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Generation of recombinant pandemic H1N1 influenza virus with the HA cleavable by bromelain and identification of the residues influencing HA bromelain cleavage

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ABSTRACT

The proteolytic enzyme bromelain has been traditionally used to cleave the hemagglutinin (HA) protein at the C-terminus of the HA2 region to release the HA proteins from influenza virions. The bromelain cleaved HA (BHA) has been routinely used as an antigen to generate antiserum that is essential for influenza vaccine product release. The HA of the 2009 pandemic H1N1 influenza A/California/7/2009 (CA09) virus could not be cleaved efficiently by bromelain. To ensure timely delivery of BHA for antiserum production, we generated a chimeric virus that contained the HA1 region from CA09 and the HA2 region from the seasonal H1N1 A/South Dakota/6/2007 (SD07) virus that is cleavable by bromelain. The BHA from this chimeric virus was antigenically identical to CA09 and induced high levels of HA-specific antibodies and protected ferrets from wild-type H1N1 CA09 virus challenge. To determine the molecular basis of inefficient cleavage of CA09 HA by bromelain, the amino acids that differed between the HA2 of CA09 and SD07 were introduced into recombinant CA09 virus to assess their effect on bromelain cleavage. The D373N or E374G substitution in the HA2 stalk region of CA09 HA enabled efficient cleavage of CA09 HA by bromelain. Sequence analysis of the pandemic H1N1-like viruses isolated from 2010 revealed emergence of the E374K change. We found that K374 enabled the HA to be cleaved by bromelain and confirmed that the 374 residue is critical for HA bromelain cleavage.

1. Introduction

Influenza virus infections remain a significant public health concern. Each year, about 36,000 excess deaths in the United States are attributed to seasonal influenza epidemics, notably in the elderly and in individuals with underlying pulmonary conditions [1]. Vaccination is the most effective means to prevent the mortality and morbidity caused by annual influenza epidemics [2,3]. The surface proteins, hemagglutinin (HA) and neuraminidase (NA), are the two major antigens that induce protective immune responses [4]. The HA is a major component of the currently licensed inactivated influenza vaccines. To keep up with constant antigenic drift of influenza viruses, influenza vaccines are updated annually to antigenically match the circulating strains. Occasionally, influenza viruses with novel antigenic characteristics infect and spread in the human population [5], resulting in influenza pandemics that cause not only morbidity and mortality but also considerable social and economic disruption. In March 2009, a novel swine-origin influenza A H1N1 virus emerged in North America and caused an influenza pandemic [6–8]. As of August 2010, the 2009 H1N1 virus has moved into the post-pandemic period after spreading to 214 countries and claiming the lives of more than 18,000 individuals worldwide (http://www.who.int/csr/don/2010_08_06/en/index.html).

The humoral immune response induced by seasonal influenza vaccines did not cross-react with the newly emerged swine-origin influenza H1N1 strain nor did it confer any significant protection against challenge with the pandemic wild-type virus [9,10]. Thus, in response to the 2009 pandemic, a live attenuated influenza vaccine (LAIV) was generated by production of a 6:2 reassortant vaccine virus that contained the HA and NA gene segments from the novel swine origin influenza A/California/7/2009 (CA09) H1N1 virus, as well as the six internal protein gene segments from the cold adapted influenza A/Ann Arbor/6/60 (AA *ca*, H2N2) master donor virus [11]. AA *ca* confers the cold adaptation (*ca*), temperature-sensitivity (*ts*) and attenuation (*att*) phenotypes to the LAIV strains [12,13]. However, the development of an LAIV against the pandemic H1N1 virus presented significant challenges when it was recognized that the vaccine virus did not replicate as efficiently as seasonal H1N1 vaccine strains in embryonated chicken eggs. Further development was required to improve vaccine virus yield for vaccine manufacture [11]. Furthermore, significant hurdles were

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also encountered in the preparation of the HA antigens to generate antiserum reagents that were necessary for vaccine characterization and safety test for product release. HA specific antibody is required for potency test of both LAIV and inactivated trivalent influenza vaccine (TIV); it is also required for neutralizing higher titers of vaccine virus for in vitro and in vivo safety testing of LAIV.

HA antigens are generally prepared by bromelain cleavage of the virions. Bromelain is the most abundant cysteine endopeptidase found in the stem of the pineapple plant (*Ananas comosus*) [14]. Bromelain cleavage at the C-terminus of the HA2 region results in the release of the HA ectodomain portion from the transmembrane and cytoplasmic tail that anchors the HA molecule into the virion [15]. The HA ectodomain released from intact virions by bromelain cleavage was successfully crystallized for X-ray analysis to solve the first crystal structures of the HA of the 1968 H3N2 pandemic virus [16]. The BHA and recombinant HA have since been used to solve the HA structures of other influenza virus subtypes including the 2009 H1N1 pandemic viruses [17–24]. Since the BHA remains as a trimer, it retains the antigenicity of the HA [25] and has been traditionally used as an antigen for producing HA-specific antiserum for vaccine product test. Surprisingly, the CA09 pandemic H1N1 virus was resistant to bromelain digestion. The resistance to bromelain cleavage was a major bottleneck for 2009 pandemic H1N1 potency reagent development [26,27]. To circumvent this challenge, a chimeric HA that bears the antigenic characteristics of CA09 HA1 with a bromelain-cleavable HA2 from the seasonal H1N1 influenza A/South Dakota/6/2007 (SD07) was constructed and a reassortant virus carrying this chimeric HA was generated. The chimeric BHA was successfully generated and used in the antiserum production for LAIV release test.

To determine the molecular basis of the inefficient bromelain cleavage of the CA09 HA, sequence comparison of the HA2 region of CA09 and SD07 revealed a total of 18 amino acid differences. These 18 amino acids were evaluated for their influence on bromelain cleavage. We identified that amino acid substitution of D373N or E374G (or D46N and E47G by HA2 numbering) in the HA2 region of CA09 greatly improved bromelain cleavage efficiency. The 2009 pandemic H1N1 virus has since been circulating in humans and has replaced previously circulating seasonal H1N1 virus [28]. Sequence analysis of the H1N1 strains isolated from 2010 revealed several amino acid changes in the HA and NA. One notable change is the E374K change in the HA. We demonstrated that the HA of a recent H1N1 virus with K374 residue could be cleaved by bromelain, confirming that K374 is critical for bromelain cleavage.

2. Materials and methods

2.1. Generation of recombinant viruses

Wild-type (*wt*) A/California/7/2009 (CA09) and A/South Dakota/6/2007 (SD07) H1N1 viruses were received from the US Centers for Disease Control and Prevention. The H1 HA and N1 NA gene segments were amplified by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the plasmid vector pAD3000 [12,29]. Site-directed mutagenesis was performed to introduce specific changes into the HA gene using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The chimeric CA09 H1 HA DNA was constructed by replacing the HA1 portion of SD07 with CA09 HA1 using the *NcoI* and *XhoI* restriction enzyme sites introduced during PCR reactions.

The 6:2 reassortant viruses were generated by co-transfecting eight cDNA plasmids encoding the HA and NA of the CA09 H1N1 virus and the 6 internal protein gene segments of AA *ca* into co-cultured 293T and MDCK cells [29]. Viruses were propagated in the allantoic cavity of 10- to 11-day-old embryonated chicken eggs. The HA sequence of each recombinant virus was confirmed by sequencing cDNA amplified from vRNA by RT-PCR.

2.2. Bromelain enzymatic cleavage of recombinant viruses and analysis of cleaved HA

To examine the bromelain cleavability of HA, recombinant viruses were concentrated and purified by gradient centrifugation prior to analysis. The allantoic fluid of influenza viruses was clarified by centrifugation at 4000 rpm for 30 min and virus particles were pelleted at 28,000 rpm for 2 h at 4 °C. The virus pellet was suspended in 45% Histodenz (Sigma–Aldrich, Inc., St. Louis, MO) in Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris and 1 mM EDTA, pH 7.2) and centrifuged through a 0–45% Histodenz gradient in TBS at 30,000 rpm for 4 h. The virus fraction was collected and pelleted in TBS at 28,000 rpm for 60 min at 4 °C. The virus pellet was re-suspended in TBS and washed once in TBS at 28,000 rpm for 60 min at 4 °C to remove residual Histodenz.

Virus suspension was diluted to a concentration of 10 mg/ml in Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 9.75) and 1 ml virus suspension was incubated with 6.5 μl of bromelain enzyme at a concentration of 50 U/ml (Sigma–Aldrich, Inc., St. Louis, MO) containing beta-mercaptoethanol (1:1000, v/v) in TE buffer for 16 h at 37 °C with gentle shaking. The reactions were subjected to centrifugation at 30,000 rpm for 2 h at 4 °C in a bench top ultracentrifuge (Beckman Optima TLX, Beckman Coulter Inc., Brea, CA). The BHA was expected to be released in the supernatant fraction and the virion particles would be pelleted. The pellet was resuspended in 0.2 ml of TE buffer. The proteins from untreated viruses, the supernatant and pellet from ultracentrifugation were analyzed by gel electrophoresis by loading 10 μl of each material on a Novex® 4–20% Tris–glycine gel (Invitrogen, Carlsbad, CA). The protein gels were either stained by Coomassie blue stain or Western blot analysis using polyclonal chicken anti-A/New Caledonia/20/99 (H1N1) antiserum followed by incubation with horse radish peroxidase (HRP)-conjugated anti-chicken antibodies (Dako, Carpinteria, CA). Protein bands were detected by a chemiluminescent detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ) and developed on an X-ray film.

2.3. Ferret studies

Groups of male and female ferrets aged 8–10 weeks old from Simonsen (Gilroy, CA) ($n = 3$/group) were used to assess immunogenicity and protective efficacy of BHA protein. The animals were housed individually and pre-screened for antibodies against circulating seasonal H1N1, H3N2, and CA09 influenza viruses by hemagglutination inhibition (HAI) assay. Ferrets were inoculated intramuscularly on days 0 and 21 with 15 μg of protein in 0.5 ml mixed with an equal volume of TiterMax adjuvant (CytRx, Norcross, GA). Ferrets were challenged with 10^7 plaque forming unit (PFU) of CA09 *wt* virus on day 42 and the lungs and nasal turbinates (NT) were harvested at 3 days post-infection. Virus titers in the lungs and NT were determined by the fifty percent egg infectious dose (EID_{50}) assay and expressed as $\log_{10}$ EID_{50}/g of tissue.

Ferrets that were assigned to the immunogenicity study were immunized with 15 μg of the BHA protein, mixed with an equal volume of complete Freund's adjuvant (CFA) (Sigma–Aldrich, Inc., St. Louis, MO) (0.5 ml total volume) on day 0 and additional doses of the BHA adjuvanted with an equal volume of incomplete Freund's adjuvant (IFA) (Sigma–Aldrich, Inc., St. Louis, MO) on days 21, 42 and 63. Sera were periodically collected on days 21, 42, 63 and 84. Pre- and post-immunization sera were assayed for antibody titers by HAI and microneutralization assay as previously described [11].