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Original Article

Prior infection with classical swine H1N1 influenza viruses is associated with protective immunity to the 2009 pandemic H1N1 virus

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Background The 2009 H1N1 pandemic emerged even though seasonal H1N1 viruses have circulated for decades. Epidemiological evidence suggested that the current seasonal vaccine did not offer significant protection from the novel pandemic, and that people over the age of 50 might were less susceptible to infection.

Objectives In a mouse challenge study with the 2009 pandemic H1N1 virus, we evaluated protective immune responses elicited by prior infection with human and swine influenza A viruses.

Results Mice infected with A/Mexico/4108/2009 (Mex09) showed significant weight loss and 40% mortality. Prior infection with a 1976 classical swine H1N1 virus resulted in complete protection from Mex09 challenge. Prior infection with either a 2009 or a 1940 seasonal H1N1 influenza virus provided partial protection and a >100-fold reduction in viral lung titers at day 4 post-infection.

Conclusions These findings indicate that in experimental animals recently induced immunity to 1918-derived H1N1 seasonal influenza viruses, and to a 1976 swine influenza virus, afford a degree of protection against the 2009 pandemic virus. Implications of these findings are discussed in the context of accumulating data suggesting partial protection of older persons during the 2009 pandemic.

Keywords Antigenicity, H1N1, hemagglutinin, immunity, influenza, pandemic.

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Introduction

Influenza A viruses are significant causes of pandemic respiratory disease and annually recurrent seasonal influenza.[1] In April 2009, a novel H1N1 influenza A virus was identified from patients in Mexico and the United States and soon spread globally.[2] In June 2009, the World Health Organization (WHO) declared the first influenza pandemic since 1968.[3] As of January 29, 2010 there have been millions of H1N1 infections and at least 14711 deaths worldwide, although the actual number of cases is likely much higher.[4] The pandemic virus is a previously unrecognized reassortant derived from two pre-existing swine influenza A virus lineages.[5]

In the present mouse challenge study, we evaluated infection-induced protection against the 2009 H1N1 pandemic influenza virus (A/Mexico/4108/09; Mex09) afforded by a pre-1957-era H1N1 virus (A/Hickox/40; H40), a contemporary H1N1 virus (A/Bethesda/NIH5009; NIH50), a contemporary H3N2 seasonal influenza A virus (A/Bethesda/NIH20/08; NIH20), and by a 1976 classical swine H1N1 virus (A/Swine/Iowa/1/76; Sw76). Classical swine lineage viruses are derived from the 1918 pandemic virus and have circulated enzootically since 1918.[6]

Materials and methods

Hemagglutinin protein sequence analysis

The sequences of selected H1 hemagglutinin (HA) proteins from 1918 to 2009 were downloaded from GenBank. The HA coding sequence of A/Bethesda/NIH50/2009 (H1N1) was determined for this study (GenBank accession number GU784795). Hemagglutinin protein sequences were aligned using the LaserGene Megalign program (DNAStar, Inc. Madison, WI, USA).

Viruses

A/Mexico/4108/09 (H1N1) (Mex09) was provided by Heinz Feldmann, NIH/NIAID (Hamilton, MT, USA). A/Hickox/40 (H1N1) (H40) was provided by Jack Bennick, NIH/NIAID (Bethesda, MD, USA). A/Swine/Iowa/1/76 (H1N1) (Sw76) was provided by Richard Webby, St. Jude Children's Research Hospital (Memphis, TN, USA). A/Bethesda/NIH20/2008 (H3N2) (NIH20) and A/Bethesda/NIH50/2009 (H1N1) (NIH50) were isolated from patients at the National Institutes of Health (NIH) Clinical Center, Bethesda, MD, USA (Protocol #07-I-0229). A chimeric virus containing the HA gene of A/South Carolina/1/1918 (H1N1) (1918) on the background of A/New York/312/2001 (H1N1) was produced by reverse genetics as previously described.[7]

Growth and titration of viruses

Viruses were passaged in Madin-Darby canine kidney (MDCK) cells in the presence of 1·0 μg/ml TPCK-treated trypsin in DMEM. Viruses were harvested between 48 and 72 hours after infection, centrifuged for 10 minutes at 500 × G and the supernatants were frozen at −80°C. For titrations by plaque assay, virus stocks were serially diluted in DMEM and added to MDCK cells grown to confluence in 12-well polystyrene plates. Each dilution was made in triplicate. Following incubation for 1 hour at 37°C, cells were washed once with 1× PBS and overlaid with 1·5 ml of agar gel (1% agar in MEM). After 2–3 days agar was removed and the cells stained with crystal violet solution [2% ethanol (v/v); 1% crystal violet (w/v)]. Virus titer was calculated by the method of Reed and Muench.[8] All work with the Mex09 and the chimeric 1918 influenza virus was performed in enhanced BSL-3 laboratories at the NIH.

Mouse infection studies

Groups of 8–10 week-old female BALB/c mice (Jackson Labs, Bar Harbor, ME, USA) were lightly anesthetized with isoflurane supplemented with O_2 (1·5 l/min) and intranasally inoculated with 50 μl sterile PBS containing 5×10^4 plaque-forming units (PFU) of either H40, Sw76, NIH50, NIH20, or PBS alone (mock infected controls). This dose was chosen based on previous experiments with the inoculating viruses where it was sufficient to cause mild illness with weight loss (data not shown). At 28 days post-inoculation, mice were challenged with 4×10^5 PFU Mex09. Body weights were measured daily and mice were humanely euthanized if they lost more than 25% of starting body weight. Lungs were collected for viral titration and pathologic examination at days 4 and 6 post-inoculation. For each virus and time point, lungs were collected from three animals for viral titration and from two animals for pathologic examination. To prevent atelectasis, lungs collected for pathology were inflated with 10% neutral buffered formalin. Lung viral titers were determined from 10% (w/v) lung suspensions by plaque assay after homogenization in sterile 1× L15 media. All experimental animal work was performed in an enhanced ABSL3 laboratory at the NIH, following approval of animal safety protocols by the NIH Animal Care and Use Committee.

Hemagglutination inhibition (HI) assay

Sera were collected 1–3 days before challenge; HI assays were performed on sera from all five individual animals in each group. Sera were treated with 3:1 (v/v) receptor destroying enzyme (RDE; Denka-Seiken Co., Nihonbashi, Japan), incubated at 37°C for 14–20 hours, heated at 56°C for 30 minutes, allowed to cool, and diluted 1:10 in PBS. Hemagglutination inhibition assay was performed in 96-well polystyrene plates by twofold serial dilution in PBS followed by addition of 4 HA units of virus to each well. Following incubation at room temperature for 15 minutes, 50 μl of 0·5% turkey red blood cells (RBC) were added to each well and incubated at room temperature for 45 minutes. Assays were read by presence or absence of teardrop shaped RBC pellets as described.[9] Data were presented as the reciprocal geometric mean titer (GMT) of the highest serum dilution completely inhibiting red blood cell agglutination. Hemagglutination inhibition assays were performed on pre-challenge sera from infected mice with the reconstructed 1918 influenza virus as above.

Pathology and immunohistochemistry

Tissue samples were dehydrated and embedded in paraffin. Five micrometer sections placed on positively charged slides were stained with hematoxylin and eosin (H&E) for histopathologic examination. Immunohistochemistry for influenza A viral antigen was performed as previously described.[7]

Results

Comparison of HA antigenic site sequences

Selected H1 HA proteins from 1918 to 2009 were aligned. The four major antigenic regions of the HA1 domain, as defined by Brownlee and Fodor,[10] are shown in Figure 1. The 2009 pandemic H1N1 HA is antigenically very similar to previous classical swine H1N1 viruses and to the 1918 virus, the likely ancestor of both human and classical swine H1N1 lineages.[11,12] In classical swine lineage HAs, the S_a and S_b sites, located near the top of the globular head of HA,[10] show strong conservation of the 1918 sequence. Of the 50 antigenic residues examined, the 2009 pandemic H1 matched the 1918 HA at 40 sites (80% identity). In contrast, the 2009 seasonal H1N1 HA used in this study contained 25 changes from the 1918 sequence (50% identity).

Hemagglutinin inhibition (HI) assays

The primary infections resulted in serum HI geometric mean titers ranging from 76·5 to 640 to the inoculating virus. Hemagglutination inhibition assays performed for

	C_b	S_a	S_b
	8 9	1 1 1 1 1	2 2
	7 2	4 7 7 7 8	0 1
		1 0 4 6 1	1 2
SC/1918	LLTASS	PN KKGSS PKLSKS	TGTDQQSLYQNA
Sw/IA/1931	---V--	-- --EN- ------	-S----------
Sw/IA/1/1976	-F-V--	-- --EN- ------	-S----------
NJ/8/1976	---V--	-- ---N- ------	-S----------
CA/07/2009	-S----	-- ---N- ------	-SA---------
Mexico/4108/2009	-S----	-- ---N- ------	-SA---------
WS/1933	--P-R-	-- ---D- ---NN-	SSN------H-V
Hickox/1940	--SKR-	-- E-DG- -N-N--	NIEN-KT--RKE
Brisbane/59/2007	-ISKE-	-- G-NGL -N----	NIGD-KA--HTE
Bethesda/NIH50/2009	-ISKE-	-- G-NGL -N----	DIGN-KT--HTE

	C_{a1}			C_{a2}	
	1 1	2 2	2 2	1 1	2
	8 8	2 2	5 5	5 5	3
	3 7	0 2	2 4	4 9	8
SC/1918	VNNKG	SSK	EPG	SYAGAS	RD
Sw/IA/1931	-----	---	---	P-----	--
Sw/IA/1/1976	-----	T--	-S-	P----N	--
NJ/8/1976	-----	---	---	P----N	--
CA/07/2009	I-D--	--R	---	PH---K	--
Mexico/4108/2009	I-D--	--R	---	PH---K	--
WS/1933	-----	--N	---	-HG-K-	--
Hickox/1940	--K-E	--N	---	-H--K-	-G
Brisbane/59/2007	A---E	--H	---	-HN-E-	--
Bethesda/NIH50/2009	A---E	--H	---	-HN-ER	--

Figure 1. Alignment of viral hemagglutinin (HA) antigenic sites. Representative human and swine H1 HA protein sequences were aligned. The four major H1 antigenic domains (C_b, C_a, S_a, and S_b) as defined by Brownlee and Fodor[10] are shown beneath the brackets. Amino acid residues (H1 open reading frame numbering, including the signal peptide) were aligned to the 1918 HA protein, and conserved residues are shown as dashes. Dashed lines separate swine lineage from human lineage HAs.

activity against the Mex09 challenge virus showed that sera from mice initially infected with seasonal human H1N1 viruses (H40 and NIH50) each had a GMT to Mex09 of 10; sera from the NIH20 H3N2 infected mice showed no cross-reactive antibodies against Mex09, while the Sw76-infected mice had a GMT of 60. Assay of pre-challenge sera against a chimeric influenza virus containing the 1918 HA showed that only sera from the Sw76 infected mice exhibited 1918 virus HA cross-reactivity, with a GMT of 60·6.

Weight loss and survival of swine 2009 H1N1 challenged mice

Following Mex09 challenge, mice previously infected with Sw76 had no illness and lost no weight (Figure 2). Mice previously infected with H1N1 viruses H40 or NIH50 showed weight loss patterns similar to each other, with a maximum loss of about 12–15% at days 3–5 post-challenge and recovery by day 14. The H3N2 NIH20 infected animals showed significant weight loss of approximately 20% with delayed recovery but no deaths. Mock infected mice lost significant weight and experienced 40% mortality.

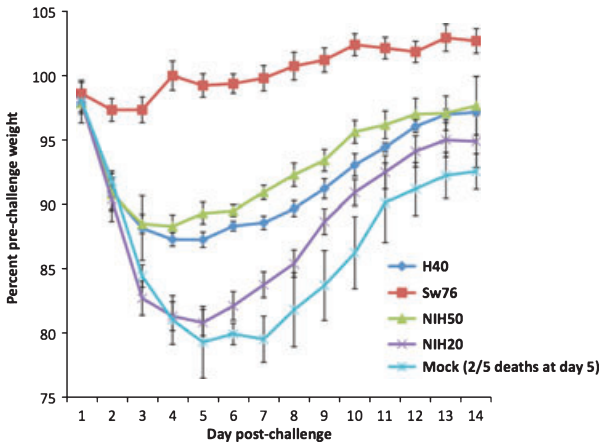

Figure 2. Weight loss of mice challenged with 2009 pandemic H1N1 virus. Mice were inoculated with 5×10^4 PFU of A/Hickox/40 (H40), A/Swine/Iowa/76 (Sw76), A/Bethesda/50/2009 (NIH50), A/Bethesda/20/2009 (NIH20) or PBS (mock). After 28 days mice were challenged with 4×10^5 PFU A/Mexico/4108/09 (Mex09) and daily weights were measured. Weights are presented as the mean percent at ''day-1'' Mex09 challenge weight. Error bars represent standard deviations of the mean.

Table 1. Replication and immunogenicity of viruses used in study

		Immune response to virus*								
		Homologous		Mex09		1918		Mex09 lung titer (Log_{10} PFU/g ± SEM)		
Virus	Subtype	No. Responding	GMT	No. Responding	GMT	No. Responding	GMT	Day 4	Day 6	Survival
A/Hickox/40 (H40)	H1N1	5/5	320	5/5	10	0/5	0	$3{\cdot}6 \times 10^3 \pm 1{\cdot}62$	BD**	5/5
A/Swine/Iowa/76 (Sw76)	H1N1	5/5	640	5/5	60	5/5	60·6	BD	BD	5/5
A/Bethesda/NIH50/09 (NIH50)	H1N1	5/5	640	3/5	10	0/5	0	$4{\cdot}4 \times 10^3 \pm 3{\cdot}21$	BD	5/5
A/Bethesda/NIH20/09 (NIH20)	H3N2	5/5	76·5	0/5	0	0/5	0	$6{\cdot}9 \times 10^4 \pm 3{\cdot}5$	$1{\cdot}5 \times 10^3 \pm 0{\cdot}13$	5/5
Mock	N/A	N/A	N/A	0/5	0	0/5	0	$6{\cdot}0 \times 10^5 \pm 3{\cdot}17$	$6{\cdot}0 \times 10^5 \pm 1{\cdot}4$	3/5

*Response to viral infection measured by hemagglutination inhibition (HI). Homologous = response to initial viral infection in each group; Mex09 = cross-reactive response to Mex09; 1918 = cross-reactive response to 1918; No. responding = number of mice in each group developing a positive HI titer; GMT = reciprocal geometric mean titer in each group.
**BD indicates below the limit of detection, which in this study was a titer of 2 (Log_{10} PFU/g).

Challenge virus pulmonary replication

Mock-infected mice had Mex09 challenge virus titers of 6×10^5 PFU/g lung tissue on days 4 and 6 post-challenge (Table 1). Mice previously infected with Sw76 showed no detectable viral replication. Prior infection with human H1N1 strains H40 or NIH50 resulted in 166-fold and 136-fold lower Mex09 virus replication at day 4 post-challenge, respectively, with titers below the limit of detection by 6 days post-challenge. Infection with the H3N2 strain NIH20 decreased viral titer 8·7-fold at day 4 and 400-fold at day 6 (Table 1).

Histopathology

Lung sections from mock-infected mice challenged with Mex09 showed moderate-to-marked bronchiolitis and alveolitis with alveolar edema and/or hemorrhage (Figure 3A). The inflammatory infiltrate was predominantly lymphohistiocytic with some neutrophils. Lung sections from mice infected with the 2008 H3N2 virus (NIH20) showed a nearly identical pathologic picture (Figure 3B). Lung sections from mice infected with the 1940 seasonal (H40) and 2009 seasonal (NIH50) H1N1 viruses showed a more focal pattern of mild-moderate bronchiolitis, alveolitis, and focal alveolar edema (Figure 3C,D); the H40 infected animals showed fewer pathologic changes than the NIH50 infected animals. In contrast, mice infected with the Sw76 virus prior to Mex09 challenge showed no evidence of alveolitis or bronchiolitis (Figure 3E), but did have prominent lymphoid aggregations in the submucosa of the bronchial tree (Figure 3F).

Discussion

Experimental protection against the pandemic H1N1 virus is of obvious interest because most humans have been exposed to circulating seasonal H1N1 and H3N2 viruses throughout their lifetimes, and because preliminary epidemiologic data from older persons in 2009 have suggested lower than expected influenza attack rates, severe disease, and death.[13] Recent studies have suggested that the current pandemic H1N1 virus predominantly infects children and young adults, with a median age of between 12 and 22.[14–16] The median age of fatal cases was recently reported as 37, with approximately 50% of fatalities in 20–49 year olds.[17] This is markedly different from seasonal influenza, in which 95% of mortality usually occurs in persons >65 years old.[18] A recent report showed that 2009 pandemic H1N1 cross reactive antibodies were detected in 6–7% of 18 to 64 year olds and in 8–33% of people ≥64 years.[19] This suggests that individuals exposed to H1N1 influenza viruses in the first few decades after the 1918 pandemic may have some degree of cross-protective immunity against the 2009 H1N1 pandemic virus. It is thus important for pandemic and vaccination planning to evaluate whether and under what circumstances past H1N1 influenza virus exposures might afford a degree of protection against the 2009 pandemic virus.

The surface glycoprotein HA contains the four major antigenic domains of influenza A viruses.[10,20] Accumulation

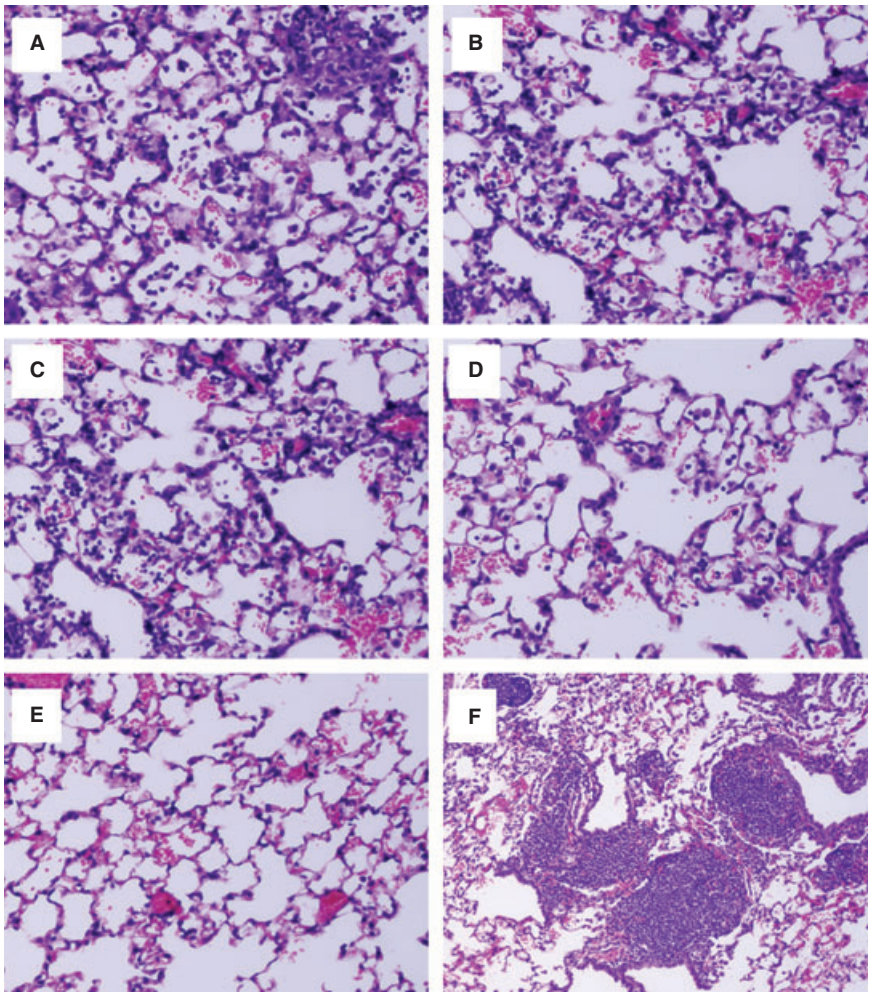

Figure 3. Pathology of mice challenged with 2009 pandemic H1N1 influenza virus. (A) Section from a mock-inoculated animal challenged with the Mex09 virus. Moderate-to-marked acute alveolitis and bronchiolitis are seen (original magnification, ×200); (B) Section from a 2008 human H3N2 (NIH20) inoculated animal challenged with the Mex09 virus. Moderate-to-marked acute alveolitis and bronchiolitis are seen (original magnification, ×200); (C) Section from a 2009 human H1N1 (NIH50) inoculated animal challenged with the Mex09 virus. Mild-to-moderate acute alveolitis and bronchiolitis are seen (original magnification, ×200); (D) Section from a 1940 human H1N1 (Hx40) inoculated animal challenged with the Mex09 virus. Focal mild acute alveolitis and bronchiolitis are seen (original magnification, ×200); (E) Section from a Sw76 inoculated animal challenged with the Mex09 virus. No significant lung pathology is noted (original magnification, ×200); (F) Section from a Sw76 inoculated animal challenged with the Mex09 virus. Prominent lymphoid aggregates in the submucosa of the bronchial tree are noted (original magnification, ×100).

of HA mutations by antigenic drift arising from population immune pressure is a significant cause of the emergence of new seasonal human influenza viruses.[21] In contrast, antigenic drift of H1N1 viruses in pigs occurs more slowly,[11] perhaps in part because of the short life span of pigs in domestic agriculture. Conservation of the S_a and S_b antigenic sites in classical swine lineage HAs might also reflect these regions being less antigenically important in antibody-mediated immune responses in swine as compared to the corresponding sites in human influenza viruses.

The 1918 pandemic H1N1 influenza virus is the likely common ancestor of both the human H1N1 and the classical swine H1N1 lineages,[11,12] both of which have evolved independently since 1918. Archaeserologic data identified high levels of cross-reactive antibody titers to the 2009 pandemic virus in persons born before 1930,[19,22] possibly indicating major H1N1 antigenic changes around the time of the severe 1928–1929 epidemic,[23] and declining titers in ever smaller percentages of seropositive persons born in the 1930s, 1940s, and later.[19] Increasing human age in 2009 should thus be highly correlated with past exposures to ever earlier drift descendants of the human 1918 virus, and thus to HAs ever more closely related antigenically to the 1918-derived classical swine lineage HAs[24] from which the HA of the 2009 pandemic virus is derived.[5] In contrast, HAs on more recent human seasonal H1N1 viruses are far more distantly related to the 1918 virus and to classical swine lineage viruses. However, it is not yet clear that these human serologic data can fully explain 2009 epidemiologic patterns, or whether there may be one or more additional age thresholds for immunologic protection corresponding to other past influenza events.

Taking data from the 1957 H2N2 pandemic as a benchmark for age-specific morbidity and mortality trends,[25] preliminary data from the 2009 pandemic[26] seem to be consistent with a protective affect against illness in persons no older than 37·5 years old, and a protective effect against pneumonia and influenza (P&I) mortality in persons older than 57·5 years old in 2009. However, such data are difficult to interpret, not only because of their

preliminary nature but also because they do not distinguish between lower attack rates, ''delayed'' infections, lower rates of clinically apparent illness, or lower rates of complications, and because a possible contribution to protection by neuraminidase or other viral antigens has not been evaluated.

It has also been speculated that decreased morbidity/mortality in older persons in the 2009 pandemic might result in part from vaccination with the 1976 ''swine flu'' vaccine,[19] an inactivated vaccine made from an influenza A H1N1 virus designated A/New Jersey/8/1976 that is antigenically very similar to the Sw76 virus used in the protection studies reported here, and whose HA is closely related to the HA on the current pandemic virus (Figure 1). The 1976 vaccine was administered to about 45 million persons,[27] most of whom were 18 years old or older at that time. Roughly 25 million of these vaccinees (57%) are believed to be alive today, approximately half being 52–65 years old and half over 65 years old (Table 2). A possible protective effect of the 1976 vaccine can hopefully be examined by ''historical'' or ''retrospective-prospective'' cohort or other epidemiologic studies. If a protective effect is found, it will be important to try to determine its mechanisms, which might be complex and include ''boosting'' of vaccine responses by infection with circulating H1N1 viruses.

The data presented in this study may provide some support for this hypothesis, as Sw76 virus infection conferred complete protection against Mex09 challenge, whereas vaccination with human H1N1 virus H40 or NIH50 provided only partial protection. Our study also provides data consistent with the archaeserologic studies described above, in which cross-reacting antibodies to the 2009 pandemic virus were detected in the sera of persons born before 1930 and likely exposed to 1918-descended H1N1 viruses in the decade after the pandemic,[19,22] since infection of mice with Sw76 resulted in cross-reactive antibodies against the reconstructed 1918 influenza virus. However, the mouse challenge studies do not fully correspond to the natural situation in humans, e.g., the 33-year gap between swine influenza vaccination in 1976 and current pandemic virus exposure, which might be associated with loss of immune memory or intermittent boosting by exposure to naturally circulating H1N1 viruses after 1977.

Table 2. Estimated number of persons alive in 2010 who had been vaccinated against influenza in the United States in 1976*

Age in 1976	Age in 2010	Alive in 2010	Percent vaccinated in 1976	Number vaccinated in 1976
≤17	34–51	77 192 149	0	0
18–20	52–54	12 923 191	0·19	2 455 406
21–25	55–59	19 517 000	0·28	5 464 760
26–29	60–63	13 816 273	0·28	3 868 556
30	64	2 943 615	0·32	941 957
31–35	65–69	12 261 000	0·32	3 923 520
36–40	70–74	9 202 000	0·32	2 944 640
41–45	75–79	7 282 000	0·32	2 330 240
46–49	80–83	4 781 546	0·32	1 530 095
50	84	950 116	0·33	313 538
51–55	85–89	3 650 000	0·33	1 120 500
56–60	90–94	1 570 000	0·33	518 100
61–65	95–99	452 000	0·33	149 160
≥66	≥100	79 000	0·33	26 070
Total		89 427 741	0·2871	25 670 542

*Derived from data published in: References [33] and [34]. The non-standard age ranges in the first and second columns reflect the original data.

The current study also revealed that mice infected with a 1940s-era H1N1 virus (H40) before Mex09 challenge had somewhat less severe pathology and lower lung titers than mice infected with a 2009 seasonal H1N1 virus (NIH50). Alignment of the antigenic sites of 1918-derived human and swine lineage H1N1 HAs demonstrates that rapid antigenic drift likely occurred in the decade after the 1918 pandemic, since the earliest isolated human H1N1 virus (A/WS/1933) had accumulated a number of antigenic differences from the 1918 virus (Figure 1). Continual antigenic drift during seasonal influenza virus circulation may partially explain why an earlier human H1N1 virus offered modestly more protection against Mex09 challenge than a contemporary seasonal H1N1 virus, and supports epidemiological findings in the 2009 pandemic that people over about age 60 may have a degree of immunologic protection. Mice inoculated with NIH20 (a 2009 seasonal H3N2) had modest decreases in viral replication after challenge with Mex09 as compared to controls, suggesting that uncharacterized immune responses may be playing a partially protective role. The observation of neutralizing antibody-independent heterosubtypic immunity to influenza viruses has been described in previous studies and are associated with cytotoxic T-lymphocyte (CTL) responses.[28–32] It must be stressed that experimental infection with live viruses, followed closely by viral challenge, is not an ideal model for studying human protection induced by live attenuated or inactivated vaccines. Further work is necessary to determine if inactivated vaccines would also elicit a similar effect and provide heterosubtypic immunity. Nonetheless, these studies are important in linking viral evolution at key antigenic sites to a role in protective heterologous immunity. Clearly, key antigenic sites on the HA protein of swine lineage influenza viruses have been preserved from 1918 to the present, and these epitopes may be susceptible to

neutralization by antibodies induced by early descendents of the 1918 human influenza virus and conceivably also by an H1N1 swine influenza vaccine administered to millions of Americans 33 years ago.

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