

Neutralizing and protective epitopes of the 2009 pandemic influenza H1N1 hemagglutinin

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Aims and Methods To facilitate antigenic characterization of the influenza A 2009 pandemic H1N1 [A(H1N1)pdm09] hemagglutinin (HA), we generated a panel of murine monoclonal antibodies (mAbs) using as the immunogen mammalian-derived virus-like particles containing the HA of the A/California/04/2009 virus. The antibodies were specific for the A/California/04/2009 HA, and individual mAbs suitable for use in several practical applications including ELISA, immunofluorescence, and Western blot analysis were identified.

Results and Conclusions As the panel of mAbs included antibodies with hemagglutination inhibition (HI) and virus neutralizing activities, this allowed identification and characterization of potentially important antigenic and neutralizing epitopes of the A/California/04/2009 HA and comparison of those epitopes with the HAs of other influenza viruses including seasonal H1N1 viruses as well as the A/South

Carolina/1918 and A/New Jersey/1976 H1N1 viruses. Three mAbs with the highest HI and neutralizing titers were able to provide passive protection against virus challenge. Two other mAbs without HI or neutralizing activities were able to provide partial protection against challenge. HA epitopes recognized by the strongest neutralizing mAbs in the panel were identified by isolation and selection of virus escape mutants in the presence of individual mAbs. Cloned viruses resistant to HI and antibody neutralization were sequenced to identify mutations, and two unique mutations (D127E and G155E) were identified, both near the antigenic site Sa. Using human post-vaccination sera, however, there were no differences in HI titer between A/California/04/2009 and either escape mutant, suggesting that these single mutations were not sufficient to abrogate a protective antibody response to the vaccine.

Keywords Hemagglutinin, monoclonal antibody, protective epitopes.

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Introduction

The novel swine-origin influenza H1N1 virus that emerged in 2009 as the first influenza pandemic of the 21st century differed in several notable ways from influenza H1N1 viruses that were circulating in the human population at that time.¹ This virus was similar in genetic make-up to triple-reassortant swine influenza viruses that had circulated in swine during the preceding 10 years but that only had sporadically infected humans.² The HA, NA, NP, M, and NS gene segments of the triple-reassortant swine influenza viruses originated from classical swine lineage viruses; the PA and PB2 gene segments were of North American avian origin; the PB1 gene segment had a human influenza virus origin. However, in the triple-reassortant pandemic 2009 H1N1 [A(H1N1)pdm09] virus, the NA and M genes had a Eurasian swine origin that had not been previously detected in swine influenza viruses isolated in the United States.^{3,4}

Because of the importance of antibodies to the virus hemagglutinin (HA) for protection against influenza, the antigenic relationship and relatedness of the A(H1N1)pdm09 virus HA to the HA of seasonal influenza viruses circulating in 2009 was of particular significance. H1N1 viruses circulated and drifted antigenically in humans from the 1918 pandemic until replaced by H2N2 in the 1957 pandemic. Upon its reemergence in 1977 and until 2009, further antigenic drift of the H1N1 HA occurred. Although having a common origin with the 1918 H1N1, the swine H1N1 HA remained relatively stable antigenically from 1930 until the late 1990s, when the swine triple-reassortants likely emerged. Subsequently, there was an increased genetic and antigenic diversity in the swine H1N1 HA.⁵ The net result of the two different evolutionary paths was a substantial antigenic divergence between circulating strains of seasonal H1N1 in humans and the swine H1N1 viruses that gave rise to the 2009 H1N1 pandemic virus.

Several reported studies have attempted to assess the extent of cross-reactivity between antibodies to the 2009 H1N1 pandemic virus and recent seasonal influenza viruses, and to earlier H1N1 isolates such as those from the 1918 H1N1 pandemic or the 1976 swine influenza outbreak in New Jersey. In most cases, there appears to be some level of 2009 H1N1 pandemic cross-reactive antibody in persons older than 55–60 years of age, corresponding with exposure to H1N1 viruses before the 1957 pandemic.^{6–8} Further, cross-reacting antibodies to the A(H1N1)pdm09 virus appear associated with 1976 swine influenza vaccination,^{8–10} consistent with the relatively close genetic relationship between those viruses. On the other hand, there are some conflicting data regarding the cross-reactivity of the 2009 pandemic virus and recent seasonal H1N1 viruses. Some studies have reported little, if any, cross-reactivity,^{6,11,12} but others have indicated some limited antibody cross-reactivity.^{13–17} Differences in study design and methodologies likely account for such different conclusions, but overall it appears that cross-reactivity of antibodies to the A(H1N1)pdm09 HA and seasonal H1N1 HA is at least limited.

To facilitate antigenic characterization of the A(H1N1)pdm09 HA, we generated a panel of murine monoclonal antibodies (mAbs) to the HA of A/California/04/2009. These hybridoma clones were generated using mammalian-derived influenza virus-like particles (VLP) containing the HA of the A/California/04/2009 virus as the immunogen. We describe the initial characterization of these mAbs, the identification of several antibodies with neutralizing and hemagglutination activity, and the evaluation of antibodies for their protective effect in mice when administered passively before influenza virus challenge. The HA epitopes recognized by strongly neutralizing mAbs were identified by the isolation of virus escape mutants. This panel of mAbs allowed comparison of antigenic and neutralizing epitopes between the A/California/04/2009 HA and the HAs of related H1N1 viruses and may help identify important HA epitopes involved in future antigenic drift.

Materials and methods

Cells and viruses

Influenza viruses were propagated in 9-day-old specific pathogen-free embryonated chicken eggs. Modified vaccinia virus Ankara (MVA) vectors expressing influenza HA have been described previously^{18,19} and were propagated in chicken embryo fibroblasts (CEF) cells and titered by plaque assay on DF-1 cells. All methods for the generation, propagation, and preparation of recombinant viruses were essentially as described by Earl *et al.*²⁰ Influenza VLP containing only the HA from the A(H1N1)pdm09 virus were prepared by MVA vector infection of Vero cells and puri-

fied on 10–45% sucrose gradients as previously described.¹⁸ Primary CEF cells were obtained from Charles River Laboratories, Wilmington, MA, USA; DF-1 and Vero cells were originally obtained from ATCC (CRL-12203 and CCL-81, respectively). All cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS (HyClone, Logan, UT, USA), 2 mm l-glutamine, and 50 µg/ml gentamicin.

mAbs to A/California/4/2009 HA

Monoclonal antibodies to A/California/4/2009 HA were prepared by Precision Antibody (Columbia, MD, USA). BALB/c mice were immunized and boosted with mammalian-derived VLP containing only the influenza A/California/4/2009 HA. When antibody titers were >1:50 000 in an HA VLP ELISA analysis, spleen cells were fused with a myeloma line. From more than 35 initial hybridoma clones, 11 were selected and expanded for further testing. mAbs from tissue culture supernatant or from mouse ascites fluids (Harlan Bioproducts for Science, Inc., Madison, WI, USA) were purified by Protein G chromatography.

Passive transfer of mAbs and animal challenge

Specific pathogen-free female BALB/c mice (Taconic Farms, Germantown, NY, USA) were housed in a BSL2 facility at CBER/FDA with *ad libitum* access to water and feed. All antibody transfers, and challenges were performed in accordance with an approved animal protocol; the procedure was similar to that described previously.⁹ At the age of 9–10 weeks, mice in groups of five were injected intravenously (i.v.) with 100 µl per mouse of either PBS or mAbs (0.5 mg/ml). At 4 hours after antibody transfer, mice were challenged intranasally with 1 LD₅₀ of wild-type A/Mexico/4108/2009 virus (50 µl/mouse) under light anesthesia of isoflurane. Body weight and mortality were monitored daily for 2 weeks after infection.

Immunofluorescence analysis

MDCK cells were infected with A/California/04/2009 over a range of multiplicities in 12-well plates. At 3 days post-infection, cells were fixed with 4% paraformaldehyde (1 hour, room temperature) in Tris-buffered saline (TBS). After washing with TBS, cells were permeabilized with 0.1% Triton-X-100 in TBS for 15 minutes, followed by paraformaldehyde quenching with TBS supplemented with 100 mm glycine. After washing, cells were incubated with primary antibody (2 µg/ml in TBS/20% fetal bovine serum/0.1% Triton-X-100) for 1 hour. Goat anti-mouse FITC-conjugated secondary antibody (Southern Biotech, Birmingham, AL, USA) was used for detection. Control antibodies were a mouse monoclonal antibody against influenza NP (BEI Resources, Manassas, VA, USA) and a monoclonal antibody to β-galactosidase (Promega, Madison,

WI, USA). Immunostained cells were observed using a Zeiss Axiovert 40 CFL microscope with an X-Cite Series 120 Q fluorescent light source. Images were acquired using Carl Zeiss Imaging System software and an AxioCam MRm camera and exported in TIFF format.

Western blot analysis

Inactivated influenza viruses were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and western blot analysis as previously described.¹⁸ Detection was by chemiluminescence (SuperSignal West Dura extended-duration substrate; Pierce, Rockford, IL, USA) using a LAS-3000 imager (Fujifilm Medical Systems, Stamford, CT, USA).

ELISA

Sucrose gradient purified VLPs or inactivated influenza viruses were used as capture antigens in ELISA as previously described.¹⁸ The endpoint titer was defined as the highest dilution of antibody that gave an absorbance (405 nm) value that was greater than that of a matched dilution of control antibody of the same isotype and was also >0.050.

Hemagglutination inhibition assay

The hemagglutination inhibition assay (HI) was performed in 96-well plates (U-bottom) by a standard method, essentially as described previously²¹ using 0.5% chicken red blood cells suspended in PBS (pH 7.2).

Human serum samples

Paired serum samples (pre- and post-vaccination) from healthy human volunteers vaccinated with 2010–2011 Southern Hemisphere trivalent influenza vaccine were kindly provided by Dr. Masato Tashiro (National Institute of Infectious Diseases, Tokyo, Japan) in support of the WHO consultation on annual influenza vaccine strain selection. A written informed consent was provided to all volunteers at the beginning of the enrollment by each physician according to the guidelines of the corresponding institutional review board. Healthy adults (20–58 years old) and elderly (62–100 years old) subjects were given a single dose (15 µg HA/strain/dose) of 2010–2011 trivalent inactivated vaccine intramuscularly. The vaccine was formulated according to the Southern Hemisphere seasonal vaccine composition containing A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008) as recommended by WHO. Sera were collected at the time of enrollment and 21 days after vaccination.

Virus neutralization assay

The influenza virus neutralization assay (VN) was performed essentially as described previously.²² The presence of virus was detected using biotin-conjugated antibody to

influenza NP (clone A; Milipore, Billerica, MA, USA) followed by HRP-labeled Streptavidin (KPL).

HA-pseudotype neutralization assay

Construction of retroviral pseudotypes expressing the influenza HA of A/California/04/2009, A/New Caledonia/20/1999, A/Solomon Islands/3/2006, A/Brisbane/59/2007, and A/New Jersey/1976 has been described previously.^{10,23} The A/South Carolina/1918 HA expression plasmid was obtained from Dr. Gary J. Nabel (National Institutes of Health, Bethesda, MD, USA). Briefly, HA pseudotypes were produced in 293T cells by cotransfection of the retroviral packing vector (pCMVDR8.2), luciferase reporter vector (pHR'CMV-Luc), human airway trypsin-like protease expression plasmid (pHAT), and HA expression plasmid, as described.²⁴ At 18 hours post-transfection, cells were fed fresh medium containing 7 mU/ml of bacterial neuraminidase from *Vibrio cholera* (Sigma, St. Louis, MO, USA) to induce the release of HA pseudotypes from the producer cells. HA pseudotypes were collected 48 h post-transfection, filtered through a 0.45-µm low protein binding filter, and used immediately or stored at –80°C. HA-pseudotype titers were determined by measuring luciferase activity in infected cells and were expressed as relative luminescence units per milliliter of HA-pseudotype supernatants (RLU/ml). For neutralization assays, HA pseudotypes containing ~15 ng/ml of p24 antigen were incubated with antibody samples for 1 hour at 37°C, and then 100 µl of pseudovirus and antibody mixture was inoculated onto 96-well plates that were seeded with 2×10^4 293T cells/well 1 day prior to infection. The antibody dilution causing a 95% reduction in RLU compared with control (IC95-neutralizing antibody titer) was used as the neutralization endpoint titer.²⁵ IC95 was calculated using GraphPad Prism software (La Jolla, CA, USA). Data reported were from at least duplicate testing of antibody samples.

Selection of escape mutants

Isolation and selection of influenza virus escape mutants to mAbs was performed similar to described methods.²⁶ Briefly, purified antibody (~2 mg) was incubated with 50 µl of A/California/04/2009 (~ 10^7 TCID₅₀/ml) virus in a total volume of 500 µl for 1 hour at room temperature, diluted with an additional 500 µl of PBS, and then 100 µl of virus-antibody mixture was injected into each of 10 embryonated chicken eggs and incubated at 35°C for 2 days. Allantoic fluid from inoculated eggs was harvested individually and tested for the presence of virus by HA and for reduced HI activity of the original monoclonal antibody. Viruses that were inhibited ~10-fold less efficiently than A/California/04/2009 were cloned by limiting dilution in embryonated chicken eggs. Consensus nucleotide sequences of viral HAs were determined by direct

DNA-sequencing of RT-PCR products and compared with those of the parental virus stock.

Three-dimensional visualization of HA

To visualize the mutations in the HA molecule of the virus escape mutants, the crystal structure of the HA of A/California/04/2009 was used (PDB ID: 3LZG; MMDB ID: 81425).²⁷ Three-dimensional visualization was conducted using Cn3D-4.3 software.²⁸

Results

Generation and initial characterization of mAbs to A/California/4/2009 HA

Mammalian-derived VLPs containing the HA of the pandemic influenza H1N1 A/California/04/2009 virus were used to immunize mice and generate a panel of hybridoma clones secreting mAbs to HA. Eleven antibodies bound to A/California/04/2009 HA in an ELISA using the H1 VLP that was the immunizing antigen for hybridoma production (Table 1) and were selected for further characterization. All of the mAbs but 2 (2E10 and 5F4) bound to virus HA when formaldehyde-inactivated A/California virus was used as the ELISA antigen (Table 1). Interestingly, one of these antibodies (2E10) also bound to VLPs containing the H5N1 A/Vietnam/1203/2004 HA (data not shown). None

of the 11 mAbs bound inactivated seasonal H1N1 virus (A/Solomon Islands/3/2006) or H3N2 (A/Uruguay/716/2007) in this type of assay (data not shown). When each mAb was used in an ELISA format to capture virus, inactivated A/California/04/2009 virus bound strongly to all of the antibodies except 2E10 and 5F4. Under the conditions used, with each mAb used at a coating concentration of 1 µg/ml and inactivated virus at a starting concentration of 2 µg/ml, there was weak binding to 2E10 and no apparent binding to 5F4 (data not shown). Isotype analysis indicated that all 11 mAbs were of the IgG2a subclass (data not shown).

Immunofluorescence microscopy was used to examine the ability of each monoclonal antibody to bind to virus-infected cells. MDCK cells were infected with A/California/04/2009, fixed at 3 days post-infection with 4% paraformaldehyde, and then stained with each monoclonal antibody (Table 1; Figure S1). Under these conditions, no fluorescence staining was detected for three antibodies (2E10, 5F4, and 1C5). Fluorescence staining was detected using the other eight antibodies, three of which (1A8, 5C12, and 4F8) appeared somewhat more intense than the other five.

Each of the 11 A/California/04/2009 mAbs was assessed for binding of HA by Western blotting under reducing and non-reducing conditions at equivalent antibody

Table 1. Characterization of mAbs to A/California/04/2009 HA

Antibody	Neutralization		ELISA		Western	
	Titer*	HI titer**	H1 VLP***	Inactivated virus	R/NR†	IF††
4F8	691	1722	256K	512K	++/++	++
1A8	453	1722	256K	256K	+/++	++
5C12	453	1218	128K	256K	++/++	++
1G10	333	320	32K	128K	++/++	+
4A10	101	4	256K	128K	++/++	+
3A7	99	6	128K	128K	++/++	+
1F11	67	9	256K	128K	++/++	+
3G4	32	19	128K	128K	++/++	+
1C5	21	7	64K	32K	+/++	--
2E10	5	11	256K	<1K	--/+	--
5F4	5	2	128K	<1K	--/--	--
β-gal	5	2	<1K	1K	NA	--

VLP, virus-like particles; GMT, geometric mean titer; mAbs, monoclonal antibodies.

*GMT of the antibody neutralization of A/California/04/2009 infection of MDCK cells determined by microneutralization assay; initial mAb concentration 4 mg/ml.

**GMT of the antibody inhibition of A/California/04/2009 hemagglutination of chicken red blood cells; initial mAb concentration 0.2 mg/ml.

***Endpoint titer – highest dilution of antibody (initial concentration of 4 mg/ml) giving an absorbance value (405 nm) >0.050 and greater than the highest dilution of a matched dilution of control antibody of the same isotype; K = 1000; antigens for capture (VLPs and inactivated whole influenza virus) used at 10 µg/ml.

†Relative intensity of monoclonal antibody binding of influenza HA in Western blot analysis under reducing (R) and non-reducing (NR) conditions.

††Relative immunofluorescence intensity of monoclonal antibody binding to A/California/04/2009-infected MDCK cells.

concentrations (Table 1; Figure 1). Seven of the mAbs bound to A/California HA well under reducing conditions, identifying an HA1 of ~55 kDa (Figure 1A). Under the same conditions, two other mAbs (1A8 and 1C5) appeared to bind to HA1 weakly; binding of HA1 by the remaining two antibodies (2E10 and 5F4) was not detectable. Under non-reducing conditions (Figure 1B), all antibodies bound HA0 (~70 kDa) except for 5F4. None of the 11 antibodies bound to the HA of pandemic H5N1 (A/Indonesia/5/05), seasonal H1N1 (A/Brisbane/59/2007), or H3N2 (A/Brisbane/10/2007) under either reducing or non-reducing conditions (Figure 1, lanes 1–3).

Antigenic characterization of pandemic H1N1 mAbs by VN and HI

The panel of HA mAbs was evaluated for their ability to neutralize virus infectivity and inhibit virus hemagglutination of chicken red blood cells (Table 1). A microneutralization assay of A/California/04/2009 was performed using all mAbs at equivalent antibody concentration (4 mg/ml). Two antibodies (2E10 and 5F4) had no neutralizing antibody activity (<1:10) at the highest concentration of antibody. A range of neutralizing activities was measured for the other mAbs with four (4F8, 1A8, 5C12, and 1G10) exhibiting noticeably higher neutralization titers than the

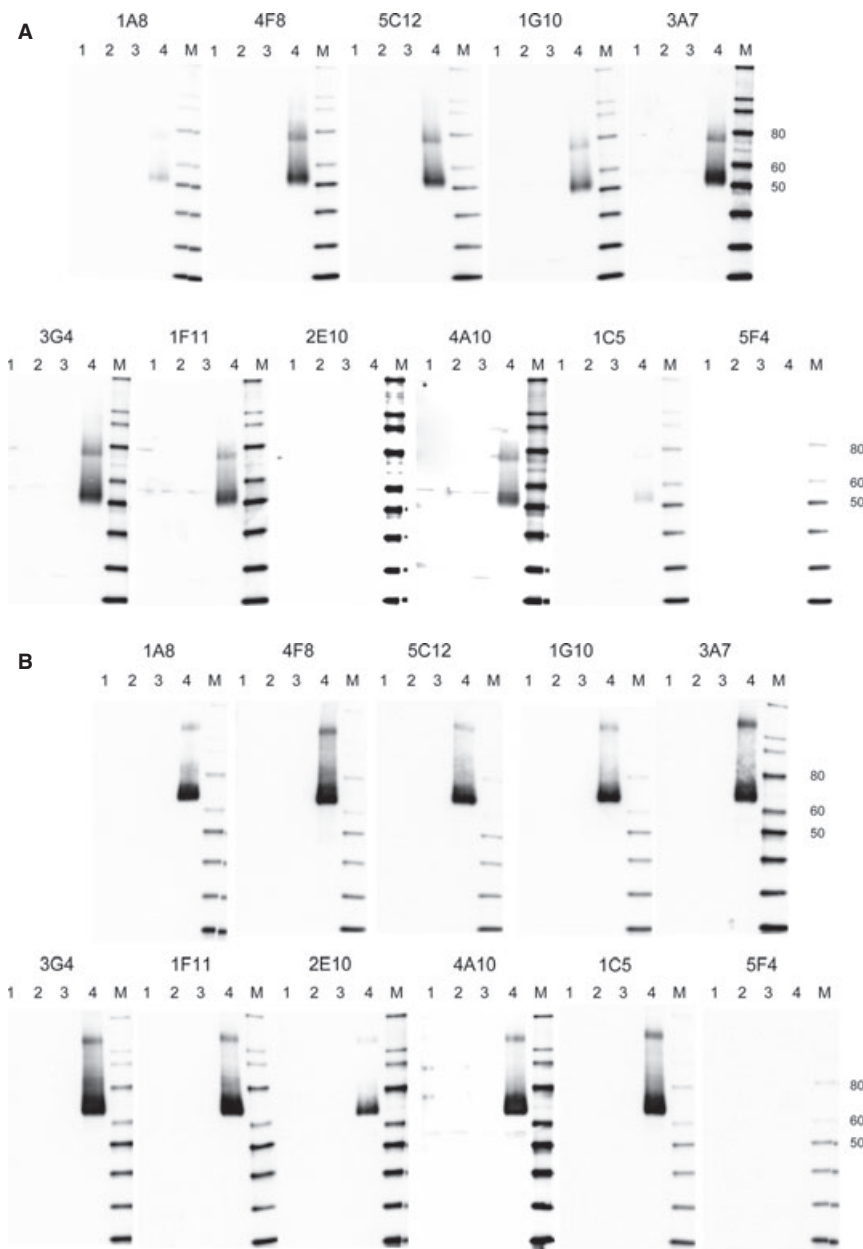


Figure 1. Monoclonal antibody detection of influenza HA in Western blot analysis. Viral proteins from inactivated influenza viruses were resolved by SDS-PAGE under reducing (A) and non-reducing conditions (B) and probed in Western blots with individual monoclonal antibodies (2 μ g/ml). Lane 1 – H5N1 (A/Indonesia/5/05); lane 2 – H1N1 (A/Brisbane/59/2007); lane 3 – H3N2 (A/Brisbane/10/2007); lane 4 – H1N1 (A/California/04/2009). M, molecular weight markers.

others. When the panel of mAbs was analyzed in a HI assay using A/California/04/2009, the four antibodies with the highest neutralization titers (4F8, 1A8, 5C12, and 1G10) also presented high levels of HI activity. Low HI titers were measured for the remaining seven H1N1 HA mAbs.

Further characterization of the neutralizing potential of the panel of mAbs was carried out using a pseudotype neutralization assay (Table 2). Retroviral pseudotypes expressing various influenza HA proteins were incubated with individual antibodies, followed by infection of 293T cells and measurement of vector expressed luciferase. In general, the results of the A/California pseudotype VN were consistent with the results of the microneutralization assay. The four antibodies with the highest pseudotype neutralization titers were the same four antibodies with the highest microneutralization and HI titers (4F8, 1A8, 5C12, and 1G10). The pseudotype VN assay also facilitated analysis of the capacity of each monoclonal antibody to neutralize other influenza HA types. These studies demonstrated that none of the A/California HA antibodies could neutralize recent seasonal H1N1 HA-containing pseudotype viruses including A/New Caledonia/20/1999, A/Solomon Islands/3/2006, and A/Brisbane/59/2007 (data not shown). On the other hand, pseudotype viruses containing the influenza HA from A/South Carolina/1918 or A/New Jersey/1976 were neutralized to some degree by mAbs 4F8, 1A8, 5C12, and 1G10, and 4F8 and 1A8, respectively, indicating the closer relatedness of these viruses to the pandemic A/California/04/2009 pandemic virus (Table 2).

Table 2. Monoclonal antibody neutralization of H1N1 HA-containing pseudoviruses

Antibody	Neutralization titer ($\mu\text{g/ml}$)*		
	A/California/2009	A/SC/1918	A/NJ/1976
4F8	0.008	0.106	7.299
1A8	0.005	0.114	1.703
5C12	0.009	0.157	>40
1G10	0.18	0.704	>40
4A10	1.98	>40	>40
3A7	0.54	28.841	>40
1F11	0.68	>40	>40
3G4	0.66	21.157	>40
1C5	4.5	33.4	>40
2E10	1.25	>40	>40
5F4	12.32	N/A	N/A

*Antibody concentration resulting in a 95% reduction in relative luciferase units of retroviral pseudotypes expressing the indicated influenza virus HA.

Protection afforded by passive transfer of pandemic H1N1 mAbs

To assess the protective capacity of the A/California/04/2009 HA mAbs, mice were passively transferred with antibodies prior to challenge with A/Mexico/4108/2009, a relatively virulent strain of the 2009 pandemic H1N1 virus. Antibodies or PBS was injected intravenously into groups of mice 4 hours before intranasal challenge with 1 LD₅₀ of wild-type A/Mexico/4108/2009. Mice were monitored for 2 weeks for morbidity (body weight loss) and mortality. Table 3 shows the results of two independent experiments evaluating the entire panel of H1N1 HA mAbs for their ability to provide passive protection against viral challenge. Transfer of PBS or an unrelated antibody (β -Gal) failed to protect mice from death (40–60% mortality) or morbidity. Average maximal weight loss in these groups approached 30% and the duration of $\geq 10\%$ weight loss persisted for nearly 10 days over the course of the 2-week observation period. In contrast, the three antibodies with the highest neutralization and HI titers (4F8, 1A8, and 5C12) provided substantial protection against challenge. None of the mice in the groups that received these antibodies died, and the average maximal body weight loss never exceeded 10%.

There was no obvious evidence of protection afforded by six of the mAbs (1G10, 4A10, 3A7, 1F11, 3G4, and 2E10) with several deaths in each group, average maximal weight loss of more than 20%, and a duration of $\geq 10\%$ weight loss of nearly 10 days. Interestingly, although neither of the mAbs 1C5 and 5F4 had appreciable neutralizing or HI titers against A/California/04/2009 (Tables 1 and 2), transfer of these mAbs provided some protection against virus challenge, with no deaths observed in these groups. The average maximal body weight loss never exceeded 15% in these mice, with only a few days in which average weight loss exceeded 10%. Follow-up experiments using different preparations and concentrations of antibody confirmed their capacity to provide some protection against challenge (data not shown).

Monoclonal antibody epitope mapping

A competition ELISA experiment was designed to determine whether the four antibodies exhibiting the highest HI activity (4F8, 1A8, 5C12, and 1G10) competed for the same epitope(s). Purified 5C12 antibody was labeled with HRP and used as a detection antibody for bound VLPs containing the HA of A/California/04/2009. The amount of each unlabeled antibody required to inhibit labeled-5C12 binding by 50% was determined. The inhibitory concentration 50% (IC₅₀) for the HI antibodies 4F8, 1A8, 5C12, and 1G10 was 0.03, 0.31, 0.12, and 0.63 $\mu\text{g/ml}$, respectively. All of the remaining H1N1 HA mAbs had an IC₅₀ >10 $\mu\text{g/ml}$.

Table 3. Protective effect of A/California/04/2009 monoclonal antibodies

Antibody	Survival		Maximal body weight loss (%)*		Duration of $\geq 10\%$ body weight loss (days)**	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
4F8	5/5	5/5	5.9 \pm 2.9	7.6 \pm 2.8	0	0
1A8	5/5	5/5	5.9 \pm 2.3	9.8 \pm 2.4	0	0
5C12	5/5	5/5	5.5 \pm 1.6	3.9 \pm 1.2	0	0
1G10	3/5	5/5	31.3 \pm 2.6	24.2 \pm 6.2	10	10
4A10	4/5	5/5	26.8 \pm 4.8	23.6 \pm 5.2	10	8
3A7	4/5	3/5	28.0 \pm 2.9	23.0 \pm 4.9	10	8
1F11	2/5	3/5	33.1 \pm 4.5	25.3 \pm 3.0	10	10
3G4	3/5	2/5	32.9 \pm 1.4	33.7 \pm 0.7	10	10
1C5	5/5	5/5	11.7 \pm 1.6	14.5 \pm 4.7	2	5
2E10	1/5	2/5	33.9 \pm 4.0	29.1 \pm 4.3	10	10
5F4	5/5	5/5	10.8 \pm 4.2	6.3 \pm 2.9	3	0
PBS	2/5	2/5	25.7 \pm 6.9	29.6 \pm 2.1	6	10
β -gal	ND	3/5	ND	29.5 \pm 1.7	ND	10

ND, not determined.

*The percent body weight loss was calculated based on the average initial body weights in each group before the challenge and was expressed as the percent of maximal body weight loss on average, plus and minus SEM.

**The duration of $\geq 10\%$ body weight loss was based on the average body weight loss of the entire group.

The results suggested that the four mAbs with the strongest HI activity were directed toward the same or a nearby epitope.

Escape mutants to A/California/04/2009 were selected by incubation of the virus with mAbs 4F8 and 5C12. Viruses that were inhibited in the HI assay ~ 10 -fold less efficiently than A/California/04/2009 were cloned by limiting dilution. Eleven independently isolated escape mutants were obtained, sequenced, and compared with the sequence of the parent virus. Two separate mutations were identified, either a D127E or a G155E (H1 numbering used throughout the text), both located near the antigenic site Sa. Figure 2A shows the sequence of the two escape mutants. Both mutations are located close to each other near the head of the HA molecule as shown in the 3-dimensional HA structure diagram (Figure 2B). Compared with A/California/04/2009, each mutant virus was more resistant to neutralization by mAbs 4F8, 5C12, and 1G10. Similarly, HI titers for these antibodies were greatly reduced for the escape mutants compared with A/California/04/2009 (Table 4). In contrast, the neutralizing activity of 4A10, which apparently does not share this epitope based on the ELISA competition assay, was not affected by either mutation. Interestingly, the same amino acid changes identified in the escape mutants were present in A/South Carolina/1/1918 (D127E – Genbank accession no. AAD17229) and A/New Jersey/1976 (D127E and G155E – Genbank accession no. ABQ44394) HAs and thus may explain the

poor neutralization of pseudotype viruses bearing these HAs by the A/California/04/2009 mAbs (Table 2). Finally, we tested whether antibodies generated by vaccination with pandemic 2009 H1N1 vaccines still recognized the D127E and G155E escape mutants. Pre- and post-vaccination sera were obtained from eight adults receiving seasonal vaccine containing the recommended A/California/07/2009-like virus strain and tested in an HI assay using A/California/04/2009 and the two A/California escape mutants with either the D127E or G155E mutation. All pre-vaccination sera had HI titers below the level of detection (1:20) to A/California/04/2009 and both A/California escape mutants. Seven of the eight post-vaccination sera had HI titers $> 1:160$ to A/California/04/2009; one post-vaccination HI titer was below the level of detection. However, there was no difference in post-vaccination HI titers between A/California/04/2009 and either escape mutant for any of the tested sera (data not shown), suggesting that the single mutations were not sufficient to abrogate a protective antibody response to the vaccine.

Discussion

This study describes the initial characterization of a panel of murine mAbs to the HA of A/California/04/2009. The antibodies exhibit a range of properties and thus have a variety of uses that will facilitate characterization of the A(H1N1)pdm09 virus HA. The panel of hybridoma clones

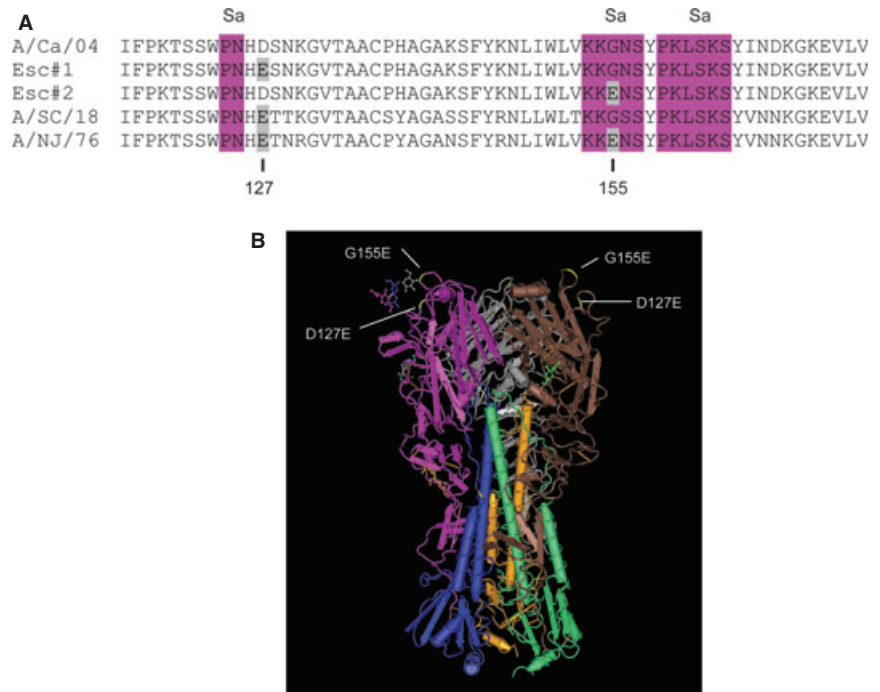


Figure 2. Location of HA amino acid changes in A/California/04/2009 escape mutants. (A) Sequence alignment of A/California/04/2009 and escape mutants selected by monoclonal antibodies to HA. The antigenic region Sa is shown in color. The amino acid changes identified in the escape mutants are shaded. Amino acid sequences in the same region of A/South Carolina/1/1918 and A/New Jersey/1976 are aligned for comparison. Amino acid numbering is as previously described.²⁷ (B) Antigenic structure of A/California HA. The locations of the two amino acid changes identified in the escape mutants are highlighted.

Table 4. Neutralization and HI titers of mAbs to A/California/04/2009 escape mutants

Virus	Neutralization titer*					HI titer**				
	4F8	5C12	1G10	4A10	5F4	4F8	5C12	1G10	4A10	5F4
A/CA/04/2009	640	381	453	48	<10	912	1024	256	4	<2
Mutant D127E	48	34	67	113	<10	8	9	7	6	<2
Mutant G155E	<40	<40	<40	57	<10	<2	<2	<2	4	<2

HI, hemagglutination inhibition; GMT, geometric mean titer; mAbs, monoclonal antibodies.

*GMT of the antibody neutralization titer of A/California/04/2009 or escape mutants D127E and G155E as determined by microneutralization assay. Limit of detection for neutralization was 40 for 4F8, 5C12, and 1G10; 10 for 4A10 and 5F4.

**GMT of the antibody HI titer of A/California/04/2009 or escape mutants D127E and G155E. Limit of detection for HI was 2.

was generated using mammalian-derived VLP containing the HA of the A/California/04/2009 virus as the immunogen. These VLP particles were produced from a recombinant MVA vector expressing only the HA of A/California/04/2009. Such VLPs have been shown to have properties in common with live influenza virus including their ability to elicit neutralizing antibodies and provide protection against influenza challenge in animals.¹⁸ By using this approach for immunization, the expressed virus HA is presented in its native form as in a virus particle. Also, because no other influenza virus proteins are present during immunization, the initial screening and counter-screening to select the desired hybridomas are simplified by

using whole influenza virus, including unrelated influenza viruses, to identify antibodies recognizing the specific HA. An immunization approach using VLPs would have an added advantage for highly pathogenic influenza viruses because there would be no virus containment issues to address during the animal procedures.

Initial characterization using ELISA, immunofluorescence, and Western blot analysis identified mAbs useful for several practical applications. By all methodologies, the panel of mAbs appeared highly specific for the HA of A/California/04/2009 with no cross-reactivity to the HA of seasonal H1N1 viruses. ELISA results using a variety of influenza viruses or VLPs as capture antigen demonstrated

sensitivity and specificity of antibody binding; no cross-reactivity of the mAbs with other seasonal H1N1 or H3N2 viruses was detected. Only one mAb, 2E10, showed some cross-reactivity with a VLP containing the HA of an H5N1 virus (data not shown). Eight of the 11 mAbs were suitable for use in immunofluorescence studies and three of these (1A8, 5C12, and 4F8) appeared particularly sensitive. In Western blot analysis under non-reducing conditions, all of the mAbs except for 5F4 bound to A/California/04/2009 HA, whereas none of the antibodies bound to HA from seasonal or pandemic influenza strains. It was possible to conclude that nine of the 11 mAbs recognized epitopes on the HA1 portion of the HA because they recognized HA1 but not HA2 under Western blot reducing conditions. Interestingly, binding of some mAbs (e.g., 1C5) was rather weak in reducing conditions compared with the binding under non-reducing conditions, suggesting that such antibodies might be directed to epitopes that are conformationally sensitive.

Hemagglutination inhibition and VN by antibodies to influenza HA are considered important functional properties that contribute to protection. We used mAbs with HI and neutralizing activity to identify and characterize potentially important antigenic epitopes of the A/California/04/2009 HA. There were four mAbs with significant measurable HI activity, and these four antibodies were the strongest neutralizing antibodies in both standard virus microneutralization assays and pseudotype neutralization assays. In general, both types of neutralization assays appeared more sensitive than HI. Whereas only four mAbs had appreciable HI activity even when the highest practical concentrations of antibody were tested, several of the other mAbs had lower but easily quantifiable neutralization activity in both assays. The absolute endpoint titers determined for the mAbs in the pseudotype neutralization assay were much higher than the corresponding titers obtained in the microneutralization assay. Similar observations have been previously reported, and the differences are likely due in part to the different methodologies of the assays, particularly in the sensitivity of virus or pseudotype virus detection. However, it has also been suggested that the greater apparent sensitivity of the pseudotype neutralization assay may be the result of a lower density of HA molecules on the surface compared with the actual virus, making the HA more accessible to antibody when expressed on the pseudotype virus.²⁴ Nevertheless, there was overall good agreement between the relative results of the two neutralization assays, particularly at the highest and lowest ranges of activity, although one mAb, 4A10, appeared to have a higher neutralization activity in the microneutralization assay relative to the other mAbs than it did in the pseudotype assay.

Although many A(H1N1)pdm09 viruses were not especially pathogenic in animal models of infection, some

viruses such as A/Mexico/4108/2009 are relatively virulent strains and can be used to assess protection afforded by vaccines and antibodies. In our mouse challenge experiments with this virus, we observed a 40% mortality and substantial morbidity as evidenced by maximal body weight loss of ~30% and almost 10 days of weight loss >10% of animal body weight. The three mAbs with the highest HI and neutralizing titers provided passive protection against virus challenge by all criteria (e.g., no deaths and no body weight loss >10%), suggesting a correlation between the functional properties of the mAbs measured *in vitro* (HI and neutralizing activity) and protection. Although mAb 1G10 had both HI and neutralizing activity, it appeared weaker in all three *in vitro* assays than the other three HI antibodies, which may explain its inability to provide protection against virus challenge in the passive transfer experiments. Interestingly, two of the mAbs with no appreciable HI activity or neutralizing activity in these *in vitro* assays showed evidence of protection in the passive transfer experiments. Although not as protective as the three HI mAbs (4F8, 1A8, and 5C12), both 1C5 and 5F4 prevented death and lessened morbidity associated with challenge compared with PBS, control antibodies, and the other six mAbs. Multiple challenge experiments using different preparations and concentrations of antibody confirmed these observations. At this time, it is not clear how these two mAbs exert their protective effect. Antiviral mechanisms of antibody activity other than those measured in traditional HI and neutralization assays have been described, including complement-dependent lysis of infected cells²⁹ and complement-mediated neutralization and enhancement of neutralization by serum components other than complement.^{30–33} We have conducted some preliminary complement-mediated neutralization experiments, and both 1C5 and 5F4 appear to have enhanced neutralizing activity in the presence of complement. However, their enhanced neutralization activities were no greater than those of the six mAbs that did not afford any protection in the passive transfer experiments (data not shown). Additional studies are planned to try to understand how 1C5 and 5F4 mAbs interact with the virus and impart a protective effect *in vivo*.

Five major antigenic sites (Sa, Sb, Ca1, Ca2, and Cb) have been described in the HA of influenza H1N1.^{34,35} To identify the HA epitopes recognized by the neutralizing mAbs in our panel, we selected virus escape mutants by incubating A/California/04/2009 in the presence of individual mAbs. Cloned viruses that were resistant to HI by antibody were sequenced to identify mutations. Although 11 independently derived virus mutants were obtained, there were only two unique mutations, both near the antigenic site Sa. All of the strong HI and neutralizing mAbs had reduced titers for both escape mutants in HI and

neutralization assays, as might be predicted from the ELISA competition studies, which suggested that the four antibodies 4F8, 5C12, 1A8, and 1G10 recognized the same or a nearby epitope on the HA. There was some indication, however, that the recognition site for these antibodies might not be identical. During the selection of escape mutants to 5C12, four of five independently isolated mutations had the D127E mutation and one isolate had the G155E mutation. In contrast, during selection of escape mutants to 4F8, all six independently isolated mutants were G155E. Interestingly, some human mAbs directed to the Sa region of the 1918 pandemic H1N1 virus HA have been shown to cross-react with the A(H1N1)pdm09 HA.³⁶ Although the epitopes recognized by the human mAbs appear to be different from the epitopes identified in the present study, the results strongly suggest that the Sa region of both H1N1 viral HAs is an important target for humoral immunity.

Since the emergence of the A(H1N1)pdm09 virus, very few genetic or antigenic changes in the virus HA have been documented. Consequently, the recommended virus strain for inclusion in the seasonal influenza vaccine has remained an A/California/07/2009-like virus for the 3 years since the pandemic, including the next 2012–2013 northern hemisphere winter influenza season.³⁷ Nevertheless, there have been some reports of virus isolates containing amino acid changes in the HA that have the potential to alter the antigenic properties of the virus. For example, only two amino acid changes in the Sa region (N125D and N156K) of virus isolates from Finland were sufficient to reduce the interaction of antibodies in convalescent sera or sera from vaccinated individuals as measured by HI.³⁸ There have also been a couple of reports describing adaptation and increased virulence of A/California/04/2009 in mice,^{39,40} and interestingly, both of the mutations identified in our mAb escape mutants (D127E and G155E) were also identified in these studies. In one study, a mouse adapted A/California/04/2009 virus contained amino acid mutations in several virus genes, including four in the HA, and exhibited increased virulence in mice. Although the mutations were not analyzed individually for their contribution to increased virulence, one of the four HA amino acid mutations was a G155E.³⁹ In another study, mutations in two amino acids in the A/California/04/2009 HA, D131E (D127E in our numbering), and S186P had a significant impact on virulence in mice.⁴⁰ By itself, the D131E (D127E) mutation appeared to result in a greater loss of animal body weight (11% versus 4% for A/California/04/2009) and increased virus titers in the lungs of mice (~0.5 log). Ferret sera to A/California/04/2009 had reduced HI titer to a virus containing the D131E (D127E) mutation. In contrast, in our limited study with human vaccine sera, there were no differences in HI titers between

A/California/04/2009 and our D127E escape mutant, likely reflecting differences between the two methodologies using post-infection ferret sera and post-vaccination human sera.

In summary, this study characterizes a panel of murine mAbs to the HA of the A(H1N1)pdm09 virus A/California/04/2009. Several antibodies with neutralizing and hemagglutination activity were identified, some of which were able to provide protection when administered passively before influenza challenge. The HA epitopes recognized by strongly neutralizing mAbs were identified by isolation of virus escape mutants and mapped near the antigenic site Sa. This panel of mAbs allowed comparison of antigenic and neutralizing epitopes of the HA of A/California/04/2009 to other H1N1 viruses, including recent seasonal isolates as well as A/South Carolina/1918 and A/New Jersey/1976 and may be useful in identifying important HA epitopes involved in future antigenic drift.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunofluorescence of A/California/04/2009-infected MDCK cells.