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Stabilizing the glycosylation pattern of influenza B hemagglutinin following adaptation to growth in eggs

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Summary The currently circulating influenza B viruses from both antigenic lineages contain an N-linked glycosylation site in the hemagglutinin (HA) protein at positions of 196 or 197. However, egg adaptation caused the loss of the glycosylation site that could impact virus antigenicity and vaccine efficacy. The effect of the 196/197 glycosylation site on influenza B virus growth and antigenicity was systemically evaluated in this study by the molecular approach. Paired recombinant 6:2 reassortant influenza B vaccine strains, with or without the 196/197 glycosylation site, were generated by reverse genetics and the glycosylation site was retained in MDCK cells. In contrast, all the viruses that contained the introduced glycosylation site were unable to grow in eggs and rapidly lost the glycosylation site once adapted to grow in eggs. We showed that glycosylation affected virus binding to the α-2,3-linked sialic acid receptor and affected virus antigenicity as tested by postinfected ferret sera. We have further identified that the Arginine residue at amino acid position 141 (141R) can stabilize the 196/197 glycosylation site without affecting virus antigenicity. Thus, the 141R could be introduced into vaccine strains to retain the 196/197 glycosylation site for influenza B vaccines.

Introduction

Types A and B influenza viruses are the major cause of human influenza epidemics. As members of the family Orthomyxoviridae, both types A and B influenza viruses contain a genome comprised of eight negative sense RNA segments. Influenza B viruses, although not as prominent as type A influenza viruses in causing influenza epidemics, frequently cause substantial morbidity and mortality in humans [1]. Since the mid 1980s, influenza B strains have diverged into two genetic lineages represented by the prototypic B/Victoria/2/87 and B/Yamagata/16/88 strains [2]. These two lineages continue to co-circulate in humans.

Influenza vaccines provide the most effective means to prevent influenza infection and epidemics. Current influenza vaccines contain antigens from two type A influenza strains

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(H1N1 and H3N2) and one influenza B strain. Vaccine composition is updated annually such that the individual components are antigenically similar to the circulating strains. FluMist®, the live-attenuated influenza vaccine licensed in the U.S., contains three 6:2 genetic reassortant viruses [3]. Each 6:2 vaccine strain contains the six internal gene segments (PB1, PB2, PA, NP, M and NS) derived from the master donor virus of *ca* A/Ann Arbor/6/60 (MDV-A) or *ca* B/Ann Arbor/1/66 (MDV-B) to confer the characteristic cold adapted, temperature sensitive and attenuation phenotypes to the vaccine strains and the hemagglutinin (HA) and neuraminidase (NA) gene segments from a currently circulating wild-type (wt) A (H1N1 or H3N2) or B virus provide antigen specificity.

Embryonated chicken eggs are currently used to manufacture influenza vaccines. However, cultivation of influenza A and B viruses in embryonated chicken eggs or chicken cells frequently selects for mutants in the HA that differ antigenically and structurally from naturally occurring viruses [4]. Amino acid substitution mutations in the HA of egg-grown influenza A viruses usually cluster at or near the receptor binding regions and may disrupt the normal glycosylation pattern of the molecule [5—7]. These changes could alter virus antigenicity, immunogenicity and vaccine efficacy [8—10]; therefore, considerable care must be exercised in the annual selection process for vaccine strains [11].

It has been reported that adaptation of certain influenza B strains in chicken eggs could select for variants that were antigenically distinct from the same isolate that was grown in mammalian cells. Several studies showed that egg adaptation of influenza B resulted in the loss of the N-linked glycosylation site (N-X-T/S) at amino acids 196—198 (B/Yamagata lineage) or 197—199 (B/Victoria lineage) in the HA1 region resulting in a significant change in the antigenicity of the molecule [12—14]. The majority of type B influenza clinical isolates that were grown in mammalian cell culture retained the glycosylation motif at the 196/197 site. Thus, it is important to understand the effect of the 196/197 glycosylation site on vaccine antigenicity and replication in eggs. We used reverse genetics technology to make live attenuated reassortant vaccine strains that contain the HA with or without the glycosylation site at 196/197 from both influenza B lineages and evaluated their biologic properties after growth in both mammalian cell culture and eggs. Our studies indicated that glycosylation at the HA 196/197 site significantly reduced virus binding to the avian receptor and hindered virus replication in eggs. The glycosylation significantly affected virus antigenicity in ferret. We further demonstrated that this glycosylation site can be retained following adaptation to eggs through introduction of a second compensatory mutation.

Materials and methods

Viruses

Wild-type (wt) influenza B viruses used in this study, B/Malaysia/2506/04, B/Ohio/1/05, B/Florida/7/04, B/Shanghai/361/02 (B/SH/361/02), B/Jilin/20/03 (B/JL/20/03), B/Jiangsu/10/03 (B/JS/10/03), were originally obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia). The HA and NA cDNAs of wt viruses were amplified by RT-PCR using vRNA as template and cloned between the two BsmBI sites in the pAD3000 vector. Recombinant 6:2 vaccine viruses were rescued using the eight plasmid transfection system [15]. Briefly, co-cultured 293T and MDCK (Madin-Darby canine kidney) cells were transfected with the six plasmids encoding the internal genes of *ca* B/Ann Arbor/1/66 (MDV-B) together with the two plasmids encoding the wt HA and NA segments. Seven days after transfection, supernatants from the transfected cells were collected and were further amplified in either MDCK cells or embryonated chicken eggs.

To generate 6:2 recombinant viruses that contained specific amino acid substitution mutations in HA, the HA cDNA was subjected to site-directed mutagenesis by QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) and viruses were produced by plasmid rescue as described above. The HA sequence of each reassortant virus was examined by sequencing analysis.

Virus growth in cells and eggs

MDCK cells originally obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). To grow influenza virus in MDCK cells, MDCK cells were infected with virus at a multiplicity of infection (MOI) of 0.004. After 60 min adsorption at room temperature, virus inoculum was removed, replaced with MEM and incubated at 33 °C. The culture supernatants were collected at 3 days of postinfection and virus titers were determined by plaque assay on MDCK cells at 33 °C [15].

Embryonated chicken eggs were obtained from Charles River SPAFAS (Franklin, CT) and incubated for 10—11 days prior to virus inoculation. Each egg was inoculated with 0.1 ml of virus at the amount of 10^2—10^5 PFU as indicated in the text and incubated at 33 °C for 3 days. Allantoic fluids were collected and virus level was examined by the hemagglutination (HA) assay using 0.5% red blood cells (RBC) from different species: turkey RBC (tRBC), guinea pig RBC (gpRBC), or horse RBC (hRBC) supplied by Colorado Serum Company (Denver, CO). Infectious virus titers were determined by plaque assay. Virus sequence was determined by sequencing the RT-PCR cDNA products amplified from viral RNAs.

Determination of HA glycosylation status by Western blotting

The difference in the number of HA glycosylation sites affects protein mobility on the polyacrylamide gel. To confirm the glycosylation status of the viruses that either contained or did not contain the 196/197 glycosylation site, virus from MDCK cell culture supernatants or from allantoic fluid was mixed with 2× Tris—glycine SDS sample buffer and electrophoresed on a Novex® 10% Tris—glycine gel (Invitrogen, Grand Island, NY). The electrophoresed