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A monoclonal antibody targeting a highly conserved epitope in influenza B neuraminidase provides protection against drug resistant strains

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ABSTRACT

All influenza viral neuraminidases (NA) of both type A and B viruses have only one universally conserved sequence located between amino acids 222–230. A monoclonal antibody against this region has been previously reported to provide broad inhibition against all nine subtypes of influenza A NA; yet its inhibitory effect against influenza B viral NA remained unknown. Here, we report that the monoclonal antibody provides a broad inhibition against various strains of influenza B viruses of both Victoria and Yamagata genetic lineage. Moreover, the growth and NA enzymatic activity of two drug resistant influenza B strains (E117D and D197E) are also inhibited by the antibody even though these two mutations are conformationally proximal to the universal epitope. Collectively, these data suggest that this unique, highly-conserved linear sequence in viral NA is exposed sufficiently to allow access by inhibitory antibody during the course of infection; it could represent a potential target for antiviral agents and vaccine-induced immune responses against diverse strains of type B influenza virus.

1. Introduction

The hemagglutinin (HA) and neuraminidase (NA) are two major glycoproteins on the surface of both influenza A and B viruses. Both HA and NA are necessary for virus proliferation. Specifically, HA mediates virus entry by binding to the sialic acid/galactose receptor of the cells while NA facilitates virus release from the cells through enzymatically cleaving the bonds between sialic acid and galactose [1,2]. Thus, the two proteins have been the main targets for vaccine-induced immune response (HA) and antiviral treatment (NA). However, both HA and NA evolve in an unpredictable fashion [3–5], presenting a daunting challenge for current vaccination and antiviral therapies. Indeed, annual formulation of vaccines with updated virus strains is necessary while drug-resistant NA mutants have been isolated from treated and untreated patients. It would be of significant interest to search for conserved viral epitopes in these two viral proteins and determine whether such epitopes could be a potential target for vaccine-induced immune responses and antiviral therapy.

While numerous studies have been published in recent years on the topic of universal HA vaccines and neutralizing antibodies, relatively little progress has been made in targeting the viral NA protein [6]. We recently identified a universally conserved peptide region within the NA protein which is located between amino acids (a.a.) 222–230 (N2 numbering). This epitope, comprised of "ILRTQESEC", constitutes part of the enzymatic active site [7,8] and is found in both influenza A and B strains. A monoclonal antibody (MAb) generated against this linear epitope was able to quantify the NA component of nine influenza A subtypes and type B in vaccine preparations by Western blot [7]. We also observed inhibitory effects of the MAb on replication of all 9 NA subtypes of influenza A viruses. Here, we investigated the inhibitory effects of the MAb on both wild type and drug-resistant influenza B viruses.

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2. Methods

2.1. Cells and viruses

Madin–Darby Canine Kidney (MDCK) cells were obtained from the American Type Culture Collection. Cells were grown and cultured in Dulbecco's Modified Eagle's Medium (DMEM) as previously described [9]. The MAb against NA (denoted as HCA-2) has been described previously [7]. The following NA inhibitor (NAI) sensitive influenza B strains were used to investigate the inhibition effect of the HCA-2 antibody: B/Brisbane/60/2008 (B/Victoria lineage), B/Hubei-Wujiagang/158/2009 (B/Yamagata lineage), B/Perth/211/2001-D197-WT Clone (B/Yamagata lineage). The following NAI-resistant viruses were also used: B/Perth/211/2001-E197-MUT clone and B/Taiwan/4/2002. The B/Perth/211/2001-E197-MUT clone virus contains a D197E NA mutation (equivalent to D198 in N2 numbering) that has been previously shown to confer oseltamivir resistance [10] while the B/Taiwan/4/2002 virus contains a D117E NA mutation (equivalent to E119 in N2 numbering) that confers both zanamivir and oseltamivir resistance [11]. Virus stocks were propagated at 35 °C in MDCK cells for 48 h. Cells were then harvested and virus was purified by ultracentrifugation over 30% sucrose cushions. Viruses were titrated in MDCK cells as previously described [12].

2.2. Enzyme linked lectin assay (ELLA)

The Enzyme Linked Lectin Assay was conducted as previously described [13]. Briefly, ninety-six well flat bottom plates were coated with 25 μg/ml of fetuin (Sigma) and stored at 4 °C for 18 h before use. Pre-determined amounts of viruses were incubated for 2 h with serial dilutions of purified monoclonal HCA-2 antibody. The fetuin coated plates were then washed with 0.5% tween/PBS and virus-antibody mixture was transferred to the wells and incubated at 37 °C for 20 h. The plates were again washed and incubated with peanut lectin (Sigma) for 2 h at room temperature in the dark. After washing, the plates were incubated with Avidin Peroxidase for 1 h in the dark at room temperature. Finally, substrate, *o*-phenylenediamine dihydrochloride (OPD) (Sigma) dissolved in citrate buffer, was then added to the wells for 10 min before the reaction was stopped with 1 M sulfuric acid and plates were read at 490 nm.

2.3. Analyses of viral growth

MDCK cells were inoculated with viral samples in serum-free DMEM at a low MOI of 0.001 for 1 h in the presence of 2 μg of TPCK-treated trypsin. The inoculum was removed by extensive washing with PBS. DMEM serum-free media with 2 μg/ml TPCK-treated trypsin and 100 μg of HCA-2 MAb was then added to the inoculated cell monolayers. Supernatant was harvested over a course of 72 h at time points 0, 6, 12, 18, 24, 36, 48 and 72 h. Growth curve analysis was performed in triplicates. Harvested media was then titrated for viral growth rate using plaque assay as previously described [12].

3. Results

Given that the HCA-2 region was essential for optimal viral function and that a MAb against this region significantly inhibited enzymatic activity of influenza A subtypes [13,24], we set out to determine whether this MAb would significantly inhibit enzymatic activity of various strains of influenza B viruses. To this end, we treated all influenza B viruses with HCA-2 MAb and then measured the enzymatic activity using the ELLA assay. As shown in Fig. 1, the NA activities of all viral strains, regardless of the influenza B lineage, or whether the strain was NAI sensitive or resistant, were substantially inhibited by HCA-2 MAb compared to normal rabbit IgG control. These data suggest that the MAb antibody not only provides protection against influenza A, but also influenza B virus infection including two commonly-isolated drug resistant strains.

Since HCA-2 MAb effectively inhibited the NA activity of all influenza B viruses regardless of antigenic lineage or NAI resistance mutation, we next determined whether the inhibitory potential of the MAb could be extended to virus replication. As shown in Fig. 2, the HCA-2 MAb significantly inhibited viral growth of all influenza B strains when compared to IgG control, demonstrating that the HCA-2 antibody significantly inhibits the growth of these NAI

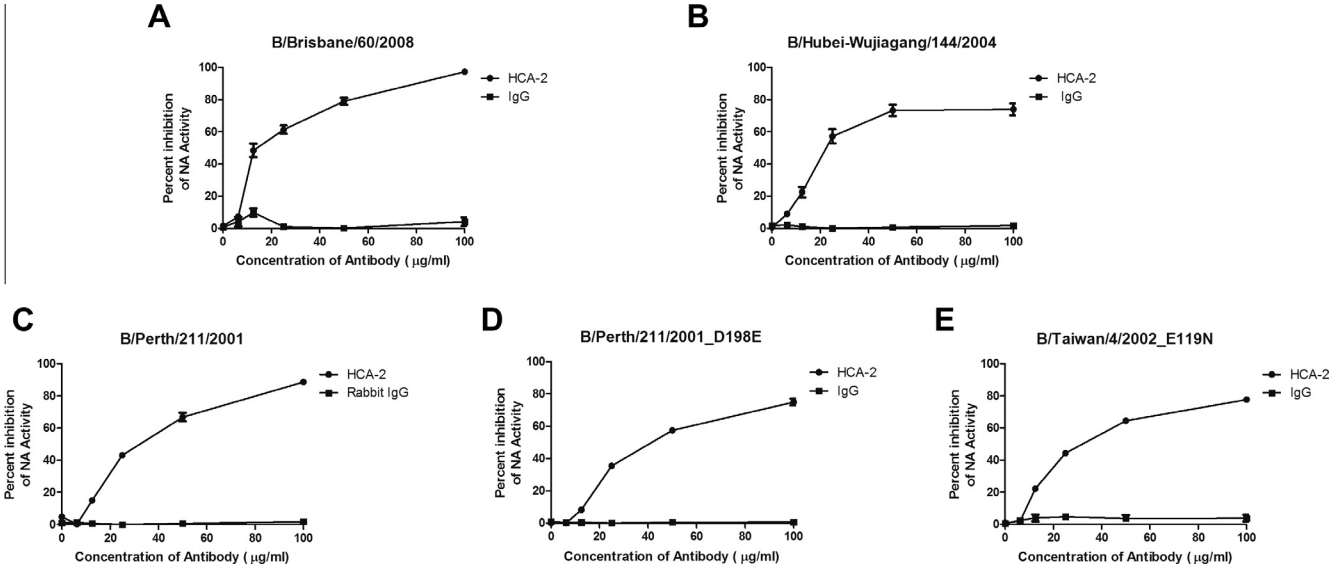

Fig. 1. HCA-2 MAb inhibits NA activity of influenza B strains representative of both lineages. HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect of NA activity. HCA-2 antibody or IgG control was incubated with either (A) B/Brisbane/60/2008, (B) B/Hubei-Wujigang/158/2009, (C) wild type drug sensitive B/Perth/211/2001 or drug resistant strains (D) B/Perth/211/2001 (E197D) and (E) B/Taiwan/4/2002 (E117 N). These mixtures were then transferred to fetuin coated plates for ELLA analysis. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

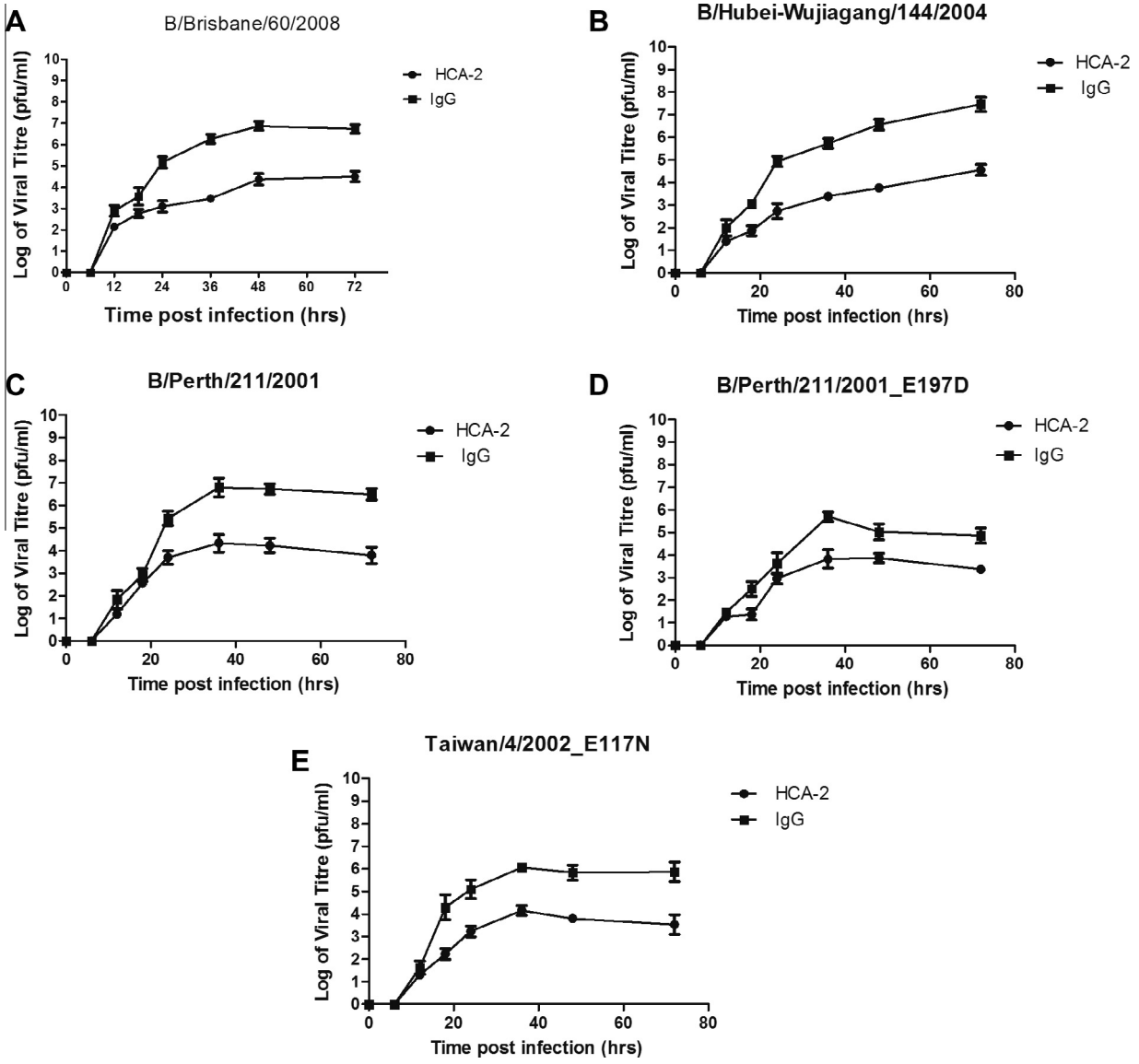

Fig. 2. HCA-2 MAb inhibits growth of both influenza B lineages. HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect against the growth of various viral strains (A–E). Data are presented as percentage of inhibition compared to virus control ± s.e.m. from three independent experiments.

resistant strains as well as the wild type strains. Therefore, the NA mutations D197E and E117D present in the NAI-resistant viruses appear to have no effect on the interactions between the MAb and NA proteins.

4. Discussion

The highly variable nature of influenza viruses presents a daunting challenge for the prevention and treatment of the disease as vaccines must be updated annually and current antivirals are prone to selecting for drug resistant variants [3]. The development of a universal vaccine and broadly-neutralizing antibodies has attracted great attention in recent years. Most of such studies have focused on the other major influenza glycoprotein hemagglutinin (HA) [6]. Specifically, as HA is largely responsible for viral entry and fusion, MAb against the only universally conserved sequence of HA found in the fusion peptide [14] provided a broad neutralization against a variation of strains [15]. Moreover, several groups have reported antibodies with wide range of cross-neutralizing activity against conformational epitopes in HA [16–20]. However, studies on NA in these areas have been very limited, with few reports on the potential role of exploiting highly or universally conserved sequences of NA as potential vaccine or therapeutic targets. The effect of the HA and NA MAbs in combination may be worth investigating in the future.

Our previous studies have revealed that a.a. 222–230 is the only universally conserved sequence in NA, with conservation rate of NA subtypes 1–9 being close to 100% [7,21]. We have also recently demonstrated that this universal epitope is critical for optimal viral function and that MAb to this region can inhibit growth of all nine influenza A subtypes *in vitro* [13,24]. In this current study we showed that the HCA-2 MAb could inhibit the NA enzyme and replication of influenza B viruses from either the Victoria or Yamagata lineages, or strains containing NA mutations that confer NAI resistance. These data suggest that similar to influenza A, the broad protection afforded by the MAb can be extended to influenza B.

Given that the MAb is targeted towards the NA proteins there was potential that NAI resistant strains with NA mutations may have been more poorly inhibited than wild type strains. Interestingly, the two amino acid mutations in the NAI resistant strains tested here (E117D and D197E) are conformationally proximal to

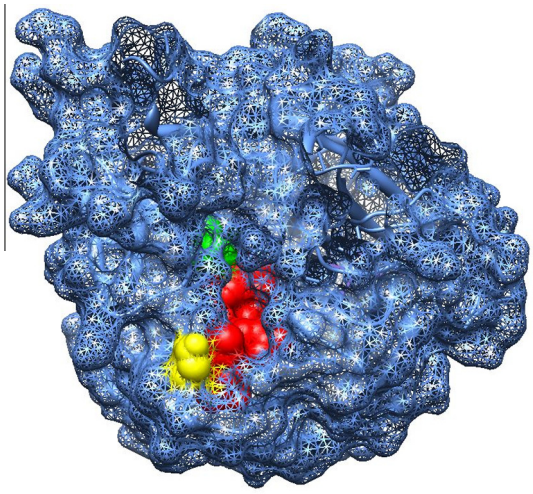

Fig. 3. Top view of neuraminidase of influenza B with the HCA-2 region and two resistance mutations highlighted. The top view of neuraminidase protein with highlighted HCA-2 region (in red). Mutated amino acids which have conferred drug resistance are in yellow (I117D) and green (D197E). Visualization and analysis were performed using the software UCSF Chimera with the protein identification number 3K36. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

the HCA-2 region [10,11,22]. Although both D197 and E117 are not in direct contact with the substrate (Fig. 3), they are located closely to surface-exposed amino acids such as I222, R224 or E227 which are known to be in direct contact with the sialic acid substrate [23] and also found to be necessary for the MAb to inhibit the enzymatic activity of the influenza A neuraminidases [13,24]. Importantly, the E117D or D198E mutations did not alter the interaction between the antibody and the NA protein, suggesting that mutations at these two positions, albeit in the conformational vicinity of the universal epitope, caused insignificant structural hindrance for the interaction between the MAb and the target epitope. Clearly more studies are needed to determine whether antibodies targeting the universally conserved epitope in viral NA could provide protection to animals from influenza in a suitable model, an issue which is not addressed in this study. Nonetheless, it could be envisaged that during the course of infection in tissue cultures, the viral peptide is sufficiently exposed to allow access by antibodies, making it an attractive target for antiviral intervention and vaccine development.

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