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Influenza A virus hemagglutinin specific antibodies interfere with virion neuraminidase activity via two distinct mechanisms

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ABSTRACT

We studied the ability of monoclonal Abs (mAbs) recognizing the major hemagglutinin (HA) antigenic sites to inhibit neuraminidase (NA) cleavage of sialic acids on fetuin. We show that virion associated-NA activity in the enzyme linked lectin assay (ELLA) is largely dependent on HA-mediated attachment of virions to immobilized fetuin. For a Sb-antigenic site specific mAb, there is a nearly perfect correlation between neuraminidase inhibition and blocking virus attachment to immobilized fetuin. By contrast, Sa-, Ca-, and Cb- antigenic site specific mAbs block NA activity in ELLA or the traditional thiobarbituric acid assay by sterically interfering with NA access to substrate. We conclude first, that ELLA with intact virus can only be used to measure anti-NA Abs if sera lack HA-specific Abs, and second, that anti-HA Abs block NA activity by both limiting virion interaction with sialic acid containing surfaces and by sterically limiting NA access to sialic acids attached to macromolecules.

1. Introduction

Influenza A virus (IAV) remains a serious human pathogen, exerting enormous health and economic costs. Virions contain two oligomeric surface glycoproteins, hemagglutinin (HA, trimeric) and neuraminidase (NA, tetrameric). Each recognizes terminal sialic acid (SA), but with opposite activities. HA attaches virus to SA on the target cell surface to initiate the infectious cycle, while NA releases virions from cell surface SA to complete the infectious cycle (Gottschalk et al., 1959; Palese et al., 1974) The functions of HA and NA must mesh to balance attachment vs. release (Banks et al., 2001; Das et al., 2013; Heaton et al., 2013; Hensley et al., 2011; Mitnaul et al., 2000; Yano et al., 2008).

The rapid antigenic evolution of HA and NA ("antigenic drift"), necessitates frequent vaccination of the human population with updated strains. The effectiveness of influenza vaccination is evaluated by levels of antibodies (Abs) specific for HA, and less often, NA. Anti-HA Abs are of paramount importance since they directly neutralize viral infectivity by blocking cellular attachment and or fusion with cellular membranes, and confer potent protection in vivo (Reading and Dimmock, 2007). Anti-NA Abs reduce IAV replication by preventing virion release from the infected cell surface, limiting infection to a single infectious cycle (Kilbourne et al., 1968; Seto and Rott, 1966). NA-specific Abs can, however, protect in vivo, and since NA evolves more slowly than HA, is receiving increasing attention as a vaccine target (Eichelberger et al., 2016; Sylte and Suarez, 2009). While NA is present in most vaccines, vaccine potency is strictly based on HA content and immunogenicity.

NA is generally 5- to 10-fold less abundant on virions than HA (Hutchinson et al., 2014), and is believed to reside in clusters of 3–5 oligomers scattered among a forest of HA spikes (Harris et al., 2006). Given the close proximity of HA and NA on the virion surface, it might be expected that Abs specific for one of the glycoproteins affect the function of the other. Indeed, decades ago, serum HA Abs were shown to inhibit NA activity, most probably by steric hindrance (Paniker, 1968; Russ et al., 1974). Such Abs might provide enhanced neutralization capacity by inhibiting viral release from infected cells. More generally, the effect of HA-specific Abs on NA function should provide insight into the organization of HA and NA on virions (Harris et al., 2006).

Two major assays are used to measure Ab-mediated NA inhibition (NI). The thiobarbituric acid assay (TBAA) chemically measures SA released from fetuin, a relatively inexpensive and highly sialylated 48 kDa glycoprotein abundant in fetal bovine serum. Due to greater safety, simplicity, and throughput, the enzyme linked lectin assay (ELLA) has largely replaced the TBAA (Couzens et al., 2014; Lambre et al., 1990; Sandbulte et al., 2009). In the ELLA, NA release of SA is measured by lectin binding to the now exposed penultimate galactose in an N-linked oligosaccharide, typically attached to fetuin. (Couzens et al., 2014).

Here we use a panel of well characterized mAbs that bind one of the four canonical antigenic sites (Sa, Sb, Ca, Cb, Fig. 1) (Caton et al.,

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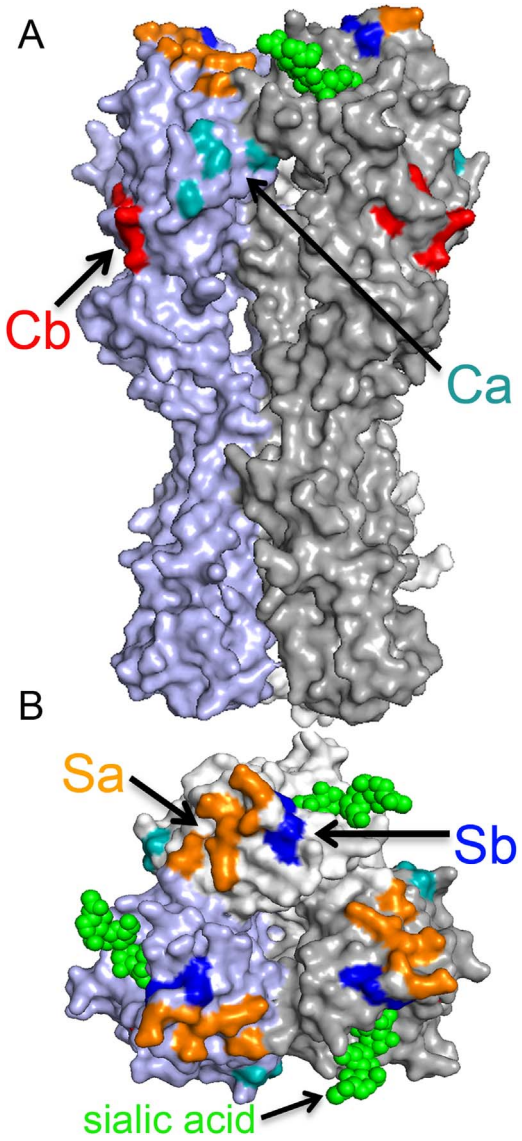

Fig. 1. Canonical antigenic sites of the HA molecule. We used Pymol software to visualize canonical epitopes on a surface representation of the HA molecule as determined by x-ray crystallography. (A) Side view of the HA trimer with Cb site (red) and Ca site (teal). (B) Top view of the HA globular head showing Sa (orange) and Sb (blue) antigenic sites. Sialic acid is shown in bright green

1982) on the HA of A/Puerto Rico/8/34 (H1N1) to explore how anti-HA Abs interfere with NA activity as measured by TBAA or ELLA. Our data provide insight into both how HA facilitates NA activity, and demonstrate that anti-HA Abs interfere with NA activity via two distinct mechanisms.

2. Results and discussion

Using ELLA, we first compared the NI activities of the anti-HA mAb panel (Fig. 2, Table 1). As with a representative anti-NA mAb used as a positive control (NA2-1C1), each of the mAbs exhibited NI (Fig. 2A), with a characteristically sharp drop occurring over a 10-fold range, consistent with simple binary law of mass action binding. Ab specificity is shown by the lack of NI activity exerted by a negative control mAb specific for M1 protein (M2-1C6). Although the HA-specific mAbs display a wide range of IC_{50}s, their *efficiency* at blocking NI (NI efficiency in Table 1), calculated as the mAb equilibrium dissociation constant K_D, (determined by ELISA) divided by the Ab concentration required for 50% NI (IC_{50}), is narrowed to a 10-fold range when we normalize for mAb binding affinities, which vary up to 200-fold (Table 1), to account for antibody occupancy on HA.

Adding 0.5% Triton X-100 (TX100) to release HA and NA from virions increased the efficiency of NA2-1C1 (anti-NA) mediated-inhibition by 14-fold. By distinct contrast, TX100 completely abrogated the NI activity of all HA-specific mAbs (Fig. 2C). This indicates that NI activity mediated by anti-HA Abs requires the physical proximity of HA and NA, and is not trivially due to mAb cross-reactive binding to NA or non-specific factors present in the mAb preparations.

To broaden these findings to a more recent human isolate, we examined the NI activity of the Ca-specific mAb H17-L10 on A/California/07/2009 (H1N1) (pdmH1N1) (H17-L10 is the only mAb in the panel that binds pdmH1N1). H17-L10 exhibits significant NI in ELLA vs. pdmH1N1 (Fig. 2E), and once again, NI activity is completely abrogated by adding TX100 to dissociate virions.

We next examined the NI activity of HA-specific mAbs in the TBAA. MAbs specific for Ca and Cb sites maintain their NI activity (Fig. 2B) relative to NA2-1C1 (though all of the mAbs demonstrated an approximate 100-fold decreased efficiency in TBAA vs. ELLA). As with the ELLA, NI activity was eradicated by TX100 treatment (Fig. 2D). H17-L10 recapitulated these properties when tested with pdmH1N1 (Fig. 2F).

In contrast to Ca- and Cb-specific mAbs, Sa- and Sb-specific mAbs exhibited more limited NI activity in TBAA, inhibiting 25% or less NA activity at the highest concentrations used (Fig. 2B). The Sb-specific mAb demonstrated a particularly large decrease in NI activity in TBAA vs. ELLA.

How to explain the greater capacity of Sa and Sb mAbs to inhibit NA in ELLA vs. TBAA? After incubating virus and mAbs with fetuin coated plates, and washing away free virus, we directly measured IAV binding to plate bound-fetuin in the ELLA using the small fluorogenic NA substrate 4-(methylumbelliferyl)-N-acetylneuraminic acid (muNANA) to detect virions via their NA activity. Importantly, due to the small size muNANA, none of the HA mAbs we tested inhibits NA cleavage of muNANA. This is expected, since even NA2-1C1 (and many other anti-NA Abs) fail to inhibit cleavage of such small substrates (Fazekas de St Groth, 1963). After recording the values, we then washed away muNANA and completed the normal ELLA.

Superimposing muNANA and ELLA Ab titration curves (Fig. 3) revealed a near perfect correlation for the IC5-4F8 (Sb) inhibition of virus binding and neuraminidase activity, consistent with the conclusion that its NI activity is nearly entirely based on blocking viral attachment, and not sterically blocking the access of fetuin to the NA active site. This is consistent with the low activity of IC5-4F8 in the TBAA (Table 1).

By contrast, each of the other mAbs tested demonstrated significant inhibition of ELLA activity when virus remained attached to plate bound-fetuin. This can be quantitated in the differences in IC_{50}s for inhibiting virion binding to fetuin vs. NA activity. The increased efficiency of blocking ELLA NA activity vs. virus attachment ranges from 2.3-fold for Y8-1A6 (Sa), 3.7-fold for H17-L10 (Ca), and 5.3-fold for H9-D3 (Cb).

Based on the ability of anti-HA Abs to inhibit NA ELLA activity by blocking virus attachment, we predicted that HA-mediated virus attachment to plate bound-fetuin enhances virion NA activity. Indeed, treating virus with TX100 to dissociate HA from NA reduces NA ELLA activity 100-fold (Fig. 3E), having no effect on NA activity measured by muNANA (Fig. 3F).

Could the enhanced NA activity of fetuin bound-virus result from conformational changes in NA that increase intrinsic enzyme activity measured by cleavage of muNANA? To test this, we added graded amounts of virus to 96-well plates coated with either fetuin or a non-glycosylated negative control protein (bovine serum albumin (BSA)), and allowed virus to bind at 4 °C before measuring NA activity with muNANA at 37 °C (Fig. 3G). This revealed no significant difference in NA activity between bound (fetuin) and unbound (BSA) virus (control