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Evaluation of the antigenic relatedness and cross-protective immunity of the neuraminidase between human influenza A (H1N1) virus and highly pathogenic avian influenza A (H5N1) virus

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ABSTRACT

To determine the genetic and antigenic relatedness as well as the cross-protective immunity of human H1N1 and avian H5N1 influenza virus neuraminidase (NA), we immunized rabbits with either a baculovirus-expressed recombinant NA from A/Beijing/262/95 (BJ/262) H1N1 or A/Hong Kong/483/97 (HK/483) H5N1 virus. Cross-reactive antibody responses were evaluated by multiple serological assays and cross-protection against H5N1 virus challenge was evaluated in mice. In a neuraminidase inhibition (NI) test, the antisera exhibited substantial inhibition of NA activity of the homologous virus, but failed to inhibit the NA activity of heterologous virus. However, these antisera exhibited low levels of cross-reactivity measured by plaque size reduction, replication inhibition, single radial hemolysis, and ELISA assays. Passive immunization with HK/483 NA-specific antisera significantly reduced virus replication and disease, and afforded almost complete protection against lethal homologous virus challenge in mice. However, passive immunization with BJ/262 (H1N1) NA-specific antisera was ineffective at providing cross-protection against lethal H5N1 virus challenge and only slightly reduced weight loss. Substantial amino acid variation among the NA antigenic sites was observed between BJ/262 and HK/483 virus, which was consistent with the lack of cross-reactive NI activity by the antibody and limited cross-protective immunity in mice. These results show a strong correlation between the lack of cross-protective immunity and low structural similarities of NA from a human seasonal H1N1 virus and an avian H5N1 influenza virus.

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Introduction

Neuraminidase (NA) and hemagglutinin (HA) are two surface glycoproteins of influenza A viruses. The HA functions as the receptor binding and membrane fusion protein as well as serving as the target for viral neutralizing antibodies (Wilson and Cox, 1990). The best understood role of the enzyme activity of NA is during the final stage of infection; NA cleaves sialic acid from the viral envelope, thus allowing release of progeny viruses from the host cell and preventing virus aggregation (Palese et al., 1974; Seto and Rott, 1966). The role of NA at the early stages of infection has also been proposed; NA may catalyze removal of "decoy" receptors on mucins, cilia, and cellular glycocalyx present on the human airway epithelium to promote access to target cells (Matrosovich et al., 2004). *In vitro*, anti-NA antibodies do not fully neutralize virus infectivity; however they can reduce viral yield (Kilbourne et al., 1968). Similarly, in animals, anti-NA antibodies are infection permissive, but NA-specific antibodies can reduce illness by diminishing viral replication and lung pathology (Johansson et al., 1989; Schulman et al., 1968). In humans, anti-NA antibodies induced by natural infection and/or immunization appear to attenuate clinical illness following subsequent exposures to influenza viruses containing the same or antigenically closely related NA (Beutner et al., 1979; Couch et al., 1974; Monto and Kendal, 1973; Murphy et al., 1972; Smith and Davies, 1976). Thus, anti-NA antibodies may modify the virulence and the epidemiological impact of influenza.

The three-dimensional structure of 7 of 10 known NA subtypes of influenza A viruses (N1, N2, N4, N5, N8, N9, and N10) and influenza B virus have been determined (Baker et al., 1987; Burmeister et al., 1992; Collins et al., 2008; Li et al., 2010, 2012; Russell et al., 2006; Varghese et al., 1983; Wang et al., 2011; Xu et al., 2008; Zhu et al., 2012). The overall structures of these NAs are very similar. Each NA monomer contains the catalytic site surrounded by multiple hypervariable loops accessible to antibodies. A total of 8 upper surface loops have been defined as antigenic regions by X-ray crystallographic

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analysis of one N2 and two N9 NA-antibody complexes; each antibody epitope consists of about four loops located at the rim of the enzyme active site (Colman et al., 1987; Malby et al., 1994; Venkatramani et al., 2006). Amino acid changes in these loops were previously observed in laboratory-derived escape mutants selected with mouse monoclonal antibodies (mAb) that inhibit NA activity of human influenza H2N2 and H3N2 viruses (Air et al., 1985; Gulati et al., 2002; Lentz et al., 1984). Similarly, amino acid substitutions in theses loops occurred naturally among viruses of the same subtype (Air et al., 1985; Colman et al., 1983; Shil et al., 2011; Xu et al., 1996), suggesting that these upper surface loops are antigenically and epidemiologically important for human H2N2 and H3N2 viruses and likely induce NA inhibition (NI) antibodies. However, until recently the antigenic determinants for N1 have not been widely studied or assessed (Wan et al., 2013).

Historically, H1N1 descendants of the 1918 pandemic virus circulated in humans through the 20th century and into the early 21st century resulting in over 20 epidemics between 1933–1957 and 1977–2009 (Centers for Disease Control and Prevention, 2010a; Francis, 1953; Katz et al., 2011; Tumpey et al., 2005). In 2009, an H1N1 virus emerged from North American swine to infect humans and possessed an NA gene segment from the Eurasian swine genetic lineage (Garten et al., 2009). Thus, most current day adults have varying levels of anti-N1 antibodies as a result of natural infection and/or vaccination with H1N1 viruses (Couch et al., 2013; Powers et al., 1996).

There is considerable concern that highly pathogenic avian influenza (HPAI) H5N1 viruses could develop the capacity to spread among humans and cause a major pandemic (WHO, 2013). The NAs of currently circulating H5N1 viruses and human H1N1 viruses are classified in the same NA subtype and low levels of anti-NA cross-reactive antibodies to H5N1 viruses have been detected in human sera of unexposed humans (Frobert et al., 2010; Sandbulte et al., 2007). However, the systematic evaluation of the antigenic and cross-protective immunity of the NAs between influenza H1N1 and H5N1 viruses is understudied.

It has been demonstrated that recombinant NA (rNA) produced by a baculovirus expression system is enzymatically functional and antigenically indistinguishable to virion NA (Johansson et al., 1995). Baculovirus-expressed rNA protein is properly folded, as confirmed by crystallization and structural analysis (Xu et al., 2008; Zhu et al., 2012). Moreover, rNA vaccines can induce NI antibodies that suppress viral replication and disease in mice (Deroo et al., 1996; Kilbourne et al., 2004). To evaluate the antigenic relationship of the NAs from human seasonal H1N1 and avian H5N1 viruses and to determine whether antibodies induced by N1 NA can provide cross-protective immunity against H5N1 virus in mice, antisera were generated by immunizations of rabbits with baculovirus-expressed rNA prepared from A/Beijing/262/95 (BJ/262) H1N1 virus or A/Hong Kong/483/97(HK/483) H5N1 virus. We compared results obtained from five different immunological assays for detecting anti-NA cross-reactive antibodies to H5N1 viruses. The amino acid sequence differences and similarities in the potential NA antigenic sites between the subtype viruses were also determined. Our results indicate that the antibodies induced by BJ/262 rNA afforded very limited cross-reactivity *in vitro* and cross-protection *in vivo* against H5N1 virus, which is consistent with the differences in their antigenic structures evaluated in this study.

Results

Antigenic relatedness between the NA of H1N1 and H5N1 viruses

Cross-reactive anti-NA antibody responses were evaluated by NI, RI, PSR, SRH, and ELISA assays using Rα raised against rNA protein from BJ/262 (H1N1) or HK/483 (H5N1) virus. Both rNA proteins generated detectable titers of anti-NA antibodies against homologous virus (Table 1). In NI tests, Rα to BJ/262 NA (Rα–BJ/262) showed substantial inhibition of NA activity of the homologous virus, but failed to inhibit the NA activity of HK/483 virus. In RI and PSR assays, Rα–BJ/262 showed substantial inhibition of BJ/262 and HK/483 viral replication in MDCK cells, but inhibition of HK/483 virus replication was 8- to 12-fold lower compared to that observed against homologous virus. In the SRH assay, Rα–BJ/262 only caused partial hemolysis of a small proportion of HK/483 virus-coated RBC cells (Table 1). Finally, vaccination with either of the rNA proteins induced substantial ELISA (IgG) antibody responses; however, titers against heterologous rNA protein were 4- to 8-fold lower than titers measured to homologous rNA (Table 1). Taken together these results demonstrate that although antisera to rNA from H5N1 or H1N1 virus generated detectable cross-reactive anti-NA antibodies, they exhibited poor inhibition of NA activity and viral replication.

In vivo cross-protective immunity of the antibodies induced by BJ/262 rNA

Next, we evaluated whether the cross-reactive antibodies induced by BJ/262 rNA could offer some level of cross-protection against H5N1 virus in mice. In the first challenge experiment, mice were injected i.p. with one dose of 0.5 ml of sera 24 h prior to challenge with 5 LD_{50} of HK/483 virus. All mice that received either Rα–BJ/262 or NRS (control) died within 8 days p.i. whereas 2 of 3 mice that received homologous Rα–HK/483 survived (data not shown). These data indicated that a single passive immunization of Rα–BJ/262 failed to provide any cross-protection against a lethal H5N1 virus challenge.

In the second challenge experiment, mice were passively immunized with three doses of anti-NA sera or NRS as the negative control. The first dose of 1 ml of sera was given 24 h prior to challenge with 3 LD_{50} of HK/483 virus; the second and the third doses of 0.5 ml of sera were given 2 and 5 days p.i. As a positive control, an additional group of mice received 0.2 ml i.p. of Rα to VN/1203 (H5) rHA (homologous HI titer = 2560) 24 h before challenge. All mice that received anti-H5 rHA were substantially protected against weight loss and survived HK/483 virus challenge (Fig. 1A and B). These mice also displayed significantly lower virus titers in lung and brain tissues compared to NRS-treated control mice (Fig. 1C). Mice that received homologous antisera to H5N1 NA (Rα–HK/483) also displayed some level of protection against H5N1 challenge; the majority (86%) of Rα–HK/483-treated mice survived challenge and exhibited significantly less weight loss ($p < 0.05$) compared to NRS-treated control mice (Fig. 1A and B). Moreover, passive immunization of Rα–HK/483 rNA resulted in at least 3000-fold less virus in lung tissues and over 100-fold less virus in brain tissues compared to that of NRS-treated mice ($p < 0.05$). However, passive immunizations with three doses of Rα–BJ/262 (H1N1) failed to provide cross-protection against death and only slightly reduced weight loss early during the course of H5N1 virus infection (Fig. 1A and B). The antiserum was also ineffective at significantly reducing virus replication in lung and brain tissues (Fig. 1C). These data demonstrated that the antibodies induced by BJ/262 rNA afforded very limited cross-protective immunity against HK/483H5N1 virus even in mice treated with multiple doses of antiserum and challenged with a lower lethal dose of virus (3 LD_{50}).

Amino acid comparisons of the NA antigenic sites between avian H5N1 and human H1N1 viruses isolated between 1934 and 2007

We performed a comprehensive sequence comparison of a panel of epidemiologically important influenza viruses and focused on residues in the globular head of NA believed to be antigenic

determinants (Colman et al., 1983). The NA crystal structure of the 1918 H1N1 virus has allowed for a more detailed analysis of the amino acids that make up the loop regions as well as its structural comparison with H5N1 NA (Xu et al., 2008). We aligned the NA sequences of 11 viruses isolated from major influenza H1N1 epidemics between 1934 and 2007 (Centers for Disease Control and Prevention, 2010a; Francis, 1953), and located the changed residues in the N1 three-dimensional structure. On the basis of their accessibility to antibodies, and their distance to the catalytic site, 20 amino acids were identified as potential key contributors to interactions with antibodies. These sites cluster preferentially into seven upper surface loops (195–202, 216–231, 243–251, 316–353, 364–374, 398–407, and 428–439). Table 2 presents these loops with the amino acid substitutions of the H1N1 viruses and Fig. 2 provides a cartoon representation of the key 20 amino acids (N2 numbering) along with the seven loops denoted in different colors. These potential antigenic sites form a nearly continuous surface across the top of the NA monomer, encircling the catalytic site pocket (shown in yellow).

Table 1
Antigenic differences between NAs of BJ/262 and HK/483 viruses.

Assays	Antigens	Antibody titers[a]		NRS
		Rα–BJ/262	Rα–HK/483	
NI	BJ/262 virus	320[b]	≤ 10	≤ 10
	HK/483 virus	≤ 10	320	≤ 10
RI	BJ/262 virus	160	10	≤ 10
	HK/483 virus	20	80	≤ 10
PSR	BJ/262 virus	2560	100	≤ 100
	HK/483 virus	200	1280	≤ 100
SRH	BJ/262 virus	194	n.d	n.d
	HK/483 virus	88[ph]	170	n.d
ELISA IgG	BJ/262 rNA	102,400	12,800	≤ 100
	HK/483 rNA	25,600	102,400	≤ 100

ph: partial hemolysis; n.d: not detectable.

[a] Rabbits were immunized with 4 doses of 100 μg of BJ/262 rNA or HK/483 rNA plus Titermax adjuvant at 1 month interval, and blood was collected 1 month after the last immunization. Normal rabbit serum (NRS) was used as a negative control.

[b] All samples were tested in duplicate or triplicate in the each assay. The homologous titers are underlined.

Next, we compared the genetic similarities between H5N1 influenza viruses circulating in avian populations since 1997 and seasonal H1N1 viruses circulating in humans between 1934 and 2007. Despite the existence of multiple clades based on HA phylogeny, the N1 NAs of avian H5N1 viruses do not exhibit progressive antigenic drift in the same way as the NA of human seasonal H1N1 viruses; only minor amino acid variations in these sites were observed (Table 2). The key amino acids within the potential antigenic sites of HK/483 and BJ/262 NA are visualized in the N1 structure showing differences and similarities (Fig. 3). The monomeric N1 structure revealed substantial differences in residue side chain composition between the H1N1 and the H5N1 viruses; only 3 amino acids located on 3 of the NA surface loops were identical (shown in red). The existence of distinct antigenic differences among the NA of the two subtype viruses most certainly accounted for the lack of cross-NI activity and cross-protection observed in mice.

Discussion

Genetic variations of the HA and NA are responsible for the appearance of epidemic and pandemic influenza viruses. The antigenic changes in the HA and NA occur independently in nature and the NA evolves at a slower rate than HA (Kilbourne et al., 2002). Although protective immunity to influenza is largely mediated by anti-HA antibodies, anti-NA antibodies may play a significant role during influenza epidemics and pandemics when major drift or shift in HA occurred without concomitant significant antigenic changes of the NA (Beutner et al., 1979; Brett and Johansson, 2005; Johansson, 1999; Kilbourne et al., 2002; Monto and Kendal, 1973; Murphy et al., 1972). As H5N1 virus remains a

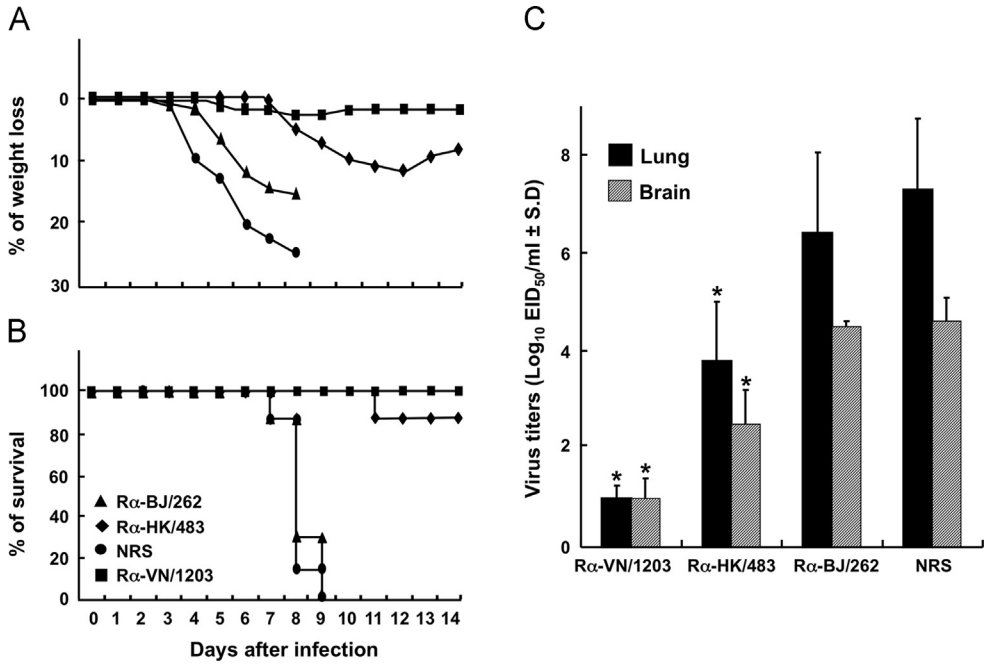

Fig. 1. Protective efficacy of influenza anti-NA antibodies against HK/483 (H5N1) virus challenge in mice. Mice (10/group) were passively immunized i.p with three doses of Rα–BJ/262 (⋆), Rα–HK/483 (♦), or NRS (●) as a negative control. Animals were given the first dose of sera (1 ml/mouse) 24 h before challenge with 3 LD_{50} (= $10^{2.3}$ EID_{50}) of a HK/483 virus; the second and the third doses of sera (0.5 ml/mouse) were given at 2 and 5 days p.i. The mice that received one dose of 0.2 ml of Rα–VN/1203 (H5) rHA (■) were used as positive controls. Mice were observed daily for weight loss (A) and survival (B) for 14 days. Virus titers in lungs and brains (C) were determined 6 days p.i. and are expressed as the $\log_{10}$ EID_{50}/ml ± S.D. of 3 mice per group. $^{*}p < 0.05$ compared to NRS group.

Table 2
Amino acid differences and similarity in antigenic sites of the NAs between human H1N1 and HPAI H5N1 viruses.

N1 numbering N2 numbering	195–202	216–231		243–251			316–353					364–374				398–407	428–439			
	200 199[a,b]	220 219	222 221[a,b]	248 247	249 248[a]	250 249[a]	329 329[a,b]	332 332	336 339[a]	339 342[b]	344 347[a]	365 368[a,b]	366 369[a,b]	367 370[a,b]	369 372[b]	396 400[a,b]	430 430[b]	432 432[a,b]	434 434[a]	435 435[a,b]
Human H1N1 viruses																				
PR/8/34	N	R	K	D	G	L	E	T	G	Y	N	H	S	S	H	M	R	K	K	–
Marton/1943	D	R	E	G	G	P	K	K	G	Y	N	N	S	S	Q	V	R	K	K	–
FM/1/47	D	R	E	G	G	P	K	K	G	Y	N	N	S	S	Q	V	R	K	N	–
Netherlands/1953	D	R	Q	D	G	P	K	K	D	N	D	N	S	S	K	M	R	R	K	T[d]
USSR/90/77	D	R	Q	D	G	P	K	K	D	N	D	N	S	S	K	M	R	R	K	T
Chile/1/83	D	R	R	N	G	P	K	K	D	T	D	N	S	S	K	M	R	R	K	T
Taiwan/1/86	N	K	R	N	G	P	K	E	N	T	D	N	S	S	K	M	R	R	N[c]	T
Texas/36/1991	N	K	R	N	G	A	K	E	N	T	D	N	R	L	K	M	R	R	N[c]	T
Beijing/262/95	N	K	R	N	G	A	K	E	N	T	D	N	R	L	K	M	R	R	N[c]	T
New Caledonia/99	N	K	R	N	G	A	K	E	N	T	D	N	R	L	K	I	L	R	N[c]	T
Brisbane/59/07	N	K	Q	N	K	A	E	E	N	T	N	N	R	L	K	I	L	R	N[c]	T
Avian H51N1 viruses																				
HK/483/97	N	R	N	N	E	Q	N	T	G	S	Y	T	S	S	S	V	R	K	K	–
Vietnam/1203/04	N	R	N	N	G	Q	N	T	G	S	Y	T	N	S	S	I	R	K	S	–
Indonesia/5/05	N	R	D	N	G	Q	N	T	G	S	Y	T	N	S	S	I	R	K	S	–
Anhui/1/05	N	R	N	N	G	Q	N	T	G	S	Y	T	N	S	S	I	R	K	S	–
Turkey/15/06	N	R	N	S	G	Q	N	T	G	S	Y	T	N	S	S	I	R	K	S	–
Hubei/1/10	N	R	N	N	G	Q	N	K	G	S	Y	T	H	S	S	I	R	K	S	–
HK/7032/12	N	R	N	N	G	Q	N	T	G	S	Y	T	H	S	S	I	R	K	S	–

[a] Amino acid variations within putative antigenic sites among human H2N2 and/or H3N2 field strains (Colman et al., 1983; Air et al., 1985; Xu et al., 1996; Shil et al., 2011).
[b] The sites were identified by genetic analysis of escape mutants isolated in the presence of mouse mAbs against N2 (Lentz et al., 1984; Air et al., 1985; Gulati et al., 2002), N8 (Saito et al., 1994), N9 (Webster et al., 1987; Air et al., 1990b; Tulip et al., 1991), or influenza B NA (Air et al., 1990a).
[c] Glycosylation sites (NXS/T).
[d] Amino acid insertion at this residue.

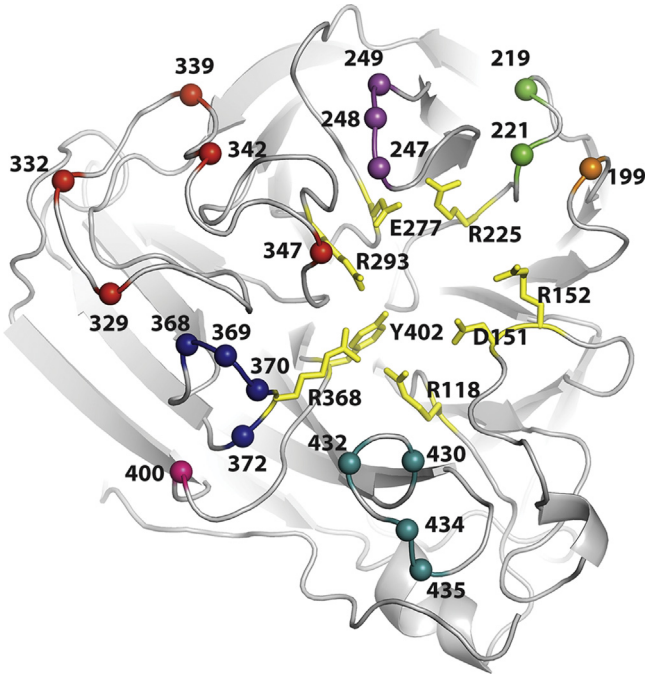

Fig. 2. Location of the potential antigenic sites of NAs of human H1N1 viruses isolated between 1934 and 2007 on the N1 NA three-dimensional structure. A total of 20 amino acids (N2 numbering) in the seven loops denoted in different colors (orange for 199, green for 219 and 221, magenta for 247–249, red for 329, 332, 339, 342 and 347, blue for 368, 369, 370 and 372, hot pink for 400, and deep teal for 430, 432, 434 and 435) are depicted on the upper NA globular head in carton model. The amino acid residues in the enzyme active site are represented as yellow sticks.

persistent threat to public health, we wanted to determine whether seasonal N1 induced anti-NA cross-reactive antibodies and protection against H5N1 virus. Overall, these results demonstrated that influenza H1N1 BJ/262 NA-specific antibodies afforded little to no cross-protective immunity against HK/483 H5N1 virus. A detailed comparison of the amino acid sequences and NA structures identified differences in putative antigenic sites between the two NA proteins.

The H1N1 subtype virus was first isolated from humans in 1933 and persisted in humans until 1957, followed by its re-emergence in 1977 (Kendal et al., 1979; Smith et al., 1933). At the time of its disappearance in 2009, a novel swine-origin H1N1 virus emerged in North America and continues to spread globally (Centers for Disease Control and Prevention, 2010b). Although H1N1 subtype viruses have caused multiple epidemics between 1933 and 2009 (Centers for Disease Control and Prevention, 2010a; Francis, 1953), the antigenic mapping of the N1 NAs have not been well studied. As a consequence of antibody selection in the human population, the H1N1 NAs showed progressive genetic changes since 1934, which has provided a unique opportunity for mapping amino acid substitutions in putative antigenic sites using epidemiologically important influenza viruses. Very recently, Wan et al. mapped antigenic domains of the N1 NA and identified N1 residues that are essential for binding of cross-reactive mouse monoclonal antibodies (Wan et al., 2013). Using data from previously published reports, we identified 20 amino acid residues on the seven upper surface loops of the N1 NA, which are mapped onto potential antigenic sites. These sites contain NA mutations found in 11 H1N1 influenza viruses that circulated in humans between 1934 and 2009. These amino acid changes are presumably accessible to anti-NA antibodies and hence, most likely to be selected by human anti-H1N1 NA antibodies. Interestingly, X-ray crystallographic analysis of N2 and N9 and NI-antibody complexes demonstrated similar antigenic sites (Colman et al., 1987; Malby et al., 1994; Venkatramani et al., 2006). NA antibody epitopes have also been identified by the use of escape mutants selected with mouse mAbs against N2, N8, N9 and/or influenza B NAs (Air et al., 1985, 1990a, 1990b; Gulati et al., 2002; Lentz et al., 1984; Saito et al., 1994; Tulip et al., 1991; Webster et al., 1987), and in H2N2/H3N2 virus escape mutants selected with human anti-N2 antibodies (Colman et al., 1983; Shil et al., 2011; Xu et al., 1996). In accordance with the

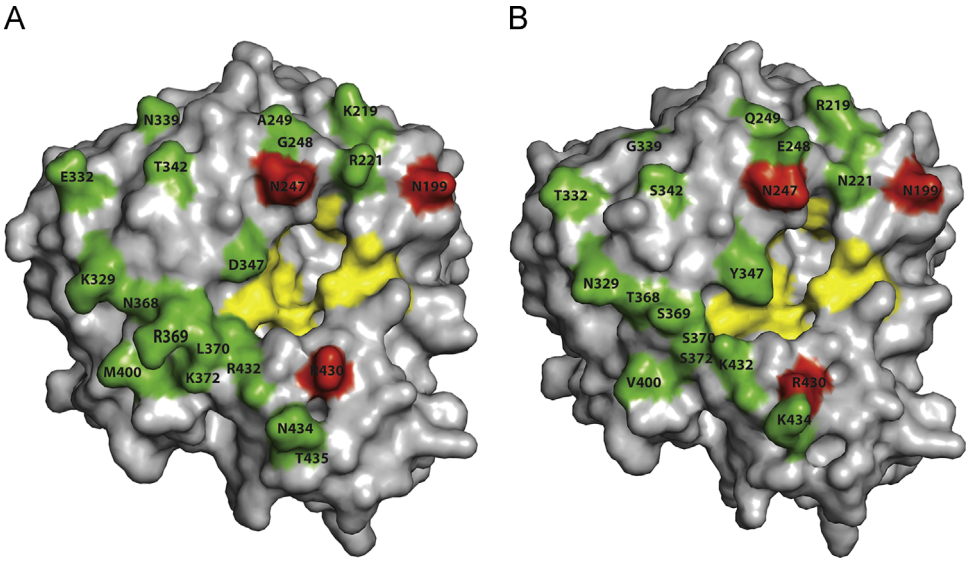

Fig. 3. Comparison of amino acid residues in the potential NA antigenic sites between HK/483 H5N1 and BJ/262 H1N1 viruses. The putative antigenic sites (N2 numbering) are shown on filled-space models of the monomeric NA structure constructed using PyMOL on BJ/262 NA (A) or HK/483 NA (B). The enzyme active site is depicted as yellow surfaces. Three of 20 sites (15%) that show the same amino acids between BJ/262 NA and HK/483 NA are given red color, and 17 of 20 sites (85%) that show amino acid variations between BJ/262 NA and HK/483 NA are given green color.

observation that all of the laboratory escape mutants can only be selected by mAbs that completely inhibit NA enzyme activity (Gulati et al., 2002; Webster et al., 1987), these antigenic sites are likely important for NI antibody recognition. Our data, along with other previously published work (Gulati et al., 2002; Saito et al., 1994; Webster et al., 1987) suggest that the location of NI antibody epitopes appear to be similar among different subtypes of influenza A viruses.

NA activity is essential for efficient influenza virus replication (Palese et al., 1974; Matrosovich et al., 2004). In the current study, the presence of HK/483 NA antibodies, containing a high level of NI antibodies to H5N1 virus, significantly reduced homologous virus replication in MDCK cells and in mouse tissues. Although low levels of cross-reactive antibodies against HK/483 virus were detected in Rα–BJ/262 by PSR, RI, SRH and ELISA assays, passive immunization with Rα–BJ/262 failed to protect mice from lethal H5N1 virus challenge. The lack of cross-reactive NI antibodies (with either rabbit serum) suggests that the protective immunity against homologous virus is mainly mediated by NI antibodies, consistent with previous reports (Johansson et al., 1998; Rott et al., 1974; Sylte et al., 2007; Webster et al., 1988). Thus, it is unlikely that the low levels of cross-reactive antibodies against H5N1 NA (detected in Rα BJ/262 by PSR, RI, SRH and ELISA assays) are binding to the globular NA head domains due to their inability to inhibit the NA activity. The lack of a cross-reactive NI antibody and cross-protection between HK/483 and BJ/262 viruses may be related to the absence of sufficient conservation in the NA antigenic structure as suggested by structural studies of NA-antibody interactions (Colman et al., 1987; Malby et al., 1994; Venkatramani et al., 2006).

A protective NI titer is not clearly defined in humans. However, multiple studies suggest that high levels of NI activity against the infecting viruses are needed to provide clinical protection in humans (Beutner et al., 1979; Kilbourne et al., 1995), in mice (Chen et al., 2000) and in chickens (Sylte et al., 2007). Although low levels of anti-H5N1 NA antibodies were detected in a small proportion of human sera (Frobert et al., 2010; Sandbulte et al., 2007) it is unclear whether they would be sufficient enough for providing cross-protection against H5N1 infection. In humans, the majority of H5N1 virus-infected cases are children and young adults many of whom likely have cross-reactive anti-N1 antibodies induced by seasonal H1N1influenza infection. This suggests that such anti-N1 antibodies may be irrelevant in the face of a severe H5N1 virus infection, which is consistent with a study demonstrating that immunization with the 2006–2007 seasonal trivalent inactivated influenza vaccine failed to induce anti-N1 cross-protection antibodies in humans (Frobert et al., 2010).

We observed a correlation between the lack of cross-protective immunity and low structural similarities between BJ/262 NA and HK/483 NA. Although these NAs are classified in the same N1 serotype, amino acid sequence similarity of NAs between them demonstrated a distant (83% identical) evolutionary relationship. Further comparison of the 20 potential antigenic sites showed that only 3 (15%) sites were shared between BJ/262 NA and HK/483 NA. Due to increasing population immunity to H1N1pdm09 since the 2009 pandemic, additional work is needed to determine whether antibodies induced by contemporary N1 NA can provide cross-protective immunity against H5N1 virus.

Materials and methods

Viruses

Influenza A viruses used in this study were A/Hong Kong/483/97 (HK/483) H5N1 virus and A/Beijing/262/95 (BJ/262) human H1N1virus. Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs under conditions that were found to be optimal for virus replication of HK/483 (37 °C, 26 h) or BJ/262 (35 °C, 48 h). Virus stocks were divided into aliquots and stored at −80 °C until use. Inactivated HK/483 virus was prepared by adding 0.1% β-propiolactone (Sigma, St. Louis, MO) to allantoic fluid and then incubating the mixture at 4 °C overnight.

Virus titrations

The hemagglutination assay was performed with 0.5% turkey red blood cells (TRBC) to quantify viral particles and hemagglutination units (HAU) were expressed as the reciprocal of the highest dilution of virus showing complete hemagglutination (Kendal et al., 1982). Fifty percent egg infectious dose (EID_{50}) and 50% mouse lethal dose (LD_{50}) were determined as previously described

(Lu et al., 1999). Fifty percent tissue culture infectious doses ($TCID_{50}$) were determined by serial titration of virus in Madin-Darby Canine Kidney (MDCK) cells and incubated at 37 °C for 3 days. After which virus titers in culture supernatants were determined by hemagglutination assay.

Preparation of rabbit polyclonal antisera

Polyclonal rabbit antisera (Rα) were generated by intramuscular and subcutaneous inoculation of adult New Zealand white rabbits with 100 μg of purified baculovirus-expressed rNA protein emulsified in Titermax adjuvant (CytRx Corporation, Norcross, GA, USA) in a 1:1 ratio. The rNA protein prepared from HK/483 and BJ/262 viruses was chosen because of its commercial availability as a tetrameric structure (Protein Sciences Corporation, Meriden, CT). The rabbits ($n=2$) were immunized with each rNA four times at 1 month intervals, and were bled for antisera 1 month after the last injection. In addition, a single rabbit was immunized with multiple doses of rHA (Protein Sciences) from A/Vietnam/1203/2004 (VN/1203) H5N1 virus emulsified in Titermax adjuvant and used as a positive control.

Antibody assays

NA and NA-inhibition assays (NI) were performed with a fetuin substrate (Aymard-Henry et al., 1973). Plaque size reduction (PSR) assays were performed as previously described (Jahiel and Kilbourne, 1966). Briefly, confluent MDCK monolayers were inoculated with 100 plaque-forming units (PFU) of virus for 1 h followed by the addition of two-fold serial dilutions of antisera incorporated into the agar overlays. Plaque assay plates were incubated for 40 h and stained with 1% (w/v) crystal violet solution. The endpoint titers were calculated as the highest serum dilution that gave 50% inhibition of mean plaque size in the presence of normal rabbit serum (NRS). NA-specific IgG enzyme-linked immunosorbent assays (ELISA) were carried out using 1 μg/ml of rNA as the coating antigen, as previously described (Katz et al., 1997). The single radial hemolysis (SRH) assay was performed as previously described (Farrohi et al., 1977). Briefly, BJ/262 or BPL-inactivated HK/483 virus was adsorbed to packed TRBCs, and then incorporated into 1% agarose gels. The anti-NA antibody titers are expressed as the area of lysis zone (mm^2). Replication inhibition (RI) assays were performed by adding 200 μl of serum–virus mixtures (100 μl of 2-fold serial dilutions of serum + 100 μl of 100 $TCID_{50}$ of virus) into MDCK monolayers. The 96-well plates were inoculated at 37 °C for 48 h. To quantify viral particles, supernatant fluids in each well were titrated for HAU by HA assays. The RI titer is expressed as the highest serum dilution which reduced $\geq$50% HA activity of the negative control sera.

Passive immunizations and challenge experiments

Six week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, MA) were injected intraperitoneally (i.p.) with one dose of 0.5 ml of sera 24 h prior to challenge with 50 μl of 5 LD_{50} of HK/483 virus. In a second experiment, 1 ml of rabbit sera was given 24 h prior to virus challenge, 0.5 ml of sera at day 2 and day 5 post inoculation (p.i.) with 50 μl of 3 LD_{50} of HK/483 virus. Mice were observed daily for weight loss and death for 14 days. Viral replication in whole lung and brain tissues was determined on day 6 p.i. and virus titers were expressed as the mean $\log_{10}$ (EID_{50})/ml (Lu et al., 1999).

Sequence alignments and NA structure modeling

NA sequences were downloaded from the Influenza Virus Resource at NCBI and Global Initiative on Sharing All Influenza Data (GISAID). A complete NA alignment of amino acid sequences was made using ClustalW (Larkin et al., 2007) and adjusted based on N2 numbering. Three-dimensional structures of NAs were generated by the Swiss Model (http://swissmodel.expasy.org) using A/Brevig Mission/1/1918 (H1N1) NA structure as a model for BJ/262 NA [PDB code 3BEQ, Sequence identity: 88%; (Xu et al., 2008)] and A/Vietnam/1203/2004 H5N1 NA structure for HK/483 NA [PDB code 3CKZ; Sequence identity: 94%; (Collins et al., 2008)]. All structure figures were generated using PyMOL software (Bramucci et al., 2012).

Statistical analysis

Student's *t* test was used to measure statistical significance of virus titers in tissues and weight loss. The log-rank test was used to measure mortality differences between antiserum treated and NRS treated mice.

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