Naturally Occurring Human Monoclonal Antibodies Neutralize both 1918 and 2009 Pandemic Influenza A (H1N1) Viruses[▽]

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The 2009 pandemic influenza A (H1N1) virus exhibits hemagglutinin protein sequence homology with the 1918 pandemic influenza virus. We found that human monoclonal antibodies recognized the Sa antigenic site on the head domains of both 1918 and 2009 hemagglutinins, a site that is hypervariable due to immune selection. These antibodies exhibited high potency against the 2009 virus *in vitro*, and one exerted a marked therapeutic effect *in vivo*.

In March and April of 2009, an outbreak of a swine-origin novel H1N1 influenza A virus began in Mexico (2). As of 29 November 2009, worldwide, more than 207 countries and overseas territories or communities have reported laboratory-confirmed cases of 2009 A (H1N1), including at least 8,768 deaths. (http://www.who.int/csr/don/2009_12_04/en/index.html). The hemagglutinin (HA) gene of the 2009 A (H1N1) strain has been present in classical swine H1N1 viruses that have circulated in pigs at least since the discovery by Shope in the 1930s (Table 1b and c) (10, 11, 16). In contrast, the HA of human H1N1 influenza viruses circulating from 1918 to 1957 and from 1977 to present drifted progressively away from the 1918 virus HA (Table 1d) (8, 14). Elderly subjects born prior to 1918 were found to have serum neutralizing antibody titers to the A/California/04/2009 (CA04) virus (5, 6).

Four major antigenic areas have been identified on the influenza virus HA protein, designated sites Sa, Sb, Ca, and Cb (1, 4). The Sa site of mid-century influenza virus H1 strains differs from that of both the 1918 and 2009 pandemic influenza viruses, but amino acid sequences in the HA protein Sa sites of these two viruses are nearly identical (Table 1a and c). We previously generated five human monoclonal antibodies (MAbs) directed against the 1918 HA by using circulating blood cells from survivors of the 1918 pandemic (20). Three of these MAbs, designated MAbs 2D1, 2B12, and 4D20, bind the Sa antigenic site. In this paper, we report that the 1918 virus-specific MAbs 2D1 and 2B12 bind and inhibit the 2009 A (H1N1) virus *in vitro* and that MAb 2D1 also exhibited a marked therapeutic effect *in vivo*.

Microneutralization assay. Influenza virus was passaged in Madin-Darby canine kidney (MDCK) cells. The titer of virus stocks was determined in MDCK cell culture monolayers by standard plaque assay. One hundred 50% tissue culture infec-

tive dose (TCID₅₀) units of virus was preincubated with dilutions of serum or MAb and then used to infect MDCK cells in 96-well plates, as described previously (20). Six replicate wells were used for each antibody dilution. Neutralizing antibody concentrations were determined and were defined as the reciprocal of the highest dilution of serum where 50% of wells were infected, as calculated by the method of Reed and Muench (9a). Specific neutralizing activity of MAbs was calculated as the lowest concentration of MAb that displayed neutralizing activity. The minimum concentration of antibody required to neutralize 100 TCID₅₀ units of A/Mexico/4108/09 virus was below 40 ng ml⁻¹ (267 pM) for 2D1 and 160 ng ml⁻¹ (1.1 nM) for 2B12 (Table 2).

HAI assays. HA inhibition activity (HAI) tests using MAbs were performed according to standard protocols (7). Briefly, serial dilutions of purified MAbs in phosphate-buffered saline (PBS) were performed from an initial concentration of 5 µg/ml. Sera were diluted initially 1:10 in receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo, Japan). Serial dilutions of mouse positive-control sera or human MAbs were preincubated with four HA units of virus per well. Turkey red blood cells (RBCs) were added to a final concentration of 0.5%, and the plate was incubated at room temperature for 30 to 60 min. MAbs 2B12 and 2D1 exhibited HAI activity (Table 2). In contrast, the three other 1918 virus HA-specific neutralizing MAbs, 4D20 (site Sa-specific) and 1F1 and 1I20 (site Sb-specific), showed no activity against the 2009 A (H1N1) virus, even when tested at 5 µg/ml (Table 2).

MAb 4D20 kinetics. Given that the Sa-specific MAb 4D20 did not bind the HA of the 2009 A (H1N1) virus, we explored the role of additional amino acid variations outside the Sa site in the alteration of binding and found that reversing either HA protein residue E77 or S78 of the 2009 novel H1N1 to the respective residue of the 1918 virus HA restored binding of MAb 4D20 by biolayer interferometry using human Fc receptor tips and recombinant secreted HA. MAb 4D20 associated more readily with the E77D mutant (k_a , 1.7×10^3 /Ms) than with the S78L mutant (k_a , 9.5×10^2 /Ms) with similar dissociation, resulting in an overall tighter binding of MAb 4D20 to the E77D mutant than that to the S78L mutant (K_D , [equilib-

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TABLE 1. Alignment of the amino acids in site Sa of the HA of representative swine or human influenza viruses from the 20th century^a

Amino acid at position in indicated site											
	Site Sa			Site Sb	Site Ca1			Site Ca2		Site Cb	
	128	156	162	186	169	206	238	140	224	77	
				*							
a) 1918 pandemic virus											
A/South Carolina/1/1918	PN	KKGSS	PKLSKS	PTGTDQQSLYQNA	VNNKG	SSK	EPG	SYAGAS	RD	DLLLTASS	
b) Representative 20th century North American swine viruses											
A/Iowa/15/1930	--	--EN-	-----	--S-----	-----	---	---	P-----	-G	-----V--	
A/Ohio/23/1935	--	---D-	-----	--S-X-----	-----	---	---	P-----	-N -G	-----V--	
A/Iowa/1945	--	---N-	-----	--S-----	-----	---	---	P-----	-G	-----V--	
A/Wisconsin/1/1957	--	---D-	-----	--S-----	-----	---	---	P-----	-N -G	E----V--	
A/Wisconsin/1/1961	--	---E-	-----	--S-----	-----	---	---	P-----	-N -G	E----V--	
A/Wisconsin/2/1966	--	-----	-----	--S-----	-----	---	---	P-----	-N -G	E--F-V--	
A/Iowa/1973	--	---N-	-----	--S-----	-----	---	---	P-----	-N -G	E--F-V--	
A/New Jersey/8/1976	-D	---N-	-----	--S-----	-----	---	---	P-----	-N -G	E----V--	
A/Wisconsin/663/1980	--	---N-	-----	--S-----	-----	---	---	P-----	-N -G	E--F-V--	
A/Iowa/24297/1991	--	---N-	-----	--S-----	I----	---	---	P-----	-N -G	E--F----	
A/Minnesota/37866/1999	--	---N-	-----	S-S-----	I---E	---	---	P-----	-N	ES-F----	
A/Minnesota/1192/2001	--	---N-	-----	S-SA-----	I---E	--R	---	PH----	--	ES-F-T--	
A/Wisconsin/87/2005	--	---N-	--I----	S-SA-----	I---E	--R	---	PH--TN	--	ES-F----	
A/Iowa/01/2006	--	---N-	-----	--S---T----	I---K	---	---	P-----	-N -N	ES-F----	
A/Ohio/01/2007	--	---N-	--IN--	S-SA-----	I---E	--R	---	PH--TN	--	ES-S----	
c) 2009 A(H1N1) human viruses											
A/California/04/2009	--	---N-	-----	S-SA----I----	I-D--	--R	---	PH---K	--	ES-S----	
A/Mexico/4108/2009	--	---N-	-----	S-SA-----	I-D--	--R	---	PH---K	--	ES-S----	
d) Seasonal human H1N1 influenza viruses											
A/Weiss/1943	-K	E-DG-	-N-KN-	SSIKE--T---KE	--K--	--N	---	-H--K-	--	ES--SER-	
A/Fort Monmouth/1/1947	-K	ETNG-	-----	SNIE--KT--RKE	-----	--N	---	-H--K-	-G	ES--SKR-	
A/USSR/92/1977	-K	E-NG-	-N----	SNIE--KTI-RKE	-----	--N	---	-HK-K-	-G	ES-VSKK-	
A/Brazil/11/1978	-K	E-NG-	-N----	SNIE--KTI-RKE	-----	--N	---	-HK-K-	-G	ES-FSKK-	
A/Chile/1/1983	-K	E-NG-	-N----	SNIE--KTI-RKE	-----	--H	---	-HK-K-	-N	ES-FSKK-	
A/Singapore/6/1986	--	E-NG-	-N----	SNIG--RAI-HTE	-----	--H	---	-HK-R-	-G	ES-FSKK-	
A/Bayern/7/1995	--	E-NGL	-N----	SNIG--RAI-HTE	-----	--H	---	-HN-K-	-G	ES-FSKE-	
A/Beijing/262/1995	--	E-NGL	-N--N-	SNIR--RAI-HTE	-----	--H	---	-HN-K-	-G	ES-ISKE-	
A/New Caledonia/20/1999	--	G-NGL	-N----	-NIGN-RA--HTE	-----	--H	---	-HN-K-	--	E--ISKE-	
A/Solomon Islands/3/2006	--	G-NGL	-N----	-NIG--RA--HTE	A---E	--H	---	-HN-E-	--	E--ISRE-	
A/Brisbane/59/2007	--	G-NGL	-N----	-NIG--KA--HTE	A---E	--H	---	-HN-E-	--	E--ISKE-	

^a Dashes in the table indicate amino acid identity with the 1918 pandemic virus sequence, shown in the first row. *, K indicates the K166 amino acid that is most frequently mutated in monoclonal-antibody-resistant mutant viruses selected with site Sa-specific neutralizing antibody 2B12.

TABLE 2. Neutralizing or HAI activity of MAbs against Mexico/4108/2009 or 1918 viruses

Antigen	Test ^c	Sp act of indicated MAb (µg/ml) ^a					Reciprocal titer of antisera ^b	
		Sa specific			Sb specific		Mouse anti-Mexico/ 4108/2009	Mouse anti-South Carolina/1/1918
		2B12	2D1	4D20	1F1	1I20		
Mex/4108/2009	Neut Ab	0.16	0.04	>	>	>	nt	nt
	HAI	0.8	0.1	>	>	>	320	160
A/SC/1/1918	HAI	0.32	0.025	0.04	0.04	0.28	35	320

^a Specific HAI or neutralization activity of MAbs was calculated as the lowest concentration of MAb that displayed inhibition of hemagglutinating or neutralizing activity. Results are the average of four tests. > indicates that activity was not detected at any concentration tested, up to 5 µg/ml.

^b Positive control mouse sera were included, and HAI activity, performed by standard methods, is expressed as the reciprocal of the highest dilution of serum inhibiting agglutination of 0.5% of turkey erythrocytes at four HA units of virus. Normal mouse serum had a reciprocal titer of less than 10. Results are the average of four tests. nt indicates not tested.

^c Neut Ab, neutralizing antibody.

TABLE 3. Therapeutic efficacy of 1918 HA-specific MABs against replication of 2009 A(H1N1) virus in mice

Antibody	Dose per mouse (μg) ^a	Lung titers ($\log_{10}$ PFU/ml) ^b	P value
2D1	200	2.3 ± 1.0	<0.0001
	20	4.9 ± 0.4	<0.0001
	2	6.7 ± 0.3	0.027
2B12	200	7.1 ± 0.2	NS
	20	7.3 ± 0.3	NS
	2	7.2 ± 0.2	NS
Control human IgG	200	7.1 ± 0.3	NS
	20	7.2 ± 0.2	NS
	2	7.1 ± 0.2	NS

^a Groups of mice were inoculated intranasally with 1,000 MID₅₀ of the California/04/2009 virus and then treated 24 h later with graded doses of 1918 HA-specific MABs or control human IgG.

^b Lung titers were determined 3 days postinoculation on tissues from four animals in each group. At each of the dose levels, the mean titer differed between control and MAb 2D1 by ANOVA. Rank sum tests also yielded similar results ($P = 0.029$, $P = 0.029$, $P = 0.08$, respectively for 200 μg , 20 μg , or 2 μg). NS indicates not significant.

rium dissociation constant], 7.2×10^{-9} M versus 1.8×10^{-8} M, respectively).

Selection and characterization of antibody escape mutants. We selected and sequenced the HA gene of new MARMs by using the wild-type (wt) A/swine/Iowa/15/1930 virus or a recombinant virus generated by reverse genetics containing the CA04 HA and NA proteins in an A/Puerto Rico/8/34 virus background (kindly provided by Peter Palese) (1, 18). Briefly, escape mutant viruses were selected by treatment of virus with excess antibody, followed by recovery of neutralization-resistant viruses in 10-day-old embryonated chicken eggs. RNA was extracted from virus-infected allantoic fluid and then cDNA was generated by reverse transcription-PCR (RT-PCR), directly cloned, sequenced, and aligned to previously determined wt virus HA gene sequences. These studies revealed that MAB 2B12 selected virus mutants containing either the K166E mutation or a novel mutation at the 125C position (S to I). The MAB 2D1 selected for the K166E or K166N mutation in the

2009 HA protein, identical to changes that mediated escape to this antibody in MARMs selected by treatment of the 1918 human or 1930 swine viruses.

Animal studies. We tested the MABs 2B12 and 2D1 for therapeutic efficacy in a nonlethal mouse model of wt CA04 virus infection (9). Female BALB/c (8-week-old) mice were inoculated intranasally with 1,000 50% median infective dose (MID₅₀) units in a 50- μl volume of the CA04 virus, as described previously (13). At 24 h after inoculation, mice were administered 200, 20, or 2 μg (approximately 10, 1, or 0.1 mg/kg) of MAB 2D1 or 2B12 or an equal volume of human IgG (Sigma) by the intraperitoneal route to each mouse, in groups of nine mice. Mice were observed for weight loss every other day for 14 days. Subsets of four animals treated with the MABs were euthanized on day 3 after infection, and whole lungs were homogenized in 1 ml of sterile PBS. Virus titer in lung tissue homogenates was determined by plaque titration in MDCK cell monolayer cultures. The limit of virus detection was $10^{0.95}$. MAB 2D1 showed a marked therapeutic efficacy when administered 1 day after virus inoculation, resulting in a 5 $\log_{10}$ PFU/ml decrease of lung virus titers of lung homogenate at the highest dose (Table 3) and the prevention of weight loss (Fig. 1). MAB 2B12 did not affect replication *in vivo* at the doses tested.

Recent studies have described some novel antibodies that recognize influenza viruses across subtypes (3, 12, 19). This report is the first to describe naturally occurring human MABs to conserved HA sequences across two pandemic viruses. Antigenic sites are defined as regions for which the binding of specific antibodies is not affected by residue changes at neighboring sites (15); however, overlap between sites can occur (1, 4). Our data suggest that the MAB 4D20 epitope overlaps both the Sa and the Cb sites and that its lack of affinity to novel H1N1 is due to changes within or adjacent to the Cb site. The Cb site was defined previously by amino acid residues 78, 79, and 80 and residues 81 to 83 of the HA (17). The variability in residue 77 and the 4D20 binding data shown here suggest that this flanking residue also contributes to the Cb antigenic site. The involvement of the residue 125C in recognition of the 2009 virus by MAB 2B12, as found in a MARM we isolated, also reveals extension of the Sa antigenic site beyond conventional definitions. These data suggest that the 2D1 MAB may be promising for diagnostic or therapeutic purposes. However, prophylactic or therapeutic use of a single MAB directed to a site that is often variable in viruses circulating in humans should be considered with caution.

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FIG. 1. Therapeutic efficacy of 1918 HA-specific MABs against disease caused by the 2009 A (H1N1) virus in mice. In each group, five mice were followed every other day for weight. MAB 2D1 at 200- μg or 20- μg doses prevented weight loss at all time points after virus inoculation, compared to the control; ($P < 0.002$ for all by analysis of variance [ANOVA]). Neither MAB 2B12 nor human immunoglobulin (Hu Ig) mediated a statistically significant effect.

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