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Monoclonal antibody against N2 neuraminidase of cold adapted A/Leningrad/134/17/57 (H2N2) enables efficient generation of live attenuated influenza vaccines

Svetlana Shcherbik[a], Paul Carney[a], Nicholas Pearce[b], James Stevens[a], Vivien G. Dugan[a], David E. Wentworth[a], Tatiana Bousse[a,*]

[a] Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, United States
[b] Battelle, Atlanta, GA 30329, United States

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ABSTRACT

Cold adapted influenza virus A/Leningrad/134/17/57 (H2N2) is a reliable master donor virus (Len/17-MDV) for preparing live attenuated influenza vaccines (LAIV). LAIVs are 6:2 reasortants that contain 6 segments of Len/17-MDV and the hemagglutinin (HA) and neuraminidase (NA) of contemporary circulating influenza A viruses. The problem with the classical reassortment procedure used to generate LAIVs is that there is limited selection pressure against NA of the Len/17-MDV resulting in 7:1 reassortants with desired HA only, which are not suitable LAIVs. The monoclonal antibodies (mAb) directed against the N2 of Len/17-MDV were generated. 10C4–8E7 mAb inhibits cell-to-cell spread of viruses containing the Len/17-MDV N2, but not viruses with the related N2 from contemporary H3N2 viruses. 10C4–8E7 antibody specifically inhibited the Len/17-MDV replication *in vitro* and *in ovo* but didn't inhibit replication of H3N2 or H1N1pdm09 reassortants. Our data demonstrate that addition of 10C4–8E7 in the classical reassortment improves efficiency of LAIV production.

1. Introduction

Influenza A virus causes substantial human disease and serious economic burdens worldwide (Gordon and Reingold, 2018; WHO, 2018). Vaccination is the most effective way to prevent influenza infections and reduce the disease severity (Houser and Subbarao, 2015; Osterholm et al., 2012). There are two major types of influenza vaccines licensed for human use: inactivated influenza vaccine, and live attenuated influenza vaccine (LAIV), which is administered intranasally. LAIVs have proven to be an effective public health tool in Russia (Aleksandrova, 1977b; Rudenko et al., 1996), USA (Ambrose et al., 2008; Bandell et al., 2011) and Europe (Bandell and Simoes, 2015). LAIV seed viruses are reassortants containing surface antigens (hemagglutinin, HA and neuraminidase, NA) seasonal influenza viruses on the backbone (include M, NA, NP, PA, PB1 and PB2 gene segments) of cold adapted master donor virus (MDV). MDV provides temperature sensitive, cold adapted and attenuated phenotype through cooperative multi-gene mutations, typically in PA, PB1 and PB2 polymerase gene segments. These genetic/phenotypic characteristics enable the LAIV reassortants to replicate efficiently at lower temperatures at the upper respiratory tract, restrict replication at the lower respiratory tract and attenuate the virus (Maassab and Bryant, 1999; Murphy and Coelingh, 2002). LAIVs confer protection by inducing neutralizing anti-HA antibodies (Belshe et al., 2000; Cox et al., 2004; Gerhard, 2001) and mucosal cellular responses. LAIVs also provides heterosubtypic protection through cross-reactive T-cell responses to conserved epitopes (Epstein and Price, 2010; Haaheim and Katz, 2011; He et al., 2006; Hoft et al., 2011).

LAIVs were developed and have been in use in Russia since 1960, they were licensed for use in North America in 2003 (Flumist), Europe has licensed LAIV (Fluenz) in 2013. Through cooperation with WHO, the production and use of LAIVs on backbone of A/Leningrad/134/17/57 (H2N2) master donor virus (Len/17-MDV) has been expanded internationally (Neuzil et al., 2012; Rudenko et al., 2011, 2016). For the preparation of seasonal LAIV seed viruses from Len/17-MDV, WHO Expert Committee on Biological Standardization recommends the conventional reassortment procedure prepared in eggs (WHO, 2013).

Rapid and efficient selection of reassortants with 6:2 genome compositions is essential for the tight schedule of vaccine production. The protocol for generating LAIVs based on Len/17-MDV was originally

* Corresponding author.
E-mail address: gsx8@cdc.gov (T. Bousse).

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developed by Institute of Experimental Medicine, Russia (Aleksandrova, 1977a; Wareing et al., 2002). The protocol includes 2 selective passages and 1 biological cloning by limiting dilution step performed under selective conditions, in the presence of anti-serum against Len/17-MDV and at low temperature (25 °C) to allow the correct 6:2 reassortants to dominate the pool. However, even under such robust selective pressure, variability of the gene segments donated by the cold-adapted donor in the vaccine reassortants was often observed. It was reported that less than 15% of LAIV reassortants derived from post- year 2000 viruses possessed the desired *wt* NA (Kiseleva et al., 2014). It has been reported that specific antibodies against HA and NA enhance incorporation of the HA and NA segments from seasonal *wt* viruses into vaccine reassortants of viruses with a A/Puerto Rico/8/1934 (H1N1) backbone (Stohr et al., 2012). To determine if addition of mAb against Len/17-MDV NA increase the efficiency of obtaining desired LAIV reassortants, we developed a new anti-neuraminidase mAb using purified Len/17-MDV NA. We found the mAb efficiently and specifically inhibit viruses containing Len/17-MDV NA (N2 subtype) both *in vitro* and *in ovo* and decreases the amount of N2 Len/17-MDV gene segment in the reassortment pool enabling the reassortants with desired NA to prevail in selection steps for production of 6:2 reassortant viruses.

2. Materials and methods

2.1. Viruses, cells, antibodies

A/Leningrad/134/17/57 (H2N2) (Len/17-MDV) was provided by BioDiem (Australia). LAIV viruses A/Texas/50/2012(H3N2)-CDC-LV4A (LV4A), A/South Africa/3646/2013(H1N1pdm09)-CDC-LV14A (LV14A), A/Hong Kong/4801/2014(H3N2) CDC-LV15A (LV15A) were generated using classical reassortment with Len/17-MDV in specific pathogen free (SPF) eggs (Charles River Laboratories Inc., USA). The viruses used in plaque assay, RG-1 (7:1 reassortant) and RG-2 (6:2 reassortant) possess HA only or HA and NA of A/Texas/50/2012 origin, respectively and the rest of the genes from Len/17-MDV. RG viruses were generated by reverse genetics as described in (Shcherbik et al., 2015). 293T, human embryonic kidney (HEK) and Madin-Darby canine kidney (MDCK London) cells were maintained in DMEM High Glucose (Life Technologies, Carlsbad, CA) supplemented with 10% (for HEK) or 5% (for MDCK) fetal bovine serum (Life Technologies), 1 × GlutaMAX (Life Technologies) and 40 μg/ml Neomycin (Sigma-Aldrich, St. Louis, MO). Ferret polyclonal antisera to recombinant HA protein of A/Japan/305/1957 (H2N2) was obtained from the International Reagent Resource (IRR, Manassas, VA).

2.2. Expression of recombinant NA proteins

Recombinant His-tagged NA from A/Anhui/1/3013 (H7N9) (recN9-NA) and A/Perth/16/2009 (H3N2) (recN2-NA) were produced in a baculovirus expression system and purified as previously described (Mishin et al., 2014; Wilson et al., 2016). The recN2-NA gene encoding residues 80–470 of Len/17-MDV were synthesized (GenScript USA Inc.) as a codon-optimized gene for insect cell expression and were subcloned into the baculovirus transfer vector pAcGP67B (BD Biosciences). The recombinant NA (recNA) protein contains an N-terminal His-tag, a tetramerization domain from the human vasodilator-stimulated phosphoprotein (Kuhnel et al., 2004) and a thrombin cleavage site (Xu et al., 2008). Secreted proteins were recovered from the culture supernatant and purified by metal affinity chromatography and size-exclusion chromatography.

2.3. Generation of mouse hybridomas secreting NA-specific mAbs

Mouse mAbs were generated using Len/17-MDV recN2-NA protein as antigen for immunization using traditional hybridoma technology at Pierce Custom Services (Thermo Fisher Scientific). Hybridomas were screened by ELISA for reactivity to Len/17-MDV recN2-NA as positive antigen and recN2-NA of A/Perth/16/2009 (H3N2) and recN9-NA of A/Anhui/1/2013 (H7N9) as negative antigens. The selected hybridomas were further subjected to screening analysis by ELISA using purified and concentrated Len/17-MDV. Positive hybridomas which best reacted to Len/17-MDV was subcloned and screened again by ELISA to purified Len/17-MDV. Antibodies were purified from tissue culture supernatants by rProtein A chromatography and supplied as 1.22 mg/ml antibody in sterile PBS with no preservative.

2.4. Enzyme-linked immunosorbent assay (ELISA) screening

Ninety-six-well, flat-bottom, nonsterile Immulon 2 HB plates (Thermo Scientific) were coated overnight with either 5 μg/ml (100 μl/well) of purified virus or 2 μg/ml (100 μl/well) of purified protein in PBS at 4 °C. The coating buffer was discarded, and the plates were blocked with 1% BSA in PBS containing 0.1% Tween 20 (TPBS; 100 μl/well) for 1 h at room temperature. For hybridoma screening analysis, 100 μl of undiluted supernatant from each hybridoma clone was added directly to wells as the primary antibody step. In the case of endpoint-titer ELISA, mAbs were added at a starting concentration of 10 μg/ml and serially diluted 1:2 in TPBS containing 1% BSA (TPBS-BSA) so that the final volume in each well was 100 μl. The plates were then incubated for 1 h at room temperature. After three washes with TPBS (200 μl/well for each wash), the plates were incubated for another hour at room temperature with 100 μl/well of secondary horseradish peroxidase (HRP)-labeled anti-mouse antibody (Abcam) diluted in TPBS-BSA. Plates were subsequently washed three times with TPBS and the reaction was developed using 100 μl per well of 0.4 mg/ml of o-phenylenediamine dihydrochloride (OPD) solution (Sigma). After a 10 min incubation at room temperature the reaction was stopped with 3 M HCl (50 μl/well), and an optical density at 490 nm was read with a Spectra Max M5 plate reader (Molecular Devices) using Soft Max Pro software (version 6.4).

2.5. Effect of mAb on the virus growth in MDCK cells and in eggs

Confluent cell monolayers were infected with viruses of interest at multiplicity of infection (MOI) of 10 and incubated at 33 °C for 1 h. After washing cells with PBS twice, MEM containing 1 μg/ml TPCK-trypsin and hybridoma supernatants (diluted 1:100) or purified mAb or PBS (control) were added to the cells. Cells were incubated for 3 days at 33 °C in 5% of CO_2 atmosphere. Supernatants of infected cells were collected and virus titers were determined as 50% tissue culture infectious dose per milliliter ($TCID_{50}$/ml) in confluent MDCK cells in 96-well microtiter plates (Corning).

For the analysis on the effect of mAb in virus growth in eggs, 10-days old embryonated hen eggs were infected with 10^3 EID_{50} of Len/17-MDV or LV4A. One hour after infection, mAb in specified amount were injected to each egg. RG-1 and RG-2 viruses were also premixed with 2 μg of 10C4–8E7 mAb before infection. The mixture was then injected to eggs, and allantoic fluids were collected at 24 h after infection. Virus titers were determined using eggs and expressed as 50% egg infectious dose per milliliter (EID_{50}/ml).

2.6. Plaque reduction assay

In pre-infection treatment studies, 50–80 plaque forming units (PFU) of RG-1 or RG-2 virus was incubated for 30 min with mAb at the indicated concentration followed by infection to a monolayer of MDCK cells in six-well plates. After 1 h incubation, cells were washed twice with PBS and overlaid with agarose supplemented with 1 μg/ml of TPCK- trypsin without antibody. On day 3 post-infection, the agarose overlay was removed, cells were fixed with 70% ethanol and plaques were visualized by staining with crystal violet. In post-infection