Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Genetic analysis of attenuation markers of cold-adapted X-31 influenza live vaccine donor strain

Yo Han Jang[a], Eun-Ju Jung[a,1], Kwang-Hee Lee[a], Young Ho Byun[a], Seung Won Yang[a], Baik Lin Seong[a,b,*]

[a] Laboratory of Molecular Medicine, Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, South Korea
[b] Vaccine Translational Research Center, Yonsei University, Seoul, South Korea

ARTICLE INFO

Article history:
Received 12 October 2015
Received in revised form 5 January 2016
Accepted 26 January 2016
Available online xxx

Keywords:
Influenza virus
Cold-adapted live vaccine
X-31
Phenotype
Nucleoprotein

ABSTRACT

Cold-adapted live attenuated influenza vaccines (CAIVs) have been considered as a safe prophylactic measure to prevent influenza virus infections. The safety of a CAIV depends largely on genetic markers that confer specific attenuation phenotypes. Previous studies with other CAIVs reported that polymerase genes were primarily responsible for the attenuation. Here, we analyzed the genetic mutations and their phenotypic contribution in the X-31 ca strain, a recently developed alternative CAIV donor strain. During the cold-adaptation of its parental X-31 virus, various numbers of sequence changes were accumulated in all six internal genes. Phenotypic analysis with single-gene and multiple-gene reassortant viruses suggests that NP gene makes the largest contribution to the cold-adapted (ca) and temperature-sensitive (ts) characters, while the remaining other internal genes also impart attenuation characters with varying degrees. A balanced contribution of all internal genes to the attenuation suggests that X-31 ca could serve as an ideal master donor strain for CAIVs preventing influenza epidemics and pandemics.

1. Introduction

The influenza virus is an important human respiratory pathogen responsible for annual epidemics and occasional pandemics [1,2]. Vaccination has been considered the most effective means to prevent influenza infections and reduce the clinical impacts made by the viral disease. In addition to the classical inactivated, killed influenza vaccine, cold-adapted influenza vaccines (CAIVs) have also been used in humans [3]. Mimicking a natural infection, CAIVs induce mucosal immunity crucial for preventing the viral infection at the initial site of viral entry and subsequent replication of the virus in the host's respiratory tracts [4,5]. Furthermore, CAIVs generate T cell-mediated immunity directed to conserved epitopes of viral proteins, thus providing cross-protection against heterologous influenza viruses [6–8]. CAIVs are a 6:2 genetic reassortant that contains two surface genes coding the hemagglutinin (HA) and neuraminidase (NA) proteins from the circulating wild type virus, and six internal genes from a cold-adapted, attenuated donor strain. Therefore, CAIVs display attenuation due to the internal genes inherited from the donor strain, while maintaining the same antigenicity to the circulating virus. It is well-documented that, during the cold-adaptation, nucleotide mutations accumulate in the viral genes, conferring the cold-adapted (ca), temperature-sensitive (ts), and attenuated (att) phenotypes to the virus [5,9]. Genetic studies using previously developed cold-adapted strains indicated that the mutations in the polymerase (PB2, PB1, and PA) genes are major contributors to the attenuation [10–16].

We recently developed a novel cold-adapted donor strain, X-31 ca virus, by serial passages in embryonated chicken eggs at progressively lower sub-optimal temperatures from 30 °C to 24 °C [17]. The X-31 ca virus exhibited the ca, ts, and att phenotypes and also genetic stability in MDCK cells and mice [17]. This donor strain served as a general platform for CAIVs against the seasonal influenza A viruses, the highly pathogenic H5N1 influenza virus, and the 2009 pandemic A/H1N1 virus [18–21]. The X-31 ca-derived CAIVs against these strains consistently demonstrated desired levels of safety, immunogenicity, and protective efficacy, offering X-31 as a suitable substrate in the development of effective and safe CAIVs. Here we report the sequence differences between the parental X-31 virus and X-31 ca virus. We also constructed a panel of reassortant viruses, each containing one or multiple gene

* Corresponding author at: Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, 50 Yonsei-ro, Seodaemun-Gu, Seoul 120-749, South Korea. Tel.: +82 2 2123 7416; fax: +82 2 362 7265.
E-mail address: blseong@yonsei.ac.kr (B.L. Seong).
[1] CJ Healthcare, Seoul, South Korea.

http://dx.doi.org/10.1016/j.vaccine.2016.01.053
0264-410X/

segments from X-31 ca and the remaining genes from A/Puerto Rico/8/34 (H1N1) (PR8) virus to determine which of each segment of the X-31 ca virus impart the ca and ts phenotypes to the PR8 virus. These viruses were evaluated for their expression of the ca and ts phenotypes in vitro and the att phenotype in mice.

2. Metarials and methods

2.1. Cells and viruses

Human embryonic kidney 293T cells and Mardin-Darby canine kidney (MDCK) cells were maintained in minimum essential media (Hyclone) supplemented with 10% fetal bovine serum (Hyclone). The influenza viruses used in this study were propagated in 11-day-old embryonated chicken eggs for three or four days. The allantoic fluid was harvested and clarified for viral titration and stored in −80 °C until use.

2.2. Sequence analysis of X-31 and X-31 ca viruses

The cDNAs of six internal viral RNA genomes were obtained by RT-PCR. The full-length of cloned genes from X-31 and X-31 ca viruses were sequenced in both directions using oligonucleotides that are complementary to every 300–500 nucleotide intervals. The nucleotide sequences of the X-31 and X-31 ca viruses have been submitted to the GenBank database under accession number AB036777 for NS, AB036778 for M, AB036779 for NP, AB036780 for PA, AB036781 for PB1, and AB036782 for PB2 of X-31 virus; DQ874873 for PB2, DQ874874 for PB1, DQ874875 for PA, DQ874877 for NP, DQ874879 for M, and DQ874880 for NS of X-31 ca virus. The GenBank accession numbers of the internal gene segments from A/Puerto Rico/8/34 (H1N1) virus are V00603 for PB2, J02151 for PB1, V01106 for PA, V01084 for NP, V01099 for M, and J02150 for NS.

2.3. Multiple alignments of amino acid sequences of influenza A/H1N1 viruses

Full-length amino acid sequences of eight internal viral proteins, PB2, PB1, PA, NP, M1, M2, NS1, and NS2 of influenza A/H1N1 viruses were obtained from Influenza Virus Resource provided by NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The total number of amino acid sequences of each protein was 8015 for PB2, 8142 for PB1, 8372 for PA, 8570 for NP, 12,336 for M1, 11,374 for M2, 9203 for NS, and 18,642 for NS2 protein, at the time of analysis. The sequences of strains derived from laboratories were excluded in the analysis. All amino acid sequences were aligned using CLUSTALW web program (http://www.genome.jp/tools/clustalw/), and the positions where amino acid substitution occurred from X-31 to X-31 ca viruses were carefully examined for variations among the sequences aligned.

2.4. Generation of reassortant influenza viruses

All reassortant viruses used in this study were generated by the plasmid-based reverse genetics system [22]. The mixture of eight plasmids each carrying the cDNA of gene segments one to eight of A/Puerto Rico/8/34 (H1N1) or X-31 ca [23] viruses were co-transfected into human embryonic kidney 293T cells using Lipofectamine™ reagent (Invitrogen) as recommended by the manufacturer. Two days after the transfection, the supernatants were collected and clarified by centrifugation at 11,000 × *g* and examined for the generation of the virus by plaque assay on MDCK cells.

2.5. Viral plaque assay

All viral titers in this study are expressed as plaque forming units (PFU) determined by viral plaque assay. MDCK cells were seeded in 6-well plates to prepare monolayers with 100% confluency. The cells were incubated with ten-fold serial dilutions of viruses on the rocker for 1 h at room temperature. After the virus solution was removed, the cells were washed twice with PBS, after which agarose/DMEM overlay was added to the wells. After the overlay turned solid, the plates were moved to a humidified incubator and incubated at 33 °C for three or four days until the viral plaques were clearly formed. The plaques were fixed by formaldehyde solution and visualized by staining with 0.1% crystal violet solution.

2.6. Characterization of phenotypes of the viruses in vitro

The ca and ts phenotypes of the influenza viruses were analyzed by comparing their titers at various temperature conditions in embryonated chicken eggs and MDCK cells. The eggs and MDCK cells were infected with 100 plaque forming units (PFU) and 0.01 multiplicity of infection (MOI) of each virus, respectively, and were incubated for three days at various temperatures. Three days later, the allantoic fluids and supernatants were harvested and clarifed, and the viral titers were determined by plaque assay on MDCK cells. The viral titers were obtained from three independent experiments and the differences in the mean viral titers between the temperatures were shown as $\Delta\log_{10}$ and presented as the parameters for the degrees of the ca and ts phenotypes.

2.7. Animal infection

The animal study was performed in strict accordance with the Korean Food and Drug Administration guidelines. Six-week-old female BALB/c mice (OrientBio, Korea) were used for analysis of virulence of the viruses. To compare the virulence characters among the wild type A/Puerto Rico/8/34 virus and the single-gene reassortants, groups of mice were infected intranasally with each virus. The mice were anesthetized before the infection with 50 μl of virus suspension. After the infection, the mice were monitored for changes in body weights and survival rates for two weeks. Mice that lost their weight by more than 25% of the starting bodyweight were euthanized by cervical dislocation and recorded as dead.

2.8. Statistical analysis

For the survival curves, statistics were claculated using a Log-rank (Mantel–Cox) test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Sequence comparison between X-31 and X-31 ca viruses

The RNA genome sequences of the six internal genes of X-31 ca virus were compared with those of the parental X-31 virus. This comparison revealed a total of 32 nucleotide substitutions in six internal genes, which constitutes 0.31% of the total number of nucleotides sequenced (Table 1). Among them, 17 nucleotide changes resulted in amino acid substitutions in seven of eight proteins encoded by the six internal genes of X-31 ca virus, four in PB2, three in PB1, two in PA, two in NP, three in M1, two in M2, and one in NS1, which all together account for 0.5% of the total amino acid residues. The X-31 virus carries the six internal genes derived from A/Puerto Rico/8/34 (H1N1) (PR8) virus [24]. We therefore determined the type of each amino acid change through multiple sequence alignment with all the sequences of A/H1N1 influenza

Please cite this article in press as: Jang YH, et al. Genetic analysis of attenuation markers of cold-adapted X-31 influenza live vaccine donor strain. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.01.053