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Capture and characterization of influenza A virus from primary samples using glycan bead arrays

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ABSTRACT

Influenza A viruses (IAVs) utilize sialylated host glycans as ligands for binding and infection. The glycan-binding preference of IAV hemagglutinin (HA) is an important determinant of host specificity. Propagation of IAV in embryonated chicken eggs and cultured mammalian cells yields viruses with amino acid substitutions in the HA that can alter the binding specificity. Therefore, it is important to determine the binding specificity of IAV directly in primary samples since it reflects the actual tropism of virus in nature. We developed a novel platform for analysis of IAV binding specificity in samples that contain very low virus titers. This platform consists of a high-density flexible glycan display on magnetic beads, which promotes multivalent interactions with the viral HA. Glycan-bound virus is detected by quantifying the viral neuraminidase activity via a fluorogenic reporter, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid. This method eliminates the need for labeling the virus and significantly enhances the sensitivity of detection.

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1. Introduction

Influenza A viruses (IAVs) are extremely diverse and exist in the abundant and widespread reservoir of migratory aquatic birds (Webster et al., 1992). It is the combination of this natural reservoir with large and globally distributed populations of susceptible farm animals (ducks, chickens and pigs) that contributes to an omnipresent threat of new emerging zoonotic viruses and IAV pandemics. A recent example was the influenza (H1N1) pandemic of 2009 caused by a triple reassortment of bird, human and pig viruses (Garten et al., 2009). Aquatic birds are thus subject to surveillance for the early detection of newly emerging strains with enhanced potential to infect humans. The host specificity of IAV is determined to a large extent by the binding specificity of the virus hemagglutinin (HA) to host glycans (Shi et al., 2014; de Graaf and Fouchier, 2014). The development of glycan arrays revolutionized analysis of IAV specificity for host glycans. Glycan arrays enable testing the binding specificity of purified HA and whole IAV to hundreds of glycans simultaneously (Arthur et al., 2014; Smith and Cummings, 2014). Pioneering work by Stevens et al. (2006) revealed that human-specific IAVs bind to host sialic acids with α2-6 glycosidic linkage to the underlying glycan (α2-6Sia), while avian adapted IAVs overwhelmingly recognize α2-3 linked sialic acids (α2-3Sia). During the 2009 pandemic, glycan microarray studies revealed the wide binding specificity of the pandemic influenza A(H1N1) virus (Childs et al., 2009).

Typically, IAVs are isolated from host animals or environmental sources and propagated in embryonated chicken eggs (‘eggs’) or cultured mammalian cells (‘cells’) prior to assessing binding specificity on glycan microarrays (Stevens et al., 2006; Eisfeld et al., 2014; Gulati et al., 2014; Heimburg-Molinaro et al., 2012). It has been noted that the HA accumulates amino acids substitutions that affect the binding specificity to sialic acids (Stevens et al., 2010; Lee et al., 2013). IAV isolates that were grown in eggs acquire specificity for binding α2-3Sia, while the same isolates grown in cultured cells have broad specificity for both α2-3Sia and α2-6Sia (Stevens et al., 2010). Therefore, analyzing the binding specificity of IAV directly from primary samples is highly valuable since it reflects the actual tropism of virus in nature.

The binding affinity of a single HA trimer to sialoglycans is low (Sauter et al., 1989), requiring multivalent interactions to achieve high avidity binding. An average-sized, single IAV virion is estimated to have 500–1000 HA trimers, and 100–500 neuraminidase (NA) tetramers, on its envelope (Taylor et al., 1987; Markovic et al., 2001; Harris et al., 2006). The HA and NA are not evenly distributed on the virion, but instead are clustered, creating patches with high local

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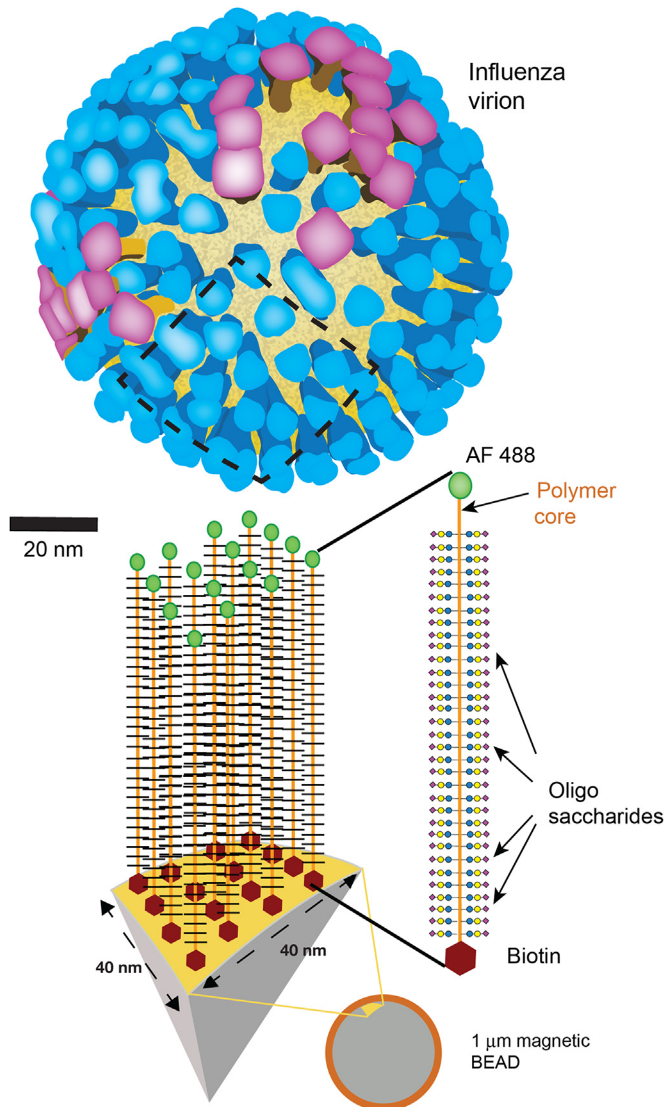

Fig. 1. Schematic representation of the 3D mucin mimetic bead array. Mucin mimetic glycopolymers are labeled with a single Alexa Fluor 488 (AF 488) fluorophore and a single biotin at the terminal ends. The glycopolymers are conjugated to streptavidin-coated magnetic bead at an average density of 27-molecules/1600 nm^2. Note that the density of HA spikes (blue) is approximately 13-molecules/1600 nm^2 and NA spikes (pink) cluster on the viral envelope (Harris et al., 2006).

densities (Harris et al., 2006). Host epithelial cells are covered with a dense matrix of glycoconjugates (glycocalyx) that can be anchored and/or secreted into the mucus layers. In vertebrates, most of these glycoconjugates carry multiple glycan chains, commonly terminating in sialic acid. Therefore, IAV likely engages multiple sialylated glycan chains on the host cell to achieve high avidity binding. Keeping this in mind, we developed a three-dimensional mucin mimetic array in which a large number of defined glycans are presented on a polymer backbone that extends from a magnetic bead core (Fig. 1). This unique high-density and flexible glycan display enhances multivalent binding by the virus, and reduces the viral titer required for reliable detection of binding (Huang et al., 2015).

Here we demonstrate the sensitivity and versatility of our novel three-dimensional mucin-mimetic array for characterizing various subtypes of avian IAV that were isolated from waterfowl. In addition, we provide a proof of principle for the analysis of IAV directly in primary samples. We analyzed swab samples collected from the cloaca of mallard ducks (*Anas platyrhynchos*) by applying them directly on the mucin mimetic beads. Thus, the array eliminates the need to amplify the virus prior to characterizing the receptor specificity, thereby limiting the risk of viral binding specificity changes due to amino acid substitutions in the HA.

2. Results and discussion

2.1. Low virus concentrations suffice for analysis on the 3D mucin mimetic array

Multivalent interactions between viral HA and the sialylated polymers are likely affected by the spacing between sialylated polymers on the beads, and both glycan density and the concentration of IAV are known to affect virus binding to glycan microarrays (Gulati et al., 2014). In order to determine the optimal density for IAV binding, the mucin mimetic polymers were conjugated to magnetic beads at densities ranging between 1–50 polymers/1600 nm^2 (Supplementary Fig. 1). The binding of A/PR/8/34(H1N1) was tested at virus concentrations of 9.2–160 HAU/ml (Fig. 2a, b and Supplementary Fig. 2). The bound virus was detected by incubation with the reporter molecule, 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU-NANA, 4MU-Neu5Ac). The viral NA cleaves sialic acid from the 4MU-Neu5Ac compounds thereby releasing a fluorescent 4-methylumbelliferone molecule (4MU). Release of 4MU (pmol/1 h) is indicative of viral activity and correlates with viral titers (Nayak and Reichl, 2004). In order to minimize interference from NA cleaving of the glycosylated beads, 6 mmol of the 4MU-Neu5Ac compounds were added to the beads. This method is highly sensitive and enables detection of IAV at concentrations as low as 5 HAU/ml (Fig. 2c). In comparison, IAV binding to the Consortium for Functional Glycomics glycan array typically requires 2500–10,000 HAU/ml IAV (Gulati et al., 2014; Lugovtsev et al., 2009; Chen et al., 2011). Virus binding to the bead array was detected when the density of polymer was larger or equal to ~12 polymers/1600 nm^2 (Fig. 2a and b). Interestingly, Harris et al. (2006) reported an average of 13 HA trimers in 1600 nm^2 of viral envelope. Beads covered with high-density polymers enabled detection of A/PR/8/34(H1N1) at concentrations as low as 9.2 HAU/ml (orange circle, Fig. 2a and b) nearly the detection limit obtained with the 4MU-NANA assay (5 HAU/ml, Fig. 2c). The same mucin mimetic polymer printed on microarray slides requires at least 25 fold more concentrated A/PR/8/34(H1N1) virus for reliable detection (Huang et al., 2015) (Supplementary Fig. 3).

2.2. Validation of the 3D mucin mimetic array

We generated a library of 43 mucin mimetic polymers conjugated to magnetic beads by incubating streptavidin-conjugated magnetic beads with 43 biotinylated mucin mimetic polymers (Fig. 3). The ligand library comprises 19 sialylated glycoconjugates (Fig. 3 1–19), and their corresponding non-sialylated backbone (Fig. 3 22–23, 29-32). In addition the library contains the ABO blood group antigens (Fig. 3 20, 24–25) and Lewis blood group glycans (Fig. 3 16–17, 22–23, 26–27), which are typically found in the mucus and mucosa layers, sialylated gangliosides (Fig. 3 9, 13–15) and monosaccharides (Fig. 3 28, 34–43). The mucin mimetic beads were analyzed by flow cytometry and the polymers density on the beads was calculated for each glycan structure. The average density was 28 glycopolymers/1600 nm^2 (Supplementary Tables 1–2 and Supplementary Fig. 4a). Two human IAV strains A/PR/8/34(H1N1) and A/Aichi/2/68(H3N2) were diluted in PBS to 64 HAU/ml, and their binding to the array was tested (Fig. 4a and b). Since virus bound to the array is quantified by measuring NA activity, we confirmed that the NA activity of both strains is comparable (Fig. 4c). In agreement with previous reports, both strains bound exclusively to glycoconjugates with terminal sialic acids in both α2-3-and α2-6-linkage to the underlying glycans (Rogers and Paulson, 1983; Cohen et al., 2013; Suzuki et al.,

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