Virology 486 (2015) 134–145

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity

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ARTICLE INFO

Article history:
Received 8 July 2015
Returned to author for revisions
30 August 2015
Accepted 31 August 2015

Keywords:
Highly pathogenic Influenza A H5N1
N-glycosylation
Hemagglutinin H5
Neutralizing antibodies
Antigenic drift

ABSTRACT

Highly pathogenic avian influenza A H5N1 viruses remain endemic in poultry in several countries and still constitute a pandemic threat. Since the early 20th century, we experienced four influenza A pandemics. H3N2 and H1N1pdm09 viruses that respectively emerged during 1968 and 2009 pandemics are still responsible for seasonal epidemics. These viruses evolve regularly by substitutions in antigenic sites of the hemagglutinin (HA), which prevent neutralization by antibodies directed against previous strains (antigenic drift). For seasonal H3N2 viruses, an addition of N-glycosylation sites (glycosites) on H3 contributed to this drift. Here, we questioned whether additional glycosites on H5 could induce an escape of H5N1 virus from neutralization, as it was observed for seasonal H3N2 viruses. Seven H5N1 mutants were produced by adding glycosites on H5. The most glycosylated virus escaped from neutralizing antibodies, *in vitro* and *in vivo*. Furthermore, a single additional glycosite was responsible for this escape.

1. Introduction

Highly pathogenic (HP) avian H5N1 influenza A epizootic episode started in Southeast Asia during the 2002–2003 winter (Li et al., 2004). Since then, this virus has spread to more than 60 countries worldwide and has remained endemic in poultry and wildfowl in several countries. To date, over 840 human cases of HP H5N1 influenza A viruses infection have been reported with a mortality rate of 59% (World Health Organization, 2015). Up to now, most of these human cases resulted from a close contact with infected animals, mainly sick poultry. Some limited human-to-human transmission cases have been reported, but this mode of infection is yet inefficient (Van Kerkhove et al., 2011; Wang et al., 2008). However, the multiplication of sporadic transmissions from poultry to humans increases the likelihood of an emergence of H5N1 virus variants able to transmit efficiently from human-to-human (Peiris et al., 2007). The introduction of such an adapted variant in the human population would represent an antigenic shift over circulating seasonal epidemic type A H1N1 and H3N2 viruses and may result in an extensive worldwide epidemics, such as the 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) or 2009 (H1N1pdm09) pandemics (Kilbourne, 2006; Smith et al., 2009).

The envelope of influenza A viruses is formed of a lipid bilayer where are anchored two types of surface glycoproteins which play essential and complementary roles in viral infection. The neuraminidase (NA) is a receptor-destroying enzyme which plays important roles in viral release and cell-to-cell spread, whereas the hemagglutinin (HA) is responsible for attachment of the virus to sialic acid-containing receptors and viral entry by membrane fusion (Gamblin and Skehel, 2010). HA is a type 1 glycoprotein that forms a homotrimer of approximately 220 kDa. In its mature form,

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http://dx.doi.org/10.1016/j.virol.2015.08.033
0042-6822/

each monomer of HA consists of two polypeptides known as HA1 and HA2 subunits that are linked by a disulfide bond (Gamblin and Skehel, 2010; Skehel and Wiley, 2000). HA is the main target of the antibody response induced during the course of a natural infection or following vaccination with inactivated virions (Johansson et al., 1987). Anti-HA antibodies are able to neutralize viral infectivity, mainly by blocking the interaction of HA with cell surface. Major antigenic sites are located around the receptor-binding site on the HA1 domain, and are named in alphabetical order from A to E for H3 and Sa, Sb, Ca1, Ca2 and Cb for H1 (Both et al., 1983; Caton et al., 1982; Wiley et al., 1981). Alternatively, antibodies that bind to the stalk region of HA can block the fusion between viral and host membranes, which is essential to initiate virus replication (Ekiert et al., 2009; Prabhu et al., 2009). Up to now, the presence of neutralizing anti-HA antibodies remains the best immune correlate for protection against influenza viruses (De Jong et al., 2003).

Viral proteins of human seasonal influenza A strains (H3N2 and H1N1pdm09), especially HA, evolve gradually through point mutation (antigenic drift) allowing the resulting variants to escape from host immunity. This continuous antigenic drift, implies regular, almost annual, updating of the vaccine composition (Boni, 2008). Since 1968 and throughout the circulation of seasonal H3N2 virus in human, the number of potential N-glycosylation sites (glycosites) (Asn-X-Ser/Thr, where X is any amino acid except Pro) on the globular head of the H3 HA increased from 2 in 1968 to 8 in 2007 (Fig. 1A) (Abe et al., 2004; Skehel et al., 1984; Zhang et al., 2004). This progressive accumulation of additional glycosites was proposed as a contributing element allowing the virus to escape antibody-mediated response, thus taking part in antigenic drift (Abe et al., 2004; Skehel et al., 1984). Indeed, the structure of N-glycans could act as a "shield", masking the access of antibodies towards antigenic sites.

In 2008, Igarashi et al. described the presence of "high potential" candidate sites for N-glycosylation on the coding sequence of several subtypes of HA. These *Cand*1, *Cand*2 and *Cand*3 sites require respectively 1, 2 or 3 nucleotide substitutions to encode an Asn-X-Ser/Thr motif (Igarashi et al., 2008). Interestingly, the authors also observed that all the glycosites that appeared on the H3 throughout the evolution of seasonal H3N2 virus in human between 1968 and 2003 derived from *Cand*1 and *Cand*2 sites. In addition, they described the presence of an important number of *Cand1* sites, on the H5 of HP H5N1 viruses (Fig. 1B).

In the present study, we undertook to evaluate if an increase in the number of glycosites on the surface of the H5 protein is likely to contribute to an antigenic drift of H5N1 virus in the same way than that observed for seasonal H3N2 viruses. Particularly, we questioned whether the sequential accumulation of additional glycosites observed on the H3 could be used as a predictive tool to anticipate a potential escape of H5N1 viruses from neutralizing antibody response in the event of their emergence in the human population. To that end, we produced A/duck/Niger/2090/06 clade 2.2 H5N1 viruses by reverse genetics with additional glycosites on H5 at *Cand1* sites, mimicking the combinatorial and kinetic accumulation of the 8N-glycosylation sites on the H3, as observed since 1968.

The phenotype of all the hyperglycosylated H5N1 viruses was characterized *in vitro*. Moreover, their genotypic stability, fitness and virulence were characterized in mice. Then, their ability to escape from neutralizing antibodies induced in mice by immunization with split-inactivated H5N1 viruses was assessed both *in vitro* and *in vivo*.

Overall, our data clearly demonstrate that the addition of glycosites on HA could be a simple way for an emerging H5N1 virus to escape from a vaccine-induced anti-H5 response and that new pre-pandemic vaccine strategies should be checked for their potential to neutralize any *Cand*1 "glyco-variant" (glyV) virus, whose viability

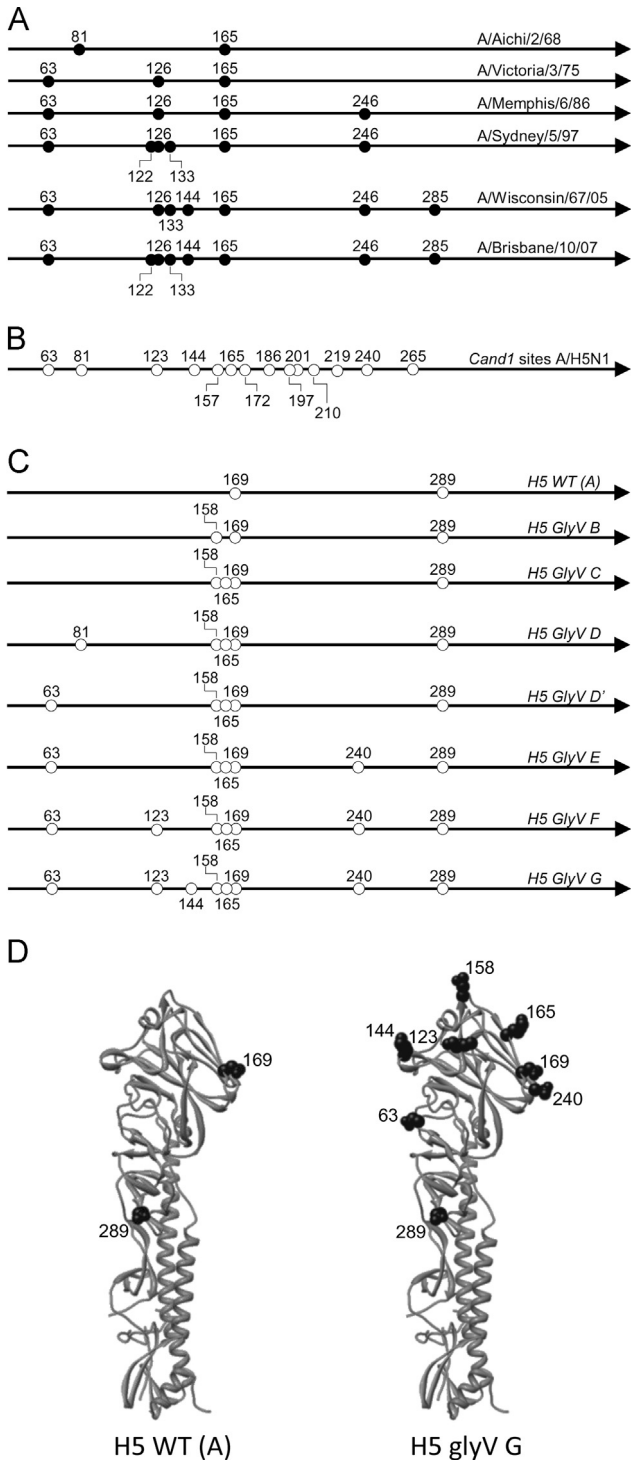

Fig. 1. Illustration of the N-glycosylation pattern of the globular head of the hemagglutinin (HA) derived from seasonal H3N2 viruses or from the A/duck/Niger/2090/2006-based glycovariants (glyV). (A) Evolution of the N-glycosylation pattern on the globular head of representative seasonal H3N2 viruses HA, isolated between 1968 and 2007. Each glycosylation site (glycosite) is represented by a black circle and its position on the amino acid sequence is indicated. (B) Localisation of the positions corresponding to *Cand*1 sites for the globular head of the A/Vietnam/1194/2004 (VN04) virus H5, as determined by Igarashi et al. (2008). Each *Cand*1 site is represented by a white circle and its position on the amino acid sequence is indicated using H3 numbering. (C) Schematic representation of the N-glycosylation profile of the globular head of the WT A/duck/Niger/2090/2006 (WT) and each of the glyV mutants H5, named from B to G. (D) Localisation of these glycosites on the 3-dimensional representation of the H5 determined by Yamada et al. (2006) for the WT virus and the most glycosylated glyV mutant (G). The asparagine acceptor of each glycosite is represented with black spheres and H3 numbering is used.