germ line genes and isotypes have been seen for light chains (LC). We therefore predicted that the heavy chain only is probably responsible for binding to HA, and randomly selected clone 1.12. For expression as an IgG1 molecule, it was cloned into pAbVec and transfected into 293T cells, as described previously (16).

Expression and purification of recombinant HAs. Recombinant HA was expressed in Sf9 cells using genetically modified baculovirus vectors as described by Ekiert et al. or Stevens et al. (7, 25). In brief, HA open reading frames were modified to contain an insect secretion leader sequence at the N-terminal end and a foldon/trimerization instead of a transmembrane and intracellular domain at the C-terminal end. Secreted recombinant protein was purified on Ni-nitrilotriacetic acid (NTA) columns (GE Healthcare) from cell supernatant harvested 4 days postinfection. To process recombinant HA protein into HA1 and HA2, 10 U of tosyl phenylalanyl chloromethyl ketone-treated trypsin (TPCK) (from bovine pancreas; Sigma-Aldrich) per 1 μg of HA was used for processing at room temperature (RT) for 1 h. Immediately after trypsinization, nonaggregated HA trimers were purified by size exclusion chromatography on a Superdex S-200 gel filtration column (GE Healthcare). All HA proteins used in phage display had an additional cysteine residue introduced at position 158 of HA1 to enable biotinylation (with EZ-link HPDP biotin; Pierce) and immobilization on streptavidin beads in an upside-down orientation.

HA-binding ELISA. Binding of IgG1 1.12 to various recombinant HAs was assessed by ELISA. To this end, high-binding, half-area plates (Costar) were coated at 4°C with 25 μl/well of 2 to 4 μg/ml HA in phosphate-buffered saline (PBS) overnight. The plates were then blocked with 60 μl of 2% low-fat dry milk in PBS for 1 h at RT. Purified IgG1 was titrated in 0.2% milk-PBS and incubated in a volume of 30 μl on the coated plates at RT for 1 h. Bound IgG1 was detected using a peroxidase-coupled goat anti-human Igκ antiserum (1:5,000; Southern Biotech). Between steps, the plates were washed 4 times with approximately 200 μl of TBST containing 0.1% Tween. The ELISA plates were then developed using Ultra TMB substrate (Pierce) for 5 to 10 min before the reaction was stopped by the addition of 2 N H_2SO_4. The optical density at 450 nm (OD_{450}) was measured in a PerkinElmer plate reader (EnVision; PerkinElmer Wallac). As a negative control, recombinantly expressed HIV-1 gp120-specific IgG1 b12 was included in all assays.

Competition ELISA. ELISA plates (half area; high binding; Costar) were coated with 25 μl/well of 2-μg/ml recombinant H1 from A/Puerto Rico/8/1934(H1N1) in PBS at 4°C overnight. The plates were then blocked with 60 μl of 2% milk in PBS at room temperature for 1 h. The blocked plates were incubated in duplicate with 30 μl of serially diluted (in PBS-0.2% milk) human IgG1 1.12 at RT for 1 h and washed 4 times with TBST. The plates were then incubated at RT for 1 h with murine IgG c179 (26) diluted in PBS-0.2% milk at 1 μg/ml and washed 4 times with TBST. Binding of human IgG 1.12 and murine IgG c179 was detected in parallel using either peroxidase-coupled goat anti-human Igκ (Southern Biotech) or rabbit anti-mouse-horseradish peroxidase (HRP) serum (Dako), respectively. The signal was developed as described for HA-binding ELISA.

Neutralization of influenza A viruses. Titrated IgG 1.12 was mixed with a fixed amount of influenza A virus corresponding to a multiplicity of infection (MOI) of 2 to 3 (~100,000 PFU/well) in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.2% bovine serum albumin (BSA) and 20 mM HEPES (D/B/H medium). Following incubation at 37°C-5% CO_2 for 2 h, the MAb-virus mixture was transferred onto PBS-washed, subconfluent Madin-Darby canine kidney (MDCK) epithelial cells (ATCC CCL-34) growing in 96-well tissue culture plates (Techno Plastic Products [TPP]). To initiate infection, cells were incubated with the antibody-virus mixture at 37°C-5% CO_2 for 1 h. The mixture was then removed, and the cells were washed with PBS before they were overlaid with D/B/H medium. After 5 to 7 h (depending on the growth kinetics of the virus isolate) at 37°C-5% CO_2, the cells were fixed with methanol, washed, and stained with a fluorescein isothiocyanate (FITC)-labeled anti-NP monoclonal antibody (ATCC; HB-65; 3 μg/ml) diluted in PBS-1% BSA at 4°C overnight. Following 4 washes with PBS, the FITC fluorescence signal was detected at 16 individual positions per well in a multilabel plate reader (PerkinElmer EnVision). For the calculation of the titers, the average values from the 16 fluorescence-measuring points were used to determine the best-fitting Hill curves using Prism 6 software (GraphPad Software). The HIV-1 gp120-specific monoclonal antibody b12 was used as a negative control.

Reversibility of neutralization. Highly concentrated (ca. 3×10^9 $TCID_{50}$/ml), sucrose cushion-purified stocks of A/Puerto Rico/8/1934 (H1N1) or unpurified A/California/07/2009(H1N1), A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007(H3N2), or A/Chicken/Vietnam/C58/2004(H5N3) virus were diluted 1:5 to 1:30 in 900 μl of D/B/H medium, depending on the titers of the stocks. To obtain a final antibody concentration of 10 μg/ml, 100 μl of the indicated MAb diluted in the same medium was added to the virus. The mixture was then incubated at 37°C-5% CO_2 for 2 h before it was combined with 120 μl of 1:10-diluted and PBS-washed MagnaBind amine-derivatized magnetic beads (Pierce) and incubated at 37°C-5% CO_2 on a rotator for 1 h. Next, 300 μl of the 1-ml bead-virus-MAb mixture was transferred into 2 individual tubes, with each tube representing one of the test conditions: no dissociation and long-term dissociation. Long-term-dissociation beads were washed 1 time with 400 μl of D/B/H medium and resuspended in 1 ml of D/B/H medium without antibody. In parallel, no-dissociation tubes were filled up to 1 ml with D/B/H medium supplemented with the appropriate MAb (final concentration, 10 μg/ml). The tubes were then incubated at 37°C-5% CO_2 on a rotator for 14 h. After this incubation, the supernatant was removed and 100 μl of D/B/H medium supplemented with 10 μg/ml of the indicated MAb was added to the no-dissociation tube. In parallel, the long-term-dissociation samples were washed with D/B/H medium

and resuspended in 100 μl of D/B/H medium, and 50 μl of resuspended beads was then transferred to MDCK cells seeded at a density of 2×10^4/well in 96-well plates 1 day before. The plates with cells were incubated at 37°C-5% CO_2 for 30 min, placed on an orbital shaker for a few seconds to resuspend the beads, and transferred back to the incubator for an additional 30 min. After this incubation, the beads were again resuspended on an orbital shaker and aspirated, and the cells were washed 1 time with PBS. Further incubation and detection of infectivity were performed as described for the neutralization assay. The HIV-1 gp120-specific antibody b12 was used as a nonneutralizing control.

Mouse protection experiments. To assess the protective capacity of MAb 1.12, 6- to 8-week-old female C57BL/6 mice were injected intraperitoneally (i.p.) in the lower right abdominal quadrant with 3 or 10 mg/kg of body weight of the indicated antibody in 200 μl PBS 24 h before the animals were anesthetized by i.p. injection with 0.05 mg/kg fentanyl, 5 mg/kg midazolam, 0.5 mg/kg medetomidin. After 5 to 10 min, when surgical tolerance was reached, the mice were weighed and allowed to aspirate 25 μl of A/Puerto Rico/8/1934(H1N1) or mouse-adapted A/Hong Kong/1/68(H3N2) (27) corresponding to 3 50% lethal doses (LD_{50}). The mice were then placed on a heating pad to prevent hypothermia, and virus was allowed to be further aspirated for 10 min before the anesthetics were antagonized by i.p. injection with 2.5 mg/kg atipamezolin, 1.2 mg/kg naloxon, 0.5 mg/kg flumazenil. Weight loss was monitored daily, and mice that had lost more than 20% (first experiment) or 15% (second experiment) of their initial body weight were euthanized and scored as dead.

Neutralization of virus particles attached to cell surfaces. MDCK cells were seeded at 2×10^4 cells/well 1 day before the experiment. To prevent virus internalization during the subsequent step, the cells were first chilled to 4°C for 15 min and then placed on ice for 15 min before they were washed with ice-cold PBS. To preadsorb virus on the cells, A/Puerto Rico/8/1934(H1N1) or A/California/07/2009(H1N1) virus at an MOI of ~3 was added to half of the plates and incubated at 4°C for 1.5 h. In parallel, the other half of the MDCK cell plates were prepared using the same procedure but without the addition of virus (virus-in-solution control sample). Both plates were then washed once with ice-cold PBS. MAbs titrated in D/B/H medium were added to the virus-preadsorbed-on-cells plate, while only ice-cold D/B/H medium was added to the virus-in-solution control plate. Both plates were then incubated at 4°C for 2 h. In parallel, A/Puerto Rico/8/1934(H1N1) or A/California/07/2009(H1N1) virus (from the same initial dilution kept at 4°C) corresponding to an MOI of ~3 was mixed with ice-cold titrated MAbs on a dilution plate and incubated at 4°C for 2 h. Both MDCK cell-containing plates were then washed once with prewarmed PBS. The virus-preadsorbed-on-cells plate was covered with 100 μl of prewarmed D/B/H medium, while the virus-in-solution plate was incubated with the virus and MAb mixture from the titration plate. Infectivity was later detected as described for the neutralization assay. The HIV-1 gp120-specific monoclonal antibody b12 was used as a control.

Competition with human sera. To establish the serum dilutions for the competition assay, the concentrations giving saturating binding were first determined by ELISA on recombinant HA from A/Puerto Rico/8/1934(H1N1). In parallel, the neutralizing activity was determined against the same isolate. Two distinct serum concentrations were chosen: first, the "optimal" dilution, which showed saturating binding but little neutralization, and second, the "neutralizing" dilution, which contained three times as much serum and therefore provided clear yet incomplete virus neutralization (see Fig. 4). To determine potential competition between sera and the monoclonal antibodies, 20 μl of concentrated, sucrose cushion-purified H1N1 A/Puerto Rico/8/1934 virus stock (ca. 3×10^9 $TCID_{50}$/ml) diluted 1:405 in D/B/H medium was combined with 20 μl of the indicated human serum at the indicated dilution and incubated at 37°C-5% CO_2 for 1 h. As a control, no serum or HIV-1-specific MAb b12 was used. Following this incubation, 20 μl of hMAb was added to the virus-serum mixture to obtain a final concentration of 1 or 10 μg/ml. This mixture was then incubated at 37°C-5% CO_2 for 1 h to allow binding of the monoclonal antibodies. To determine the residual infectivity after the incubation, 50 μl was transferred to MDCK cells and incubated at 37°C-5% CO_2 for 1 h to initiate infection. All subsequent steps were performed as described for staining in neutralization assays.

Affinity measurement. For determination of the affinities of MAbs, a CM5 Biacore chip was covalently coated with goat anti-human Fc (Bethyl Laboratories; A80-104 A) at 0.1 mg/ml to a final density of 1,342 response units (RU) before purified MAb 1.12 or 3.1 at 0.01 mg/ml was captured to a level of 120 RU each. After recording association and dissociation sensograms of recombinant HA concentration series (0.625, 1.25, 2.5, 5, and 10 nM for HA1; 4 and 5 nM and 5, 10, 20, 40, and 80 nM for HA3 and HA12, respectively) at a flow rate of 30 μl/min, the data were fitted to a simple 1:1 binding model (T100 Evaluation Software; Biacore) and the k_{on} (association constant), k_{off} (dissociation constant), and K_D (equilibrium dissociation constant) were calculated.

Ethics statements. All human samples used for this study were obtained from adult volunteers in the context of a broader, accompanying study (EK-17-42) that has been reviewed and approved according to Swiss human subject legislation by the ethics commission of the Canton of Zurich, Switzerland. Written informed consent has been obtained from all participating volunteers.

All animal experiments performed for this study have been reviewed and approved according to the Swiss National Animal Welfare Act under license number 121/210 by the Cantonal Veterinary Office of Zurich, Switzerland.

RESULTS

To assess the functional, kinetic, and steric properties of HA stem-reactive antibodies, a test panel of three heterosubtypic MAbs was compiled (hMAbs 1.12, 3.1, and FI6). One of these antibodies, hMAb 1.12, was isolated for this study from a healthy donor using phage display. The second antibody, hMAb 3.1 (16), is genetically related to the well-characterized hMAb FI6 (9) but of more limited breadth (primarily H1a clade). hMAb 1.12 was very remarkable in that it neutralized multiple influenza A virus strains belonging to HA subtypes 1 through 15. Moreover, hMAb 1.12 was found to bind recombinant HA protein from the recently isolated H17 and H18 subtypes (Fig. 1a and b). Binding-competition assays revealed that 1.12 recognizes an epitope overlapping the previously described epitope for the stem-reactive prototype hMAb c179 (Fig. 1c) (5, 28). Like other hMAbs, the *in vivo* prophylactic protective efficacy of hMAb 1.12 was evaluated in C57BL/6 mice (Fig. 2a). To this end, mice were injected intraperitoneally with 10 or 3 mg/kg of hMAb 1.12 24 h before intranasal infection with 3 LD_{50} of A/Puerto Rico/8/1934(H1N1) or A/Hong Kong/1/1968(H3N2). The experiment was conducted with 5 mice per group and a 20% weight loss abortion criterion (Fig. 2a) and repeated with 5 mice per group and a more stringent abortion criterion of 15% maximal weight loss [only for A/Puerto Rico/8/1934(H1N1)] (not shown). For A/Puerto Rico/8/1934(H1N1), when data from both experiments were pooled, it was found that at 10 mg/kg, 8 out of 10 animals were protected, whereas at 3 mg/kg, 9 out of 10 mice still survived. In the case of A/Hong Kong/1/1968(H3N2), all mice were protected at 10 mg/kg and 4 out of 5 at the 3-mg/kg dose. The surviving mice did not show apparent signs of morbidity and displayed only moderate weight loss. Interestingly, the time point of antibody application had an influence on protection by MAb 1.12. Intraperitoneal application 3 h before infection conferred the lowest level of protection, whereas intravenous injection 2 h before infection was protective to all animals at both tested doses (15 and 5 mg/kg; 3 mice per group). To further understand the aforementioned differences,

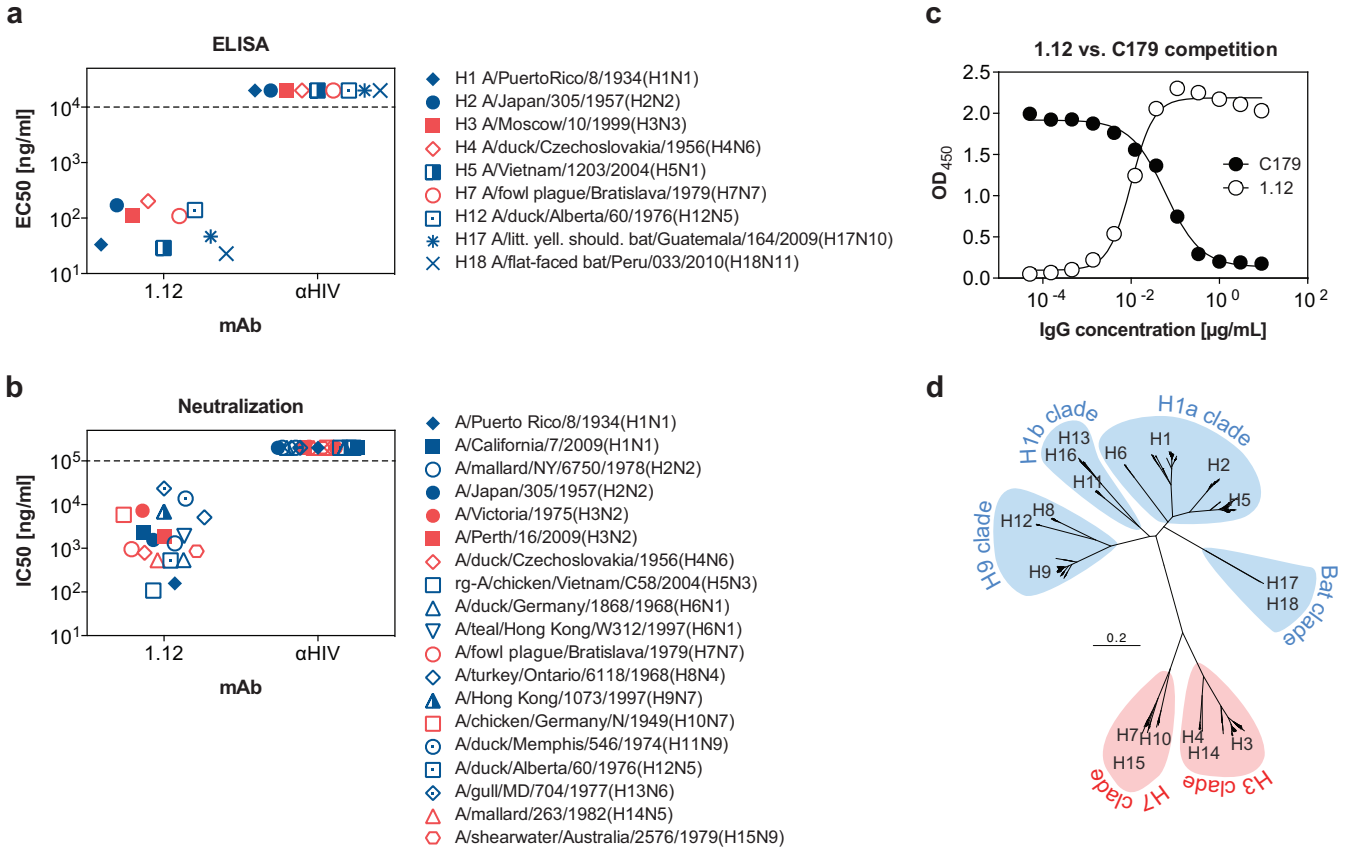

FIG 1 Specificity of hMAb 1.12. (a) Half-maximal binding concentrations (EC_{50}s) of hMAb 1.12 to recombinantly expressed HA proteins from 9 subtypes. The data represent the means of EC_{50}s obtained in two independent experiments. (b) Half-maximal neutralizing concentrations (IC_{50}s) of MAb 1.12 to a panel of 19 viruses from 15 subtypes. The HIV-1 gp120-specific MAb b12 was used as a negative control in both experiments (αHIV). A representative of at least 2 independent, consistent experiments performed in triplicate is shown. Avian viruses are depicted with open symbols, human isolates with solid symbols, and bat isolates with asterisk-like symbols. Isolates with zoonotic potential are designated with mixed symbols. Isolates from phylogenetic group 1 are depicted in blue and those of phylogenetic group 2 in red. A dashed line is used to indicate the detection limit of the assay. (c) The epitope recognized by hMAb 1.12 was roughly evaluated in a binding-competition ELISA using HA stem-reactive hMAb c179. ELISA plates coated with purified HA from A/Puerto Rico/8/1934(H1N1) were incubated with titrated amounts of hMAb 1.12, washed, and later incubated with a fixed concentration (1 μg/ml) of the murine hMAb c179. Binding of both antibodies was then detected using species-specific secondary antibodies. (d) Phylogenetic tree for all 18 HA subtypes. At total of 1,339 arbitrarily chosen recent nonidentical HA amino acid sequences (from the year 2000 to the present for frequent isolates; from 1985 to the present for rare isolates) were aligned using Muscle 3.8 (34). The tree was built using "neighbor" from the Phylip 3.69 software package (http://evolution.genetics.washington.edu/phylip.html) and illustrated as a rooted tree in FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree). Phylogenetic group 1 is indicated in blue and group 2 in red.

mice were injected intravenously with 5 or 15 mg/kg of hMAb 1.12. Serum antibody titers determined after 3 days were found to be a third of the serum antibody concentration measured 1 h after injection (data not shown).

The reversibility of heterosubtypic neutralization was then assessed by preincubation of A/Puerto Rico/8/1934(H1N1), A/California/07/2009(H1N1), A/Brisbane/59/2007(H1N1), A/Brisbane/10/2007(H3N2), or A/Chicken/Vietnam/C58/2004(H5N3) virus in solution with 10 μg/ml of one of the hMAbs from our panel or with strain-specific, apically binding MAbs H36-4 (29) and 30D1 (30) (Fig. 2b). The preincubated virus particles were then captured on magnetic beads, and two different dissociation protocols were performed: no dissociation, where captured virus was incubated in the presence of the antibody for 17 h, and long-term dissociation, where antibodies were allowed to dissociate for 14 h before the virus was added to the cells. As depicted in Fig. 2b, even after a prolonged dissociation time of 14 h, virus neutralization was indistinguishable from that of virus permanently incubated in the presence of the antibodies [with the exception A/Brisbane/10/2007(H3N2) neutralization by hMAb FI6, showing partial dissociation of the antibody]. Minor differences seen in A/California/7/2009(H1N1) can largely be attributed to poorer capturing of these virions on beads, and therefore to a poorer signal-to-background ratio, resulting in more noise for the isolate. Nonetheless, it can be concluded that the heterosubtypic epitope is very accessible on free virus particles in solution and that both homo- and heterosubtypic neutralizations are virtually irreversible.

To test whether hMAbs have access to their epitope after virus attachment to the cell surface, we compared the neutralizing activities of hMAbs 1.12, 3.1, and FI6 against both free and cell-attached A/Puerto Rico/8/1934(H1N1) and A/California/07/2009(H1N1). As depicted in Fig. 3, neutralization of cell-attached virus was found to be considerably less (about 2 orders of magnitude or more, depending on the antibody and isolate used) efficient than that of free virions. This was seen for all stem-reac-

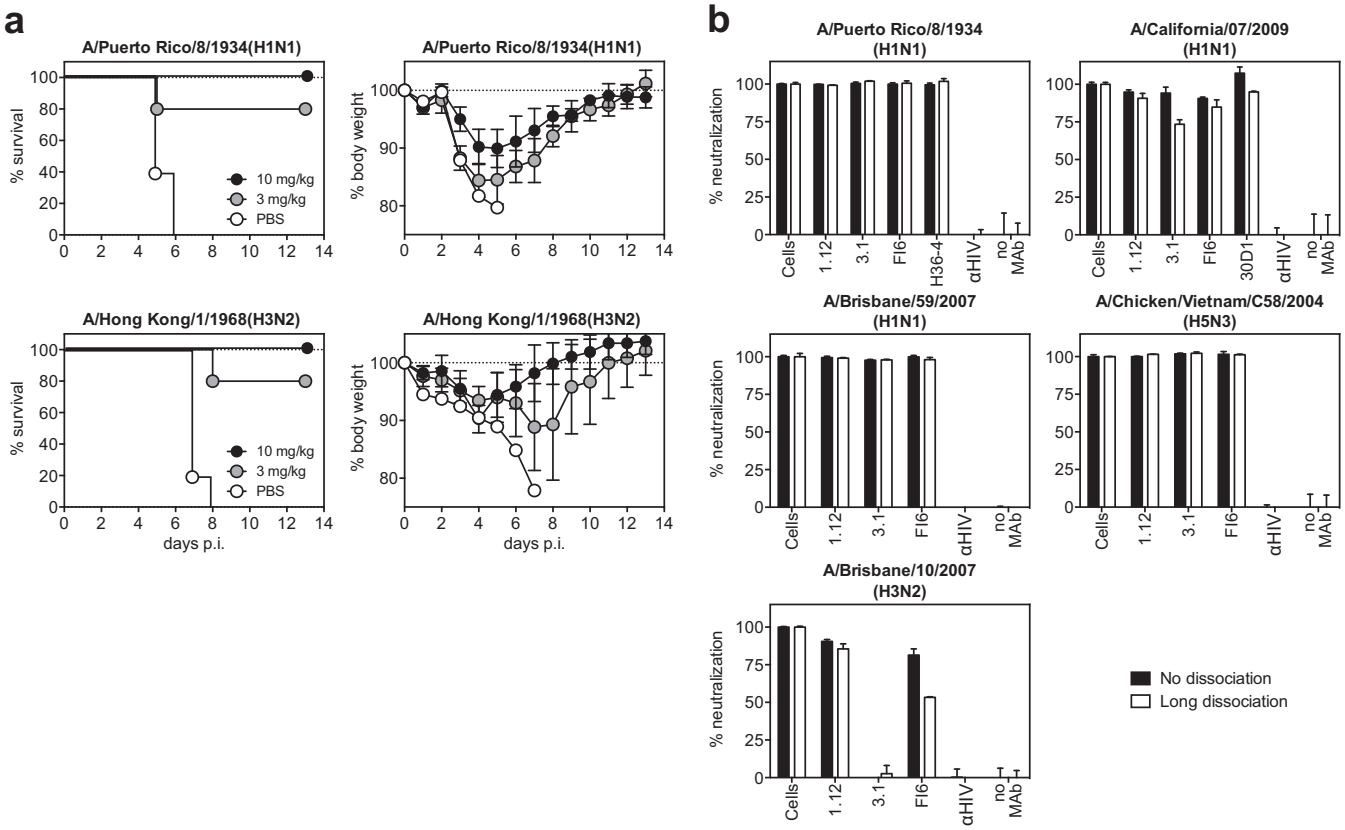

FIG 2 Protection of mice and reversibility of neutralization. (a) Passive immunization of mice. Animals were injected intraperitoneally with the indicated dose of hMAb 1.12 in PBS or with PBS alone (control group) 24 h prior to intranasal infection with a lethal dose of A/Puerto Rico/8/34(H1N1) or mouse-adapted A/Hong Kong/1/1968(H3N2). Mice that dropped below 80 or 85% of their initial body weight were scored as dead and euthanized. For A/Puerto Rico/8/1934(H1N1), pooled data from two separate experiments are shown, each performed with 5 C57BL/6 females per group. One experiment was performed with 15% and one with 20% weight loss as the abortion criteria. For mouse-adapted A/Hong Kong/1/1968(H3N2), only the depicted experiment with a 20% weight loss abortion criterion was performed. (b) Reversibility of neutralization. Three HA stem-reactive antibodies were incubated at a concentration of 10 μg/ml with A/Puerto Rico/8/1934(H1N1), A/California/07/2009(H1N1), A/Brisbane/59/2007(H1N1), A/Brisbane/10/2007(H3N2), or A/Chicken/Vietnam/C58/2004(H5N3) virus (amounts corresponding to an MOI of ~ 30) before the MAb-virus mixture was captured on magnetic beads. The beads were then processed so that no-dissociation and long-term-dissociation conditions were applied. In the last step, the residual infectivity of each sample was measured on MDCK cells. The HIV-1 gp120-specific MAb b12 was used as a negative control (αHIV). Uninfected and infected cells were included as positive and negative controls, respectively. Representatives of at least 2 consistent experiments (performed in duplicate) are shown. The error bars indicate standard deviations.

tive hMAbs tested, suggesting that these antibodies cannot effectively access their epitopes on hemagglutinin spikes on cell-bound virus. In contrast, the strain-specific and apically binding MAbs 30D1 and H36-4 were both able to neutralize cell-bound virus almost as efficiently as free virus (~11- to 13-fold differences versus 93- to 2,119-fold in the case of hMAbs).

The protective capacity of heterosubtypic antibodies has primarily been determined by passive immunization of seronegative animals. However, since it has been speculated that apically binding strain-specific antibodies may sterically restrict access to the membrane-proximal heterosubtypic epitopes, we tested the neutralizing activities of the antibodies against A/Puerto Rico/8/1934(H1N1) virus particles saturated with human serum antibodies (Fig. 4). To this end, 7 sera that displayed high binding but poor neutralizing titers against this isolate were identified during an independent study (29). For 4 of these sera, we were able to determine concentrations that were saturating but subneutralizing (Fig. 4). Indeed, when virus was incubated with these human sera at the corresponding concentrations (or left mock treated), no reduction in the neutralizing activity resulted from saturation of the virions with human serum antibodies. When both serum and our MAbs were applied at subneutralizing concentrations, additive neutralization was observed (Fig. 4). Thus, strain-specific human serum antibodies do not interfere with neutralization by stem-reactive hMAbs.

DISCUSSION

With this study, we introduced a new hMAb whose breadth is exceeded only by that of hMAb CR9114 (6). Quite interestingly, the two antibodies share common features in that both are encoded by the V_H1-69 V gene-joined J_H6, and both display an extraordinary stretch of four or five tyrosine residues in HCDR3 of the heavy chain. Although the structure of hMAb 1.12 has not been solved yet, it is safe to assume that, like other V_H1-69-encoded antibodies, the central contacts are made by the heavy chain. Indeed, during phage display, many phages that used the 1.12 heavy chain in combination with different light chains were isolated. Unfortunately, and in contrast to hMAb CR9114, hMAb 1.12 does not recognize HA from the influenza B virus genus, but so far, the molecular reasons for this difference re-

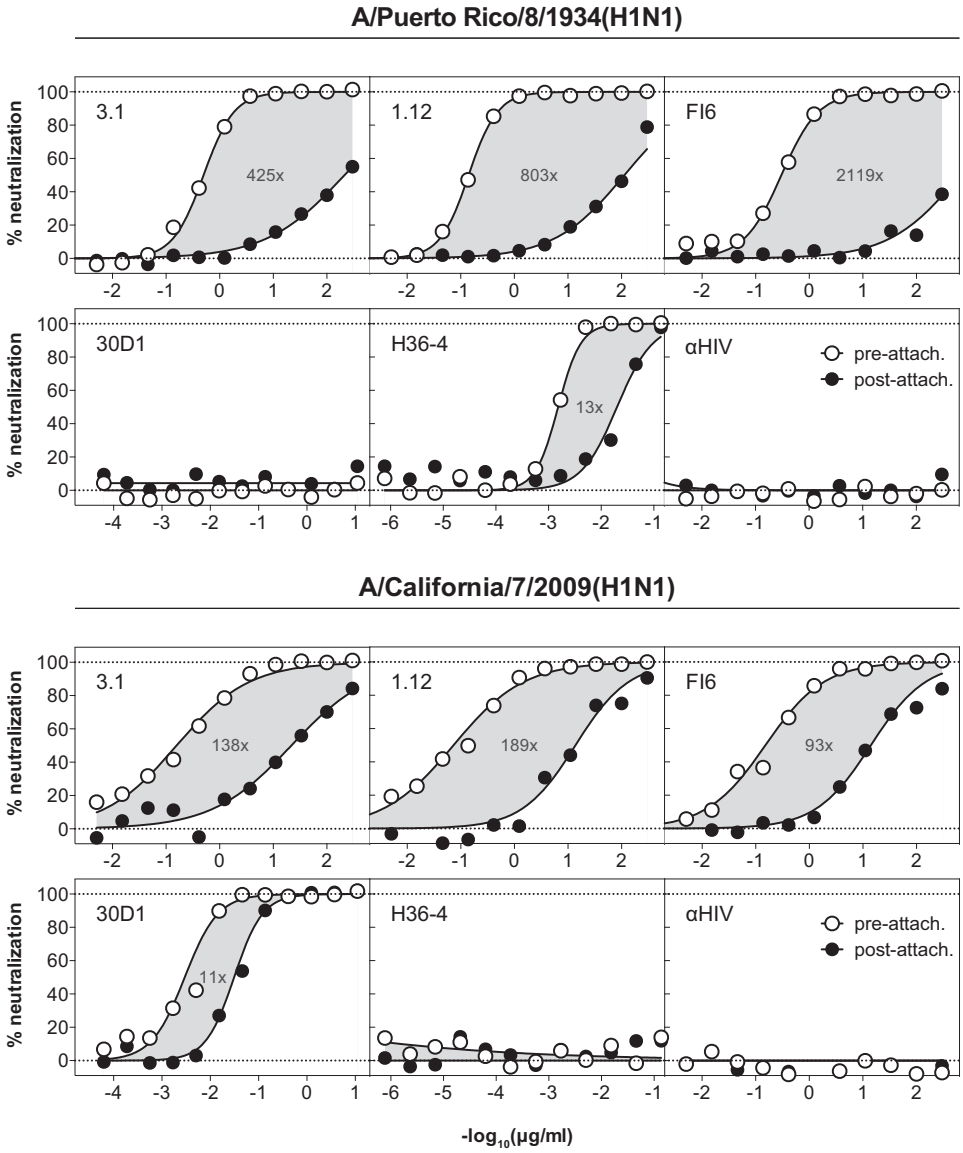

FIG 3 Neutralization of virus attached to cell surfaces. A/Puerto Rico/8/1934(H1N1) or A/California/7/2009(H1N1) virus was preadsorbed to MDCK cells at 4°C to avoid virus internalization. The attached viruses were then incubated with titrated amounts of HA stem-reactive (3.1, 1.12, and FI6) or hemagglutination-inhibiting (30D1 and H36-4) MAbs, and residual infectivity was detected. As a control, viruses were mock incubated in cell-free medium before titrated amounts of the MAbs were added. The HIV-1 gp120-specific MAb b12 (αHIV) was used as a nonneutralizing control. Gray areas highlight the differences between the neutralization curves, and numbers indicate the fold difference in the corresponding IC_{50}s. Representatives of at least 2 independent, consistent experiments (performed in duplicate) are shown.

main elusive. In our hands, hMAb FI6, which genetically is more closely related to hMAb 3.1 than to 1.12, struggled to neutralize A/Chicken/Germany/N/1949(H10N7) and to some degree also A/fowl plague/Bratislava/1979(H7N7), but otherwise, it performed comparably in *in vitro* neutralization assays.

We found that neutralization by hMAbs and strain-specific monoclonal antibodies was virtually irreversible. In the cases of hMAbs 1.12 and 3.1, the slow equilibrium dissociation constant (Table 1) (16) observed in Biacore most likely enables these antibodies to stay attached for a long time. In addition, the dense packing of HA proteins on the virus envelope (31) and the membrane-proximal location of the epitope are likely to further slow down dissociation in that detached antibodies probably reattach rather than diffuse into the supernatant. Induction of conformational changes rendering the HA protein nonfunctional, as postulated for gp120-specific MAbs (32), appears unlikely, as no evidence for antibody-induced conformational changes was found in crystal structures. Strain-specific antibodies, which typically are 1 or 2 orders of magnitude more potent than stem-specific antibodies, were found to neutralize virus virtually irreversibly.

So far, we have no explanation for the poor neutralization of cell-attached virus. Based on the postulated aggregation of an estimated six HA molecules required for fusion (three of which have to undergo a conformational change [33]), we concluded that a cluster of HA molecules is formed whose center is not accessible to hMAbs. Accordingly, three or more HA spikes can still undergo

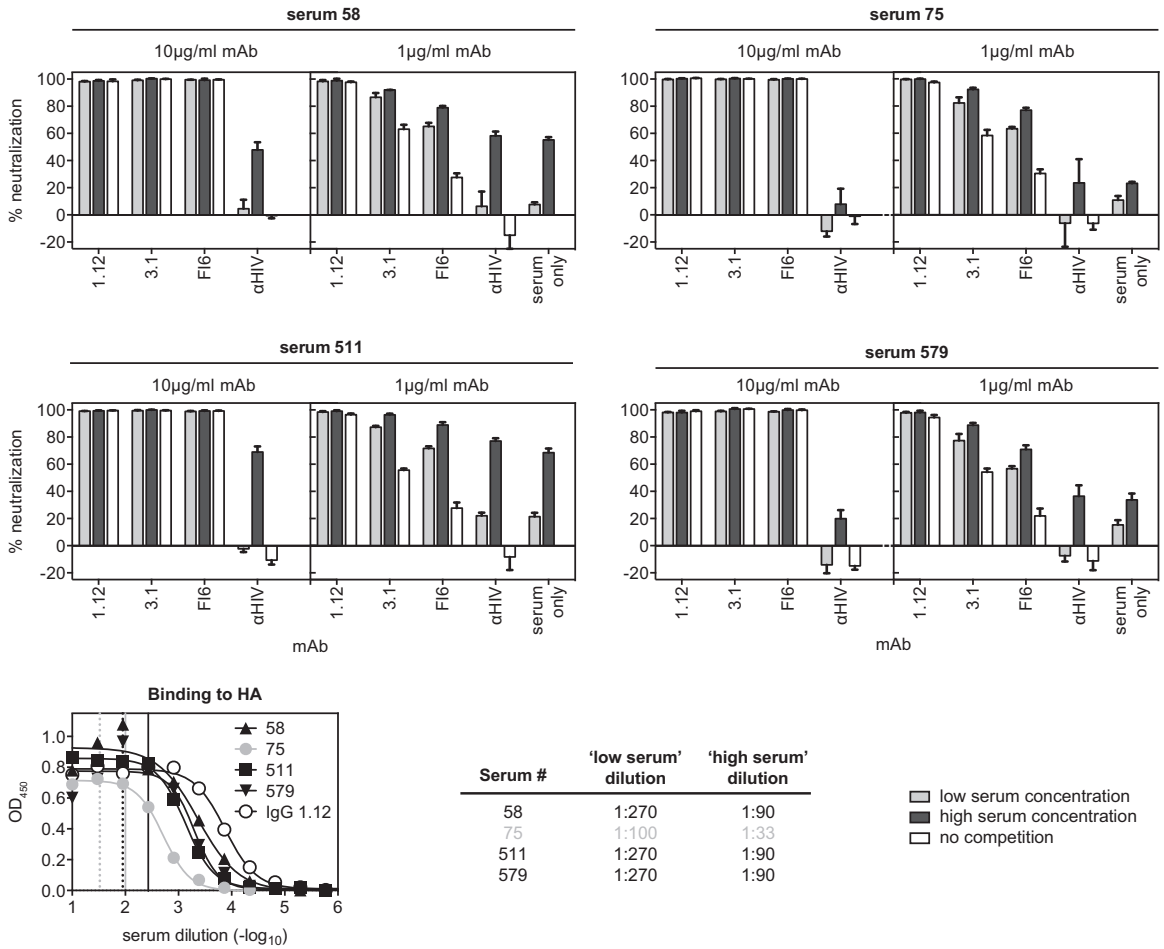

FIG 4 Competition with human sera. A/Puerto Rico/8/1934(H1N1) virus was first incubated with sera from the indicated donors for 1 h before the indicated hMAbs were added at a concentration of 10 μg/ml. Residual infectivity of the sample was evaluated and compared to the infectivity of samples without preincubation with human serum. The low serum concentration corresponds to the dilution giving saturated signals in ELISA while having only minor neutralizing activity against the virus. The high serum concentration is three times higher than the low concentration. Representatives of 2 independent, consistent experiments (performed in triplicate) are shown. The error bars indicate standard deviations.

the confirmation change required for infection (33). We believe that the lower impact of prior cell attachment on the neutralizing activity of hemagglutination-inhibiting antibodies originates in the poor affinity of the HA protein for its receptor: apically binding antibodies should have no problem with displacing dissociated receptors if they are sufficiently affine and concentrated. Based on present data, it is not clear why cell-attached A/California/7/2009(H1N1) was less resistant to heterosubtypic neutralization than A/Puerto Rico/8/1934(H1N1). We believe that the extensive adaptation of A/Puerto Rico/8/1934(H1N1) to cell culture probably contributed to this observation.

The densely packed arrangement of the HA and NA spikes on the surfaces of influenza A virions (31), in combination with the membrane-proximal location of the stem epitope, led to speculations that access to these epitopes could be difficult. Moreover, it has been reasoned that this access may even be further restricted if the virions are saturated with preexisting homotypic antibodies that are binding to the strain-specific apical epitopes. If true, this hypothesis could pro-

TABLE 1 Binding avidities for MAb 1.12[a]

Isolate	k_{on} (1/mol·s)	k_{off} (1/s)	K_D (M)
A/Puerto Rico/8/1934(H1N1)	4.24×10^5	$\leq 5.0 \times 10^{-5}$	$\leq 1.2 \times 10^{-10}$
A/Moscow/10/1999(H3N2)	2.95×10^4	$\leq 5.0 \times 10^{-5}$	$\leq 1.7 \times 10^{-9}$
A/Duck/Czechoslovakia/1956(H4N6)	5.81×10^5	2.9×10^{-4}	5.0×10^{-10}
A/Vietnam/1203/2004(H5N1)	1.06×10^6	$\leq 5.0 \times 10^{-5}$	$\leq 4.7 \times 10^{-11}$
A/Duck/Alberta/60/1976(H12N5)	1.08×10^5	6.6×10^{-5}	6.1×10^{-10}

[a] hMAb 1.12 was immobilized on an anti-F_c-coated CM5 chip before binding of soluble recombinant HA was assessed, and binding constants were calculated on a Biacore instrument.

vide an explanation for the poor immunogenicity of the epitope in humans, but it would also call into question the efficacy of passive or active immunization targeting this epitope in humans, as strain-specific antibodies can be found in most individuals. However, we showed that neither concern proved to be relevant *in vitro*. Accordingly, we do not predict that a universal influenza virus vaccine or therapy based on stem-reactive hMAbs would be impaired by the presence of preexisting strain-specific humoral immunity. However, their action may be limited against cell-associated viruses.

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