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Universal anti-neuraminidase antibody inhibiting all influenza A subtypes

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ABSTRACT

The only universally conserved sequence amongst all influenza A viral neuraminidase (NA) is located between amino acids 222–230 and plays crucial roles in viral replication. However, it remained unclear as to whether this universal epitope is exposed during the course of infection to allow binding and inhibition by antibodies. Using a monoclonal antibody (MAb) targeting this specific epitope, we demonstrated that all nine subtypes of NA were inhibited *in vitro* by the MAb. Moreover, the antibody also provided heterosubtypic protection in mice challenged with lethal doses of mouse-adapted H1N1 and H3N2, which represent group I and II viruses, respectively. Furthermore, we report amino acid residues I222 and E227, located in close proximity to the active site, are indispensable for inhibition by this antibody. This unique, highly-conserved linear sequence in viral NA could be an attractive immunological target for protection against diverse strains of influenza viruses.

1. Introduction

Current seasonal influenza vaccines are designed to provide strain-specific protection against two circulating subtypes of influenza A virus (H1N1 and H3N2) and one influenza B virus. The main target in these vaccines is the hemagglutinin (HA) for which there are at least 16 subtypes. HA has been identified to primarily mediate entry into host cells. Thus, neutralizing antibodies induced by vaccines can block viral entry by either preventing the attachment of the virus to sialic acid receptors on host cells or by interfering with HA-mediated viral fusion (Kida et al., 1983; Yoden et al., 1986).

Neuraminidase (NA) is another major glycoprotein on the surface of the virus of which there are at least 9 known subtypes (Baker et al., 1987; Webster and Bean, 1978). Recently, a highly divergent influenza A virus was isolated from little yellow shouldered bats (designated as H17N10). However, the N10 is a NA-like protein which does not possess sialidase activity (Tong et al., 2012). NA exerts its primary functional role during viral exit from the infected cells by cleaving the sialic acid receptors to facilitate the release of viral particles (Skehel and Wiley, 2000; Air and Laver, 1989; Palese et al., 1974). This role is supported by reports that NA-defective virus, or wild type viruses in the presence of NA inhibitors, form aggregations of viruses on the apical surface of the cells (Griffin et al., 1983; Liu et al., 1995; Hashem et al., 2009; Govorkova and McCullers, 2013). NA may also promote the breakdown of mucus in the respiratory tracts and the release of virus from sialic acid containing inhibitors, thus facilitating viral diffusion and access to the respiratory tract during infection (Matrosovich et al., 2004) and contributing to the entry and fusion of the influenza virus into host cells (Su et al., 2009). In contrast to HA, NA antibodies are not known to neutralize viral infectivity, but they have been shown to reduce viral yield by inhibiting NA enzymatic activity and thus to contribute to protection against influenza infection (Johansson et al., 1989; Qiu et al., 2006; Chen et al., 2000, 2005).

Both HA and NA proteins are highly variable being subject to antigenic drift and shift (Johansson and Brett, 2007; Air, 2011;

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Hashem et al., 2011; Ilyushina et al., 2012). Therefore, heterosubtypic protection against influenza A viruses is largely believed to be mediated by cross-reactive cytotoxic T cells against the highly conserved internal proteins rather than by antibodies against HA or NA (Epstein et al., 2005; Takada et al., 2003; Rimmelzwaan et al., 2007). However, recent reports have identified various broadly neutralizing antibodies targeting conserved epitopes in HA (Thosby et al., 2008; Thomson et al., 2012; Yoshida et al., 2009; Ekiert et al., 2009, 2011). Moreover, Sandbulte et al. (2007) and Marcelin et al. (2011) have shown that cross-reactive anti-neuraminidase antibodies elicited by seasonal H1N1 infection could not only contribute to protection against H1N1 but also provide cross-protection against H5N1 and H1N1pdm09 viruses, respectively. However, an antigenically conserved sequence affording heterosubtypic inhibition across all subtypes of NA has not been reported (Marcelin et al., 2012; Johansson and Brett, 2007).

We recently identified a universally conserved peptide located between amino acids (a.a.) 222–230 (N2 numbering). This epitope, comprised of "ILRTQESEC", constitutes part of the enzymatic active site (Gravel et al., 2010; Varghese et al., 1983), and plays critical roles in viral replication (Doyle et al., 2013). Using a monoclonal antibody (MAb) generated against this peptide, we found that this peptide epitope could be an attractive antiviral target.

2. Materials and methods

2.1. Antibodies against the universally conserved epitope in NA

A rabbit MAb against the highly conserved sequence was generated (Gravel et al., 2011, 2010; Chun et al., 2008). The MAb, denoted as HCA-2 MAb, was used in all experiments reported here.

2.2. Viruses

Influenza A strains used to investigate the heterosubtypic inhibitory effect of HCA-2 MAb were propagated at 37 °C in the allantoic cavities of 10-day-old embryonated hen eggs for 24 h (hrs). Allantoic fluid was clarified by centrifugation, aliquoted and stored at −80 °C until used. Viruses were titrated in MDCK cells as previously described (Hashem et al., 2009).

2.3. Mutagenesis and reverse genetics

The influenza A/Puerto Rico/8/34(H1N1) virus 8-plasmid reverse genetics system was kindly provided by Dr. Richard Webby (St. Jude Children's Research Hospital, Memphis, TN). The Stratagene Quick change II mutagenesis kit (La Jolla) was used to generate substitution mutations by alanine within the NA plasmid, which were sequenced to ensure the presence of each substitution. Wild type (wt) and each mutant virus were generated and purified as previously reported (Hoffmann et al., 2000). To ensure that no additional mutations occurred in the NA during generation or passaging, vRNA was extracted and sequenced.

2.4. NA enzyme linked lectin assay (ELLA)

ELLA was performed as described (Lambre et al., 1991). 96-well flat plates were coated with 25 μg/ml of fetuin (Sigma) and stored at 4 °C for 18 h before use. The titrated virus was incubated for 2 h with serial dilutions of the purified MAb. The fetuin coated plates were then washed in PBS with 0.5% Tween, followed by transferring the virus-antibody mixture to the wells and subsequent incubation at 37 °C for 20 h. The plates were again incubated with biotinylated-peanut lectin (sigma) for 2 h at RT in the dark. Avidin-peroxidase conjugate was added and incubated for 1 h in the dark at RT. Finally, the substrate, o-phenylendiamine dihydrochloride (OPD) (Sigma) was used to develop colorimetric reaction.

2.5. Virus inhibition assay

Unless specified, MAb was serially diluted and mixed with equal volume of virus at an MOI of 0.001 in a total volume of 60 μl and were incubated at 37 °C for 1 h. The antibody-virus mixture was transferred to confluent MDCK cells in 96-well plates and incubated at 37 °C for 20 h. Cell monolayers were washed twice with PBS, fixed with ice-cold 80% acetone for 10 min and viral nucleoprotein (NP) was quantified by indirect ELISA as described in standard microneutralization assay (Hashem et al., 2009); the NP ELISA was chosen over $TCID_{50}$ or plaque assays as the ELISA was more accurate, faster and reading could be automated (Hashem et al., 2009).

2.6. Animal study

Female, 19–21 g, Swiss-Webster (CD-1) mice were purchased from Charles River Laboratories International (Wilmington, MA). All *in vivo* research was performed in accordance with the guidelines of the Canadian Council on Animal Care. All efforts were made to minimise suffering; mice were humanely euthanised upon experimental endpoint (when infection resulted in greater than 25% body weight loss accompanied by respiratory distress). The mice ($n = 5$) were injected intraperitoneally with either 60 mg/kg of the rabbit MAb or rabbit IgG control on days −2, −1 and 0 relative to infection. On day 0, mice were intranasally challenged with lethal doses of $\sim 5 \times LD_{50}$ of influenza A/FM/1/47/MA (H1N1) or influenza A/HK/1/68-MA20 (H3N2) as previously determined in Swiss Webster CD-1 mice (Ping et al., 2011; Smeenk et al., 1996). Mice were weighed daily and clinically monitored for signs of illness for 12–14 days.

3. Results

3.1. HCA-2 MAb inhibits diverse strains of influenza viruses

The MAb was determined for its inhibitory activities against all 9 NA subtypes of influenza viruses. As shown in Fig. 1, the MAb resulted in a significant decrease in NA enzymatic activity of all NA subtypes in ELLA assay. Specifically, HCA-2 MAb inhibited NA activity in a dose response manner in which enzymatic activity of all NA subtypes were reduced by 80–100% at the highest concentration of the MAb in comparison to the IgG control which did not cause any reduction in NA activity. This effective inhibition of NA enzyme activity prompted us to investigate whether the MAb could inhibit viral replication in culture.

As shown in Fig. 2, the *in vitro* growth of viruses containing any of the 9 NA subtypes was substantially inhibited by HCA-2 MAb compared to normal IgG control. Specifically, coincubation of the highest concentration of the MAb with the viruses on MDCK cells resulted in 80–90% inhibition in viral replication. On the other hand, a slightly lower percentage of inhibition (~70%) against N4 and N5 strains was detected. The reason for the slightly lower rate of inhibition remains to be fully understood but is likely to be multi-factorial. For example, the balanced action between HA and NA, which is known to affect viral replication, might vary between the different strains used in this study (Wagner et al., 2002). Moreover, amino acid substitutions within the HCA-2 region (i.e.: for L223M in H8N4 and E229S in H6N5) as well as variation in sequences outside the universal epitopes could alter the susceptibility of NA to inhibition (Table 1). Nonetheless, this substantial inhibition was further reflected in the IC_{50} values of the HCA-2 MAb required to

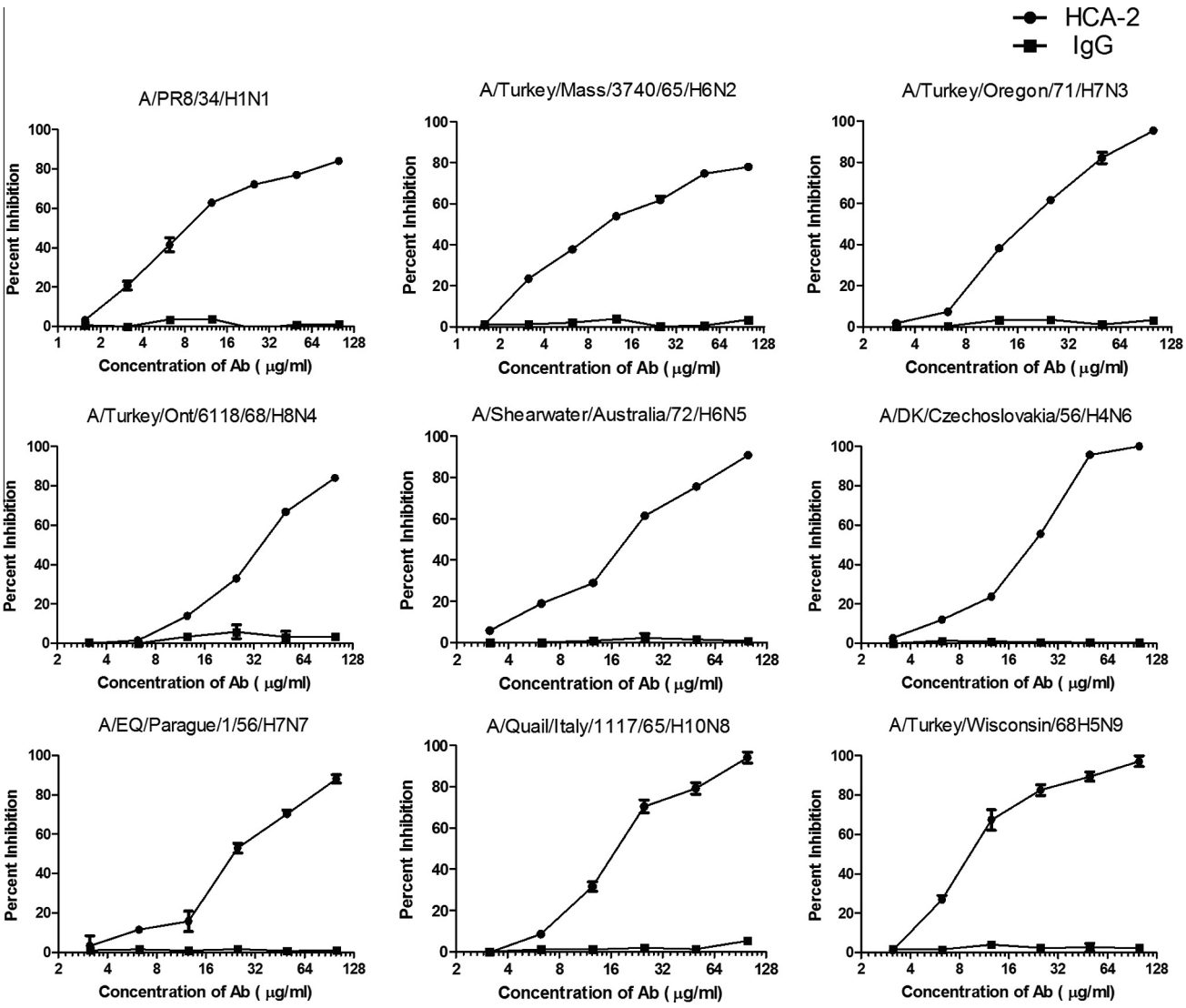

Fig. 1. HCA-2 MAb inhibits NA activity across all nine NA subtypes. MAb inhibitory effect on NA enzymatic activity was determined by ELLA assay. Fetuin coated plates were incubated with HCA-2 MAb or normal rabbit IgG control and virus containing one of the nine NA subtypes. Data are presented as a percentage of inhibition compared to virus control ± s.e.m. from three independent experiments done in duplicates.

inhibit viral growth which ranged between 2.6 and 18.2 μg/ml depending on the subtype (Table 2).

We next investigated whether this protection could be extended to a murine model. Two mouse-adapted viruses were used, with H1N1 and H3N2 representing group I and group II NAs, respectively. Upon passive transfer of the antibodies to naïve mice, we observed that the HCA-2 MAb treatment but not the normal IgG control protected 60% and 40% of the mice after challenge with H1N1 and H3N2 viruses, respectively (Fig. 3a). This protection was also observed in animal weight loss, as protected animals maintained their weight, in contrast the mice from the control group lost more than 20% of their total body weight within 8 days, suffered severe clinical symptoms and died between 8 and 10 days after viral challenge (Fig. 3b). These data support the *in vitro* findings that the universal MAb against NA can provide heterosubtypic protection against virus infection. Notably, the HCA-2 MAb failed to provide complete protection *in vivo* (40–60%) which might be due to the fact that MAb is of rabbit origin. However, the level of inhibition seen with this rabbit MAb is comparable to other reports in which murine polyclonal antibodies are used to protect mice in passive transfer studies (Marcelin et al., 2011; Sandbulte et al., 2007). It is also of note that the murine polyclonal antisera in these previous studies were obtained from mice which were immunized with an antigen of the same virus subtype which was used in the subsequent challenging. Thus these studies only examined the protection afforded by antibodies within the same subtype of NA (Sandbulte et al., 2007; Marcelin et al., 2011; Wan et al., 2013). Broadly inhibitory MAb against specific NA subtype was reported recently, but the MAbs were targeting the N1 subtype (Wan et al., 2013). In contrast, the present data clearly show that HCA-2 MAb is protective against diverse influenza A virus subtypes due to its highly conserved target peptide in the NA protein.

3.2. Identification of key a.a. in the NA epitope involved in HCA-2 MAb-mediated viral inhibition

To determine the key amino acid residues within the HCA-2 region which contribute to the observed antibody inhibitory effects, we tested the MAb against various mutant viruses which have a single alanine substitution within, or a deletion of the HCA-2 region. We observed that viruses with deleted HCA-2 region or alanine substitution at any of the following residues; L223, R224, C230 were non-viable (viruses could not be rescued), indicating the critical roles of these residues (Doyle et al., 2013). The

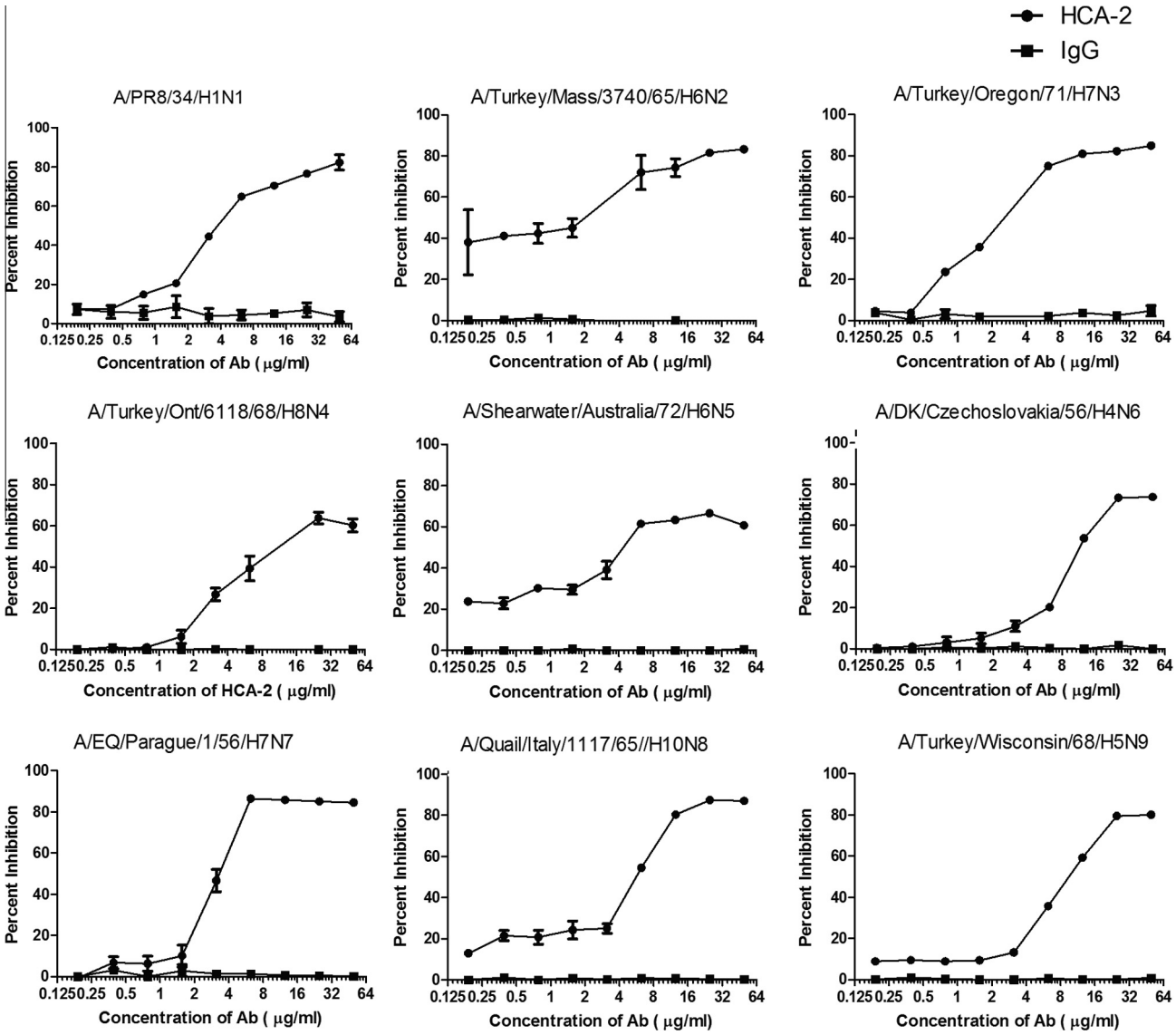

Fig. 2. HCA-2 MAb shows broad inhibitory effect against all influenza NA subtypes. HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect against H1N1, H6N2, H7N3, H8N4, H6N5, H11N6, H7N7, H10N8 or H5N9 in virus inhibition assay as described in Section 2. Data are presented as percentage of inhibition compared to virus control ± s.e.m. from three independent experiments done in duplicates.

Table 1
The conservation rate of the HCA-2 epitope in each of the NA subtypes.

NA subtype	HCA-2 sequence conservation rate (%)[a]	Note	Total NA homology against PR8/H1N1 (%)[b]	Consensus HCA-2 sequence[c]
N1	99.75		–	ILRTQESEC
N2	98.5		44	ILRTQESEC
N3	99.7		53	ILRTQESEC
N4	100	All N4 sequences have L223M	49	IMRTQESEC
N5	100	All N5 sequences have S229	44	ILRTQESSC
N6	99.8		45	ILRTQESEC
N7	99.4		55	ILRTQESEC
N8	99	All N8 have S229	47	ILRTQESSC
N9	99.4		48	ILRTQESEC
N10	100		30	GLRLPNSEC

[a] Conservation rate of HCA-2 sequence for each subtype.
[b] NA homology was determined between the specific strain used in this work and the A/PR8/34(H1N1).
[c] HCA-2 consensus sequence in each NA subtypes.

remainder of the mutants with alanine substitution at positions I222, T225, Q226, E227, S228 and E229 were all successfully rescued. Interestingly, HCA-2 MAb inhibited the *in vitro* growth of T225A, Q226A, S228A and E229A variants to levels that were similar to the wt virus, however, this inhibitory effect of the MAb was largely abolished against I222A and E227A mutants (Fig. 4),

Table 2
IC_{50} of HCA-2 MAb in inhibiting influenza viral growth for each individual virus strains, with statistical comparisons were made against A/PR8/34/H1N1.

Virus	IC_{50} (μg/ml)
A/PR8/34/H1N1	5.30
A/Turkey/Mass/3740/65/H6N2	8.49*
A/Turkey/Oregon/71/H7N3	2.56
A/Turkey/Ont/6118/68/H8N4	16.03*
A/Shearwater/Australia/72/H6N5	18.2*
A/DK/Czechoslovakia/56/H4N6	12.68*
A/EQ/Paraguay/1/56/H7N7	2.67
A/Quail/Italy/1117/65/H10N8	5.43
A/Turkey/Wisconsin/68/H5N9	10.16*

* Represents a p-value <0.05.

suggesting the critical need for the two residues in antibody-mediated interaction. This was also reflected in the IC_{50} values of the HCA-2 MAb against the different variants, which ranged from 0.95 to 2.17 μg/ml for the majority of the mutants but increased to 181 μg/ml and 106 μg/ml for I222A and E227A respectively (Table 3).

4. Discussion

The importance of NA in inducing protective antibodies has been well documented (Marcelin et al., 2012). Recent publications suggest that polyclonal antibodies against NA can induce cross-protection against diverse strains within the same subtype (Marcelin et al., 2011; Sandbulte et al., 2007), but NA heterosubtypic protection, i.e., antibody generated by immunization of one NA subtype capable of protecting against a different NA subtypes, has not yet been reported (Johansson and Cox, 2011). This lack of NA specific heterosubtypic protection is mainly attributed to genetic variation combined with immune pressure resulting in marked structural variability (Colman, 1992; Colman et al., 1983). It would be of significant interest to search for universally conserved epitopes.

We recently identified a universally conserved NA peptide sequence (222–230: I-L-R-T-Q-E-S-E/S-C), in which all residues show 99.1–100% conservation rate, except for E229 which shows conservation at 94.5%, across all NA sequences (Gravel et al., 2010; Hashem et al., 2011). It should be noted that at a.a. position 229, E constitutes 94.5 while S constitutes 5.5% and the S229 are presented in all N5 and N8 strains. Within N1, S229 exists in only 0.02% of the strains (Table 1). Furthermore, this sequence is only partially conserved in the NA-like N10 subtype from little yellow shouldered bats in which the only two available sequences in the GenBank show the following substitutions I222G, T225L, Q226P, E227N with remaining residues being conserved (Table 1).

More recently, we found that this universal epitope plays crucial roles during viral replication by stabilizing the enzyme structure and providing a favourable environment for maximal enzymatic activity (Doyle et al., 2013). However, it remained unknown as to whether this epitope could be subjected to attack by inhibitory agents during the course of infection and whether the MAb, generated using the peptide as the immunogen and bind the NA proteins in ELISA, could inhibit virus replication. In this communication, we show that all nine subtypes of viruses were inhibited by this MAb, revealing that during the course of infection, this universal epitope is exposed sufficiently in cultures to be attacked by macromolecules such as an antibody.

It is noted that *in vitro* inhibitory rate of the MAb against most viruses was around 90% and this was largely in agreement with the magnitude of inhibition of NA enzymatic activity (Supplementary information, Fig. S1). The slightly lower inhibition rates (~70%) against N4 (H8N4) and N5 (H6N5) viruses compared to the others remain to be fully understood, but is likely to be due to multiple factors including the differences in a.a. sequences within the HCA-2 region which could result in varied degrees of interaction with the MAb. Moreover, it is not completely unexpected that less potent protection against the virus infection in the mouse than that observed in *in vitro* tissue cultures, given that the MAb is of rabbit

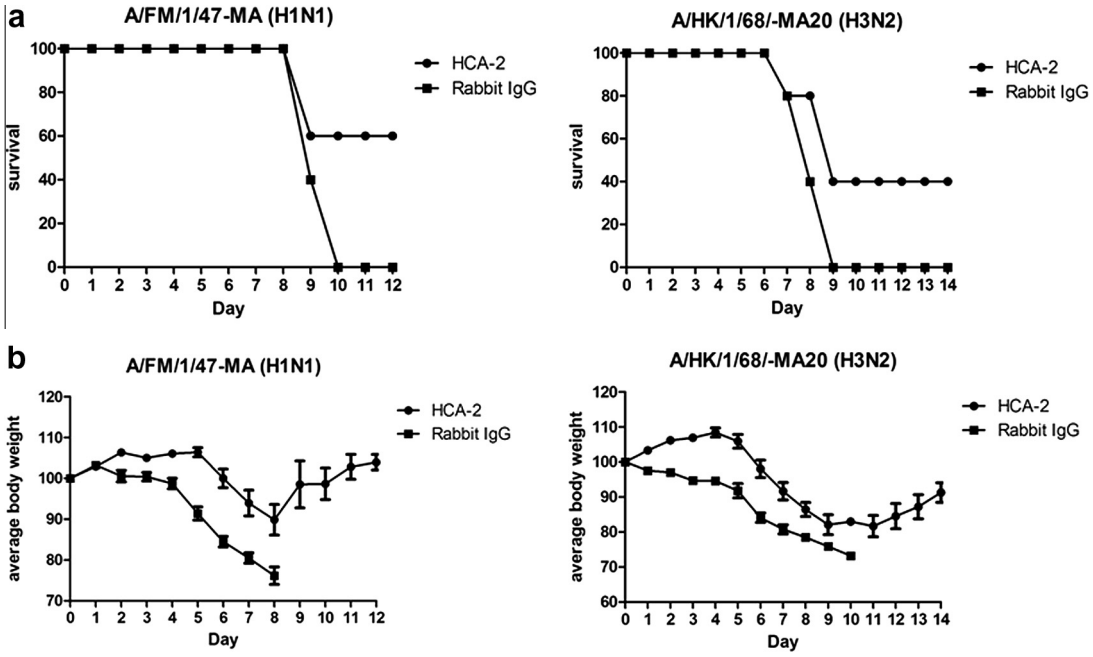

Fig. 3. *In vivo* effect of HCA-2 MAb. MAb (60 mg/kg) were passively transferred to naïve (n = 5) mice via intra-peritoneal injection on days −2, −1 and 0 relative to challenge. On day 0, mice were intranasally challenged with lethal doses of ~5 × LD_{50} of influenza A/FM/1/47/MA (H1N1) or influenza A/HK/1/68-MA20 (H3N2). Mice were weighed and monitored daily for signs of illness for 12–14 days. Panels depict survival curves post-challenge (a) and body weight (b) with both influenza viruses.

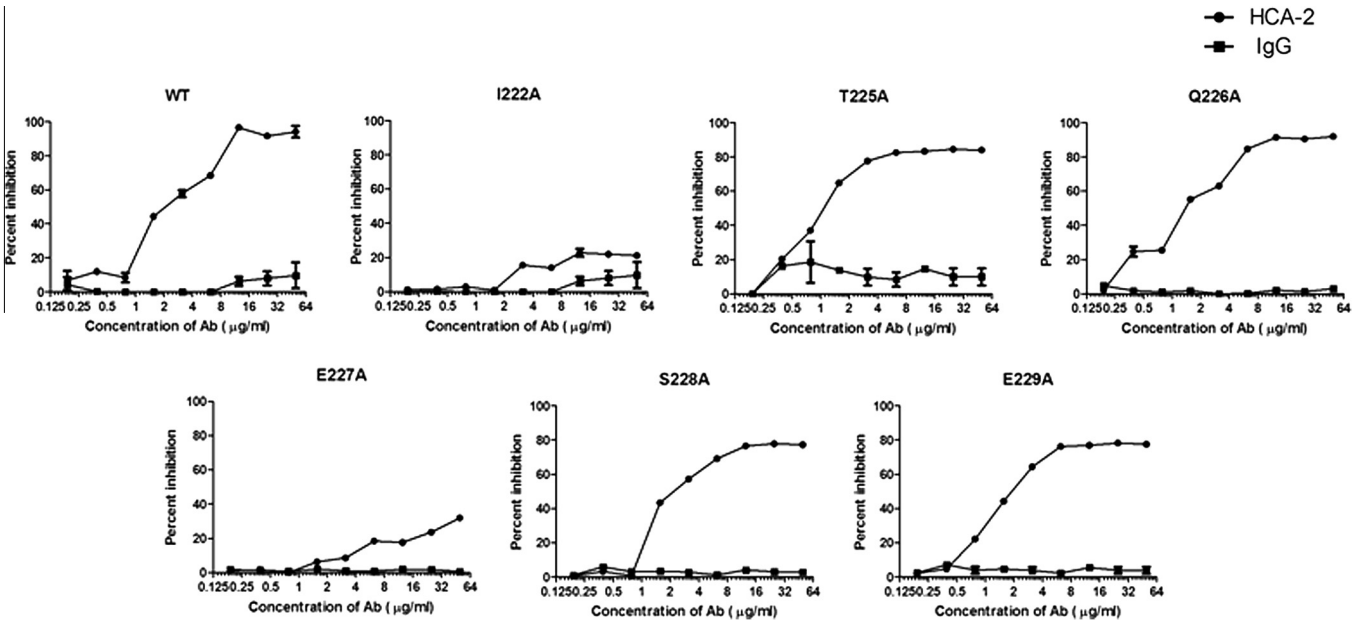

Fig. 4. Critical amino acid residues for HCA-2 MAb inhibitory effect. HCA-2 MAb or normal rabbit IgG were tested for their inhibition against viruses containing the wild type NA sequence or any of the mutated NA sequences in a influenza A/Puerto Rico/8/34 (H1N1) backbone. Data are presented as percentage of inhibition compared to virus control ± s.e.m. from three independent experiments.

Table 3
IC_{50} values of HCA-2 MAb against viral variants determined by the inhibition against the NA enzymatic activity, with statistical comparisons were made against the WT.

Variant	IC_{50} (μg/ml)
WT	1.82
I222A	181.93**
T225A	0.95
Q226A	1.60
E227A	106.26**
S228A	2.27
E229A	1.87

** Represents a *p*-value <0.01.

origin. However, the *in vivo* efficacy of HCA-2 MAb could be improved by combination therapy with NA inhibitors such as oseltamivir or zanamivir, use of a higher dose of the Mab or intranasal versus systemic intraperitoneal administration (Weltzin and Monath, 1999; Seiler et al., 2000).

Interestingly, a similar *in vivo* protection rates (40–60%) of NA-specific antibodies has been reported by others using polyclonal subtype-specific antibodies (Sandbulte et al., 2007; Marcelin et al., 2011). However, these investigators used polyclonal antisera obtained from animals vaccinated with whole NA proteins. Noticeably, such polyclonal antisera were reported to inhibit multiple strains only within the same subtype while this report is the first showing a MAb to afford heterosubypic protection against remarkably diverse strains of viruses across different subtypes.

It is of note that a.a. residues I222 and E227 were found to be indispensable for inhibition by the HCA-2 MAb. These two residues were highly conserved. Specifically, based on sequences of circulating strains deposited on GenBank/GISAID, only 0.18% (16/8725) of N1 or N2 strains had mutations at residue I222 (6 × I222T, 6 × I222R, 2 × I222K and 2 × I222X), while none of the strains (n = 8741) contained a mutation at E227. As the two mutants I222A and E227A, but not the others, were found to be resistant to the MAb-mediated inhibition, it could be reasonably envisaged that binding of the MAb to the two a.a. would disrupt functional activity of the enzyme. Previously we have used alanine scanning to demonstrate the contributing role of each a.a. within the HCA-2 region to viral function as a whole. Indeed, we found that the I222A and E227A variants demonstrated a decreased replication capacity and a decrease in NA enzymatic activity. Thus, these data suggest that these amino acids are important for optimal enzyme function. While it would be interesting to explore the potential of inducing HCA-2 escaping mutants with the MAb *in vitro*, we are unable to isolate such escapee so far, which further suggests that escape mutants are likely to be unfit and might not have the ability to spread widely within the community. Furthermore, it not clear to us yet whether L223, R224, C230 have critical roles in the interaction with the MAb since replacing these residues with alanine resulted in non-viable viruses (Doyle et al., 2013).

While available anti-NA drugs exert their antiviral activities through their direct binding to enzymatic active site (Kim et al., 2013; McKimm-Breschkin, 2013) the exact mechanism by which HCA-2 MAb inhibits influenza virus remains to be fully understood. However, given the size differences between small molecule inhibitors and antibody, it can be envisaged that the insertion of the complementarity determining regions (CDRs) of the antibody into NA active site and/or their direct interaction could interfere with NA function.

5. Conclusions

In conclusion, during the course of infection, this universal peptide could be sufficiently exposed to allow access by macromolecules as large as an antibody, making it worthwhile being considered for future antiviral intervention and vaccine development. This notion could be substantiated by the recent findings that the epitope is crucial for effective virus replication (Doyle et al., 2013).

Competing financial interests statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.09.018.

References

Air, G., 2011. Influenza neuraminidase. Influenza Other Respir. Viruses 6, 245–256.
Air, G.M., Laver, W.G., 1989. The neuraminidase of influenza virus. Proteins 6, 341–356. http://dx.doi.org/10.1002/prot.340060402.
Baker, A.T., Varghese, J.N., Laver, W.G., Air, G.M., Colman, P.M., 1987. Three-dimensional structure of neuraminidase of subtype N9 from an avian influenza virus. Proteins 2, 111–117. http://dx.doi.org/10.1002/prot.340020205.
Chen, Z., Kadowaki, S., Hagiwara, Y., Yoshikawa, T., Matsuo, K., 2000. Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase. Vaccine 18, 3214–3222.
Chen, J., Fang, F., Li, X., Chang, H., Chen, Z., 2005. Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-expressing DNAs by electroporation. Vaccine 23, 4322–4328.
Chun, S., Li, C., Van Domselaar, G., Wang, J., Farnsworth, A., Cui, X., Rode, H., Cyr, T.D., He, R., Li, X., 2008. Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine 26, 6068–6076. http://dx.doi.org/10.1016/j.vaccine.2008.09.015.
Colman, P.M., 1992. Structural basis of antigenic variation: studies of influenza virus neuraminidase. Immunol. Cell Biol. 70, 209–214. http://dx.doi.org/10.1038/icb.1992.26.
Colman, P.M., Varghese, J.N., Laver, W.G., 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature 303, 41–44.
Doyle, T.M., Jaentschke, B., Van Domselaar, G., Hashem, A.M., Farnsworth, A., Forbes, N.E., Li, C., Wang, J., He, R., Brown, E.G., Li, X., 2013. The universal epitope of influenza A viral neuraminidase fundamentally contributes to enzyme activity and viral replication. J. Biol. Chem. 288 (25), 18283–18289. http://dx.doi.org/10.1074/jbc.M113.468884.
Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., Wilson, I.A., 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.
Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.
Epstein, S.L., Kong, W.P., Misplon, J.A., Lo, C.Y., Tumpey, T.M., Xu, L., Nabel, G.J., 2005. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 23, 5404–5410. http://dx.doi.org/10.1016/j.vaccine.2005.04.047.
Govorkova, E., McCullers, J., 2013. Therapeutics against influenza. Curr. Top. Microbiol. Immunol. 370, 273–300.
Gravel, C., Li, C., Wang, J., Hashem, A.M., Jaentschke, B., Xu, K.W., Lorbetskie, B., Gingras, G., Aubin, Y., Van Domselaar, G., Girard, M., He, R., Li, X., 2010. Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences. Vaccine 28, 5774–5784. http://dx.doi.org/10.1016/j.vaccine.2010.06.075.
Gravel, C., Li, C., Wang, J., Hashem, A.M., Jaentschke, B., Van Domselaar, G., He, R., Li, X., 2011. Quantitative analyses of all influenza type A viral hemagglutinins and neuraminidases using universal antibodies in simple slot blot assays. J. Vis. Exp. 50. http://dx.doi.org/10.3791/2784.
Griffin, J.A., Basak, S., Compans, R.W., 1983. Effects of hexose starvation and the role of sialic acid in influenza virus release. Virology 125, 324–334.
Hashem, A.M., Flaman, A.S., Farnsworth, A., Brown, E.G., Van Domselaar, G., He, R., Li, X., 2009. Aurintricarboxylic acid is a potent inhibitor of influenza A and B virus neuraminidases. PLoS One 4, e8350. http://dx.doi.org/10.1371/journal.pone.0008350.
Hashem, A.M., Doyle, T.M., Van Domselaar, G., Farnsworth, A., Li, C., Wang, J., He, R., Li, X., 2011. Recent developments in bioinformatics analyses of influenza A virus surface glycoproteins and their biological relevance. Curr. Bioinformatics 6, 415–426.
Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U.S.A. 97, 6108–6113. http://dx.doi.org/10.1073/pnas.100133697.
Ilyushina, N.A., Bovin, N.V., Webster, R.G., 2012. Decreased neuraminidase activity is important for the adaptation of H5N1 influenza virus to human airway epithelium. J. Virol. 86, 4724–4733. http://dx.doi.org/10.1128/JVI.06774-11.
Johansson, B.E., Brett, I.C., 2007. Changing perspective on immunization against influenza. Vaccine 25, 3062–3065. http://dx.doi.org/10.1016/j.vaccine.2007.01.030.
Johansson, B.E., Cox, M.M., 2011. Influenza viral neuraminidase; the forgotten antigen. Expert Rev. Vaccines 10 (12), 1683–1695.
Johansson, B.E., Bucher, D.J., Kilbourne, E.D., 1989. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. J. Virol. 63, 1239–1246.
Kida, H., Webster, R.G., Yanagawa, R., 1983. Inhibition of virus-induced hemolysis with monoclonal antibodies to different antigenic areas on teh hemagglutinin molecule of A/seal/Massachusetts/1/80 (H7N7) influenza virus. Arch. Virol. 76, 91–99.
Kim, J., Resende, R., Wennekes, T., Chen, H., Bance, N., Buchini, S., Watts, A., Pilling, P., Streltsov, V., Petric, M., Liggins, R., Barrett, S., McKimm-Breschkin, L., Niikura, M., Withers, S., 2013. Mechanism-based covalent neuraminidase inhibitors with broad-spectrum influenza antiviral activity. Science 340 (6128), 71–75.
Lambre, C.R., Terzidis, H., Greffard, A., Webster, R.G., 1991. An enzyme-linked lectin assay for sialidase. Clin. Chim. Acta 198, 183–193.
Liu, C., Eichelberger, M.C., Compans, R.W., Air, G.M., 1995. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J. Virol. 69, 1099–1106.
Marcelin, G., DuBois, R., Rubrum, A., Russell, C., McElhaney, J., Webby, R., 2011. A contributing role for anti-neuraminidase antibodies on immunity to pandemic H1N1 2009 influenza A virus. PloS one 6 (10), e26335.
Marcelin, G., Sandbulte, M.R., Webby, R.J., 2012. Contribution of antibody production against neuraminidase to the protection afforded by influenza vaccines. Rev. Med. Virol. 22, 267–279.
Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J. Virol. 78, 12665–12667. http://dx.doi.org/10.1128/JVI.78.22.12665-12667.2004.
McKimm-Breschkin, J., 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. Influenza Other Respir. Viruses 1, 25–36.
Palese, P., Tobita, K., Ueda, M., Compans, R.W., 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 61, 397–410.
Ping, J., Keleta, L., Forbes, N., Dankar, S., Stecho, W., Tyler, S., Zhou, Y., Babiuk, L., Weingartl, H., Halpin, R., Boyne, A., Bera, J., Hostetler, J., Fedorova, N., Proudfoot, K., Katzel, D., Stockwell, T., Ghedin, E., Spiro, D., Brown, E., 2011. Genomic and protein structural maps of adaptive evolution of human influenza A virus to increased virulence in the mouse. PLoS one 6 (6), e21740.
Qiu, M., Fang, F., Chen, Y., Wang, H., Chen, Q., 2006. Protection against avian influenza H9N2 virus challenge by immunization with hemagglutinin- or neuraminidase-expressing DNA in BALB/c mice. Biochem. Biophys. Res. Commun. 343 (4), 1124–1131.
Rimmelzwaan, G.F., Fouchier, R.A., Osterhaus, A.D., 2007. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr. Opin. Biotechnol. 18, 529–536.
Sandbulte, M.R., Jimenez, G.S., Boon, A.C., Smith, L.R., Treanor, J.J., 2007. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med. 4 (2), e59.
Seiler, P., Senn, B.M., Klenerman, P., Kalinke, U., Hengartner, H., Zinkernagel, R.M., 2000. Additive effect of neutralizing antibody and antiviral drug treatment in preventing virus escape and persistence. J. Virol. 74 (13), 5896–5901.
Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. 69, 531–569. http://dx.doi.org/10.1146/annurev.biochem.69.1.531.
Smeenk, C., Wright, K., Burns, B., Thaker, A., Brown, E., 1996. Mutations in the hemagglutinin and matrix genes of a virulent influenza virus variant, A/FM/1/47-MA, control different stages in pathogenesis. Virus Res. 44 (2), 79–95.
Su, B., Wurtzer, S., Rameix-Welti, M.A., Dwyer, D., van der Werf, S., Naffakh, N., Clavel, F., Labrosse, B., 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell–cell fusion and virus entry by the viral neuraminidase (NA). PLoS One 4, e8495. http://dx.doi.org/10.1371/journal.pone.0008495.
Takada, A., Matsushita, A., Ninomiya, Y., Kawoaka, H., Kida, K., 2003. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine 21, 3212–3218.
Thomson, C.A., Wang, Y., Jackson, L.M., Olson, M., Wang, W., Liavonchanka, A., Keleta, L., Silva, V., Diederich, S., Jones, R.B., Gubbay, J., Pasick, J., Petric, M., Jean, F., Allen, V.G., Brown, E.G., Rini, J.M., Schrader, J.W., 2012. Pandemic H1N1 influenza infection and vaccination in humans induces cross-protective antibodies that target the hemagglutinin stem. Front. Immunol. 3, 87. http://dx.doi.org/10.3389/fimmu.2012.00087.
Thosby, M., van den Brink, E., Jongeneelen, M., Poon, L.L., Alard, P., Cornelissen, L., Bakker, A., Cox, F., van Denventer, E., Guan, Y., 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PloS one 3 (12), e3942.

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A.A., et al., 2012. A distinct lineage of influenza A virus from bats. Proc. Natl. Acad. Sci. U.S.A. 109, 4269–4274.

Varghese, J.N., Laver, W.G., Colman, P.M., 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature 303, 35–40.

Wagner, R., Matrosovich, M., Klenk, H.D., 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev. Med. Virol. 12, 159–166. http://dx.doi.org/10.1002/rmv.352.

Wan, H., Gao, J., Xu, K., Chen, H., Couzens, L.K., Rivers, K.H., Easterbrook, J.D., Yang, K., Zhong, L., Rajabi, M., Ye, J., Sultana, I., Wan, X.F., Liu, X., Perez, D.R., Taubenberger, J.K., Eichelberger, M.C., 2013. Molecular basis for broad neuraminidase immunity: conserved epitopes in seasonal and pandemic H1N1 as well as H5N1 influenza viruses. J. Virol. 87 (16), 9290–9300.

Webster, R.G., Bean Jr, W.J., 1978. Genetics of influenza virus. Annu. Rev. Genet. 12, 415–431. http://dx.doi.org/10.1146/annurev.ge.12.120178.002215.

Weltzin, R., Monath, T.P., 1999. Intranasal antibody prophylaxis for protection against viral disease. Clin. Microbiol. Rev. 12 (3), 383–393.

Yoden, S., Kida, H., Kuwabara, M., Yanagawa, R., Webster, R.G., 1986. Spin-labeling of influenza virus hemagglutinin permits analysis of the conformational change at low pH and its inhbition by antibody. Virus Res. 4, 251–261.

Yoshida, R., Igarashi, H., Ozaki, N., Kishida, D., Tomabechi, H., Kida, K., Ito, A., Takada, A., 2009. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. PLoS Pathog. 5 (3), e1000350.