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Cell-cultured, live attenuated, X-31ca-based H5N1 pre-pandemic influenza vaccine

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ABSTRACT

The manufacture of influenza vaccines has traditionally depended upon a method using embryonated hen's eggs. However, concerns regarding the potential shortage of the influenza substrate in the event of a pandemic has led to the development of cell culture-derived vaccines, which offers shorter lead-in times and greater production flexibility. We examined optimal conditions for the production of reassortant X-31ca-based H5N1 cold-adapted live attenuated influenza vaccine (rH5N1ca) cultured in mammalian cell lines. During ten passages in MDCK cells, the rH5N1ca vaccine maintained cold-adapted and temperature-sensitive phenotypes, and no mutations occurred in the hemagglutinin and neuraminidase surface antigens, demonstrating genetic and phenotypic stability. Single immunization in mice with the rH5N1ca induced robust antibody responses and protected the mice against lethal challenge. Stable maintenance of attenuation phenotypes and immunogenicity of the rH5N1ca from cell-culture suggest that they can be produced as a stockpile for pandemic preparedness as an alternative to current egg-based production.

1. Introduction

Influenza viruses are responsible for annual epidemics as well as occasional pandemics. Vaccination remains the primary and most effective method to prevent and control influenza infections. Currently, the majority of influenza vaccines are produced in embryonated hen's eggs. Although well established and standardized, the egg-based vaccine production presents several disadvantages. First, the circulation of highly pathogenic avian influenza (HPAI) H5N1 in poultry farms is likely to jeopardize the timely supply of egg substrates in sufficient quantities. Moreover, wild type isolates often require adaptation in eggs to enable their growth in high yields, a process that risks contamination by avian pathogens in the eggs supply or microbial contaminants and results in antigenic changes that lower vaccine efficacy (McQuillan et al., 2009). Finally, due to potential anaphylactic responses in egg-allergic individuals, the ACIP (Advisory Committee on Immunization Practices) recommends medical supervision for individuals with a severe allergy to egg proteins before vaccination (Centers for Disease and Prevention, 2012). Therefore, there is an urgent need to develop alternative manufacturing platforms to increase the availability of influenza vaccines for seasonal and pandemic preparedness (Liu et al., 2009).

As an alternative to the egg-based process, cell culture presents several key features that render it a suitable platform. Growth of human influenza viruses in cultured cells typically results in fewer adaptive mutations than in embryonated eggs, alleviating concerns for the change of virus antigenicity (Schild et al., 1983). In addition, cell cultures can be cryopreserved, reconstituted, and scaled up at any time. For this purpose, the continuous mammalian cell lines MDCK (canine kidney cells), Vero (monkey kidney cells), and PER.C6 cells of human origin have been used successfully to produce seasonal and prepandemic influenza vaccines (Cox et al., 2009). The MDCK cell line reliably supports a high yield of influenza viruses (Hussain et al., 2010) and thus has been favored in many influenza laboratories for decades for epidemiological and diagnostic studies (Gregersen et al., 2011). The Vero cell line usually produces a lower titer of influenza viruses but has also been approved by regulatory authorities for the manufacture of viral vaccines against polio, rabies, and Japanese encephalitis viruses (Lee and Hu, 2012). As an attractive alternative to the inactivated influenza vaccines, cold-adapted live attenuated influenza vaccines (CAIVs) have been used in humans since 2003 against annual seasonal influenza virus infections. Furthermore, CAIVs against a pandemic or potential pandemic strains, such as the pdmH1N1 and HPAI H5N1, have been developed and evaluated for their safety and efficacy in animal models (Chen et al., 2011; Desheva et al., 2006; Fan et al., 2009; Jang et al., 2012; Suguitan et al., 2006; Yang et al., 2011).

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However, CAIVs have been produced predominantly by egg-based facilities, and the potential merits of using cell-culture methods have been explored only recently with A/Ann/Arbor-based vaccine strains (Liu et al., 2009).

Here we explored the potential of the cold-adapted influenza vaccine (CAIV) strain, X-31ca, as a safe and protective cell-cultured live vaccine donor strain. The X-31ca strain has been used as a master donor for the production of reassortant vaccines including the seasonal trivalent influenza vaccine (Jang et al., 2014) and the H5N1 pre-pandemic vaccine (Jang et al., 2013b), as well as the 2009 H1N1 pandemic vaccine (Jang et al., 2013a). Recent studies also showed that the genetic mutations accumulated during cold-adaptation contribute to the attenuation phenotypes of the X-31 ca, supporting its general favorability as a CAIV against many other strains as well (Jang et al., 2016; Lee et al., 2016). We established optimum conditions for the growth of the reassortant X-31 H5N1 cold-adapted (rH5N1ca) live attenuated influenza virus, including cell lines, infection doses, and trypsin concentration. We verified that the vaccine maintained the cold-adaptive (*ca*), temperature-sensitive (*ts*) and attenuated (*att*) phenotypes and was free from contaminations of microorganisms or other viruses. We compared the immune responses in mice immunized with either cell-cultured vaccines or recombinant HA protein as subunit vaccine. Both vaccines induced a substantial level of antibodies in sera, but the cell-cultured rH5N1ca produced much more pronounced secretory antibodies in the bronchoalyeolar lavage fluid (BALF) and nasal wash. Single immunization with the cell-cultured rH5N1ca provided an excellent protection against lethal challenge, demonstrating safety, immunogenicity, and protective efficacy of the vaccine.

2. Results

2.1. Optimum condition for the production of rH5N1ca influenza vaccine

To test the production of influenza vaccine in mammalian cell lines, we chose MDCK and Vero cells, the two most commonly used cell lines. First, we screened the seeding doses for maximal growth titer of the rH5N1ca vaccine. Both MDCK and Vero cells were infected with the multiplicity of infection (MOI) of 0.1, 0.01, 0.001, or 0.0001 of rH5N1ca, and were cultured with MEM containing 5 μg/ml of trypsin at 30 °C. The supernatant was harvested every 24 h and viral titer was determined using a plaque assay on MDCK cells at 33 °C. In MDCK cells, the growth rates were similar irrespective of infection doses, with the peak titer up to 7.7 $\log_{10}$ PFU/ml by 0.0001 MOI at 96 h (Fig. 1A). In Vero cells, the growth rates were dependent on the initial infection dose, whereas the final viral titers were approximately 6.0 $\log_{10}$ PFU/ml across three infection doses, 0.1, 0.01, and 0.001 MOI (Fig. 1B). Based on the results of growth rate and peak titer, 0.0001 MOI and 0.1 MOI were used for subsequent vaccine production.

An important prerequisite for the functional replication of influenza viruses in mammalian cells is the cleavage of the precursor HA_0 into HA_1 and HA_2 (Klenk et al., 1975), for which in cell cultures the protease trypsin is added. Since trypsin activity varies considerably depending on the cell type, we next examined the concentration of trypsin required for optimal growth of the rH5N1ca vaccine strain in MDCK and Vero cells. The cells were infected with rH5N1ca at a dose that produces the highest yield (0.0001 and 0.1 MOI for MDCK and Vero cells, respectively), and treated with trypsin in concentrations of 0.1, 0.2, 1, 2, 5, or 10 μg/ml in culture media. In MDCK cells, 0.1 or 0.2 μg/ml of trypsin failed to support the culture, whereas peak titer was achieved with 2 μg/ml trypsin at 72 h (Fig. 1C). The peak yield was similar across all trypsin concentrations 1 μg/ml or greater. Growth pattern in Vero cells was rather independent on trypsin concentration, and was similar across all six concentrations (Fig. 1D). Overall, these results show that viral productivity in MDCK cells is approximately 10- to 100-fold higher than in Vero cells. Based on this finding, our stock of rH5N1ca was made from the supernatant of MDCK cells infected at MOI of 0.0001 and cultured in the presence of 2 μg/ml trypsin at 30 °C, where the viral titer reached 8.03 $\log_{10}$ PFU/ml.

2.2. Immunogenicity of rH5N1ca influenza vaccine in mice

To evaluate the immunogenicity of the cell-cultured vaccine, mice were immunized with rH5N1ca with various doses (10^2, 10^3, 10^4 and 10^5 PFU). Sera were collected at 2, 3, and 4 weeks post-infection and the antibody titers were determined by the hemagglutinin inhibition (HI) assay. HI antibody titers increased in a time- and dose-dependent manner (Fig. 2A). The antibody titer induced by the lowest dose (10^2 PFU) of rH5N1ca was ≥40 after three weeks post-infection. Similar to HI titers, neutralization (NT) titers also showed time- and dose-dependence (Fig. 2B). In parallel, another group of mice was immunized intraperitoneally with the HA protein at various doses (1, 5 and 10 μg of HA protein with alum) or with PBS, then subsequently boosted with the same dose two weeks after the first vaccination. The HA protein also induced time- and dose-dependent sera antibodies (Fig. 2C), as represented by both HI and NT titers (Fig. 2D). These results demonstrate that a single immunization with the cell-cultured rH5N1ca induces robust neutralizing antibodies equivalent to those of a boost immunization with the recombinant HA.

BALF and nasal wash from mice either immunized with a single dose of cell-cultured rH5N1ca or boost immunized with the HA protein were collected and examined for their mucosal secretory IgA antibodies. As analyzed by the ELISA assay, the IgA antibodies increased in a time- and dose-dependent manner in both BALF and nasal wash. However, in contrast to rH5N1ca, HA protein barely induced any IgA antibodies in both the BALF (Fig. 3A and C) and nasal wash (Fig. 3B and D). These results confirm and further support the robust mucosal immune response associated with live vaccines (Barria et al., 2013; Boyce et al., 1999), which is usually much stronger than those induced by recombinant vaccine.

2.3. Protective efficacy against wild type influenza virus

To confirm the protective efficacy of the vaccine, mice were challenged with 5 MLD_{50} of the wild type influenza virus, A/aquatic bird/Korea/w81/05 (H5N2), four weeks after vaccination with the cell-cultured rH5N1ca. Without exhibiting any visible clinical symptoms (Fig. 4A), all mice survived the challenge, even at the lowest vaccination dose (10^2 PFU) (Fig. 4B). In contrast, mice immunized with the HA recombinant antigen showed clinical signs of 10–15% weight loss within seven days of the challenge, and depending on the vaccination dose even after boosted immunization (Fig. 4C and D). These results show that the cell-cultured rH5N1ca LAIV provides more efficient protection than the recombinant HA antigen.

3. Discussion

As an alternative to the traditional egg-based production, cell-culture methods are becoming the favored influenza vaccine platform (Barrett et al., 2010). While other technologies obviating the need for virus cultures, such as baculovirus derived recombinant proteins, virus-like particles, DNA vaccine, or vectored vaccine using adenovirus, alphavirus, NDV, VSV, and poxvirus, are also under evaluation (Audsley and Tannock, 2008; Ulmer et al., 2006). MDCK, Vero and PER.C6 cell lines are widely used for manufacturing influenza vaccines at high yields. For instance, the MDCK-derived inactivated influenza vaccine (Optaflu) is currently licensed in Europe and was shown to be comparable with egg-derived TIV in terms of safety, immunogenicity, and efficacy in children and adults up to 50 years of age (Groth et al., 2009; Reisinger et al., 2009; Szymczakiewicz-Multanowska et al., 2009; Vesikari et al., 2012).