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Immunization with a low dose of hemagglutinin-encoding plasmid protects against 2009 H1N1 pandemic influenza virus in mice

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A B S T R A C T

A vaccine against the novel pandemic influenza virus (2009 H1N1) is available, but several problems in preparation of vaccines against the new emerging influenza viruses need to be overcome. DNA vaccines represent a novel and powerful alternative to conventional vaccine approaches. To evaluate the ability of a DNA vaccine encoding the hemagglutinin (HA) of 2009 H1N1 to generate humoral responses and protective immunity, BALB/c mice were immunized with various doses of 2009 H1N1 HA-encoding plasmid and anti-HA total IgG, hemagglutination inhibition antibodies and neutralizing antibodies were assayed. The total IgG titers against HA correlated positively with the doses of DNA vaccine, but immunization with either a low dose (10 μg) or a higher dose (25–200 μg) of HA plasmid resulted in similar titers of hemagglutination inhibition and neutralizing antibodies, following a single booster. Further, 10 μg plasmid conferred effective protection against lethal virus challenge. These results suggested that the DNA vaccine encoding the HA of 2009 H1N1 virus is highly effective for inducing neutralizing antibodies and protective immunity. DNA vaccines are a promising new strategy for the rapid development of efficient vaccines to control new emerging pandemic influenza viruses.

1. Introduction

The outbreak of a novel, epidemic, 2009 H1N1 influenza virus (2009 H1N1) in April 2009 attracted global attention due to its initial high morbidity and rapid spread. This novel virus arose from genomic segmental reassortment and the majority of the population were immunologically naïve to this new virus and therefore susceptible to infection, especially adolescents and children (Dawood et al., 2009; Hancock et al., 2009). The seasonal influenza vaccines widely used offered little protection against this novel virus (Garten et al., 2009; Pascua et al., 2009). A vaccine against 2009 H1N1 has been developed; however, several problems relating to vaccine preparation remain to be addressed. These include shortening the time from vaccine strain selection to vaccination, increasing the production capacity and broadening the vaccine-induced immune responses (Huang, 2009; Palache, 2008). Furthermore, the global manufacturing capacity for influenza vaccines is limited, inadequate and not readily augmented (Dawood et al., 2009).

DNA vaccines represent a novel and powerful alternative to conventional vaccines, with the advantages of being noninfectious, extremely stable and inexpensive (Kim and Jacob, 2009; Liu, 2003). Most importantly, DNA vaccines can be designed easily and rapidly based on the genome of an emerging pathogen or bioterrorism agent (Kim and Jacob, 2009; McDonnell and Askari, 1996). Hemagglutinin (HA) is the key surface glycoprotein that mediates influenza virus infection, and antibodies against HA generally neutralize viral infectivity, presumably by interfering either with virus attachment to sialic acid receptors on the host cell surface or with the subsequent process of fusion between viral and endosomal membranes (Couceiro et al., 1993; Johansson et al., 1989; Johansson and Kilbourne, 1993). DNA encoding HA from different influenza virus strains has been shown to induce high titers of antibodies in species ranging from mice to monkeys (Lalor et al., 2008; Ljungberg

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et al., 2002; McCluskie et al., 1999; Olsen, 2000; Webster et al., 1994; Zheng et al., 2009). In this study, the ability of a DNA vaccine expressing the HA from 2009 H1N1 to generate humoral responses in mice was evaluated. The results showed that although the total IgG against 2009 H1N1 HA correlated positively with the dose of DNA used, both the hemagglutination inhibition antibodies and neutralizing antibodies induced by immunization with between 10 μg and 200 μg of HA plasmid, reached similar titers after a single booster. Furthermore, immunization with 10 μg HA plasmid protected mice against lethal virus challenge. These findings suggest that a low dosage of 2009 H1N1 HA DNA vaccine can induce sufficient protective immunity in mice.

2. Materials and methods

2.1. Cells

Human alveolar epithelial type-II A549 cells, Madin–Darby canine kidney (MDCK) cells and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS).

2.2. Plasmids

The cDNA fragments encoding the full-length HA and neuraminidase (NA) proteins of the 2009 pandemic H1N1 influenza virus A/California/05/2009 were synthesized and inserted into the expression vector pVRC to produce recombinants expressing HA and NA, as described previously (Zhang et al., 2009, 2010).

2.3. Assay of HA expression

293T cells were transfected with HA expression plasmids or empty vector using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent. 48 h later, the cells were fixed with cold methanol, and then probed with sera from a 20-year-old female student in the laboratory who was inoculated with the 2009 H1N1 influenza vaccine one month prior to blood collection. A serum sample obtained prior to vaccination was used as a negative control. The secondary antibody was FITC-conjugated goat anti-human total IgG (Jackson ImmunoResearch, West Grove, PA, USA). Cell nuclei were stained with DAPI. Fluorescently labeled cells were visualized by fluorescence microscopy. This experiment was approved by the Ethical Committee of the Second Military Medical University, China and the informed consent of the student was obtained.

2.4. DNA immunization

The HA-encoding plasmid and empty vector were propagated in *Escherichia coli* strain XL1-blue and purified using Qiagen Giga columns (Hilden, Germany), according to the manufacturer's instructions. Seven-week-old female BALB/c mice were purchased from SIPPR-BK Experimental Animal Co. Ltd. (Shanghai, China). These mice were injected with various dosages (10, 25, 50, 100, 200 μg) of 2009 H1N1 HA-encoding plasmid, in a total volume 100 μl, into both tibialis anterior muscles. Control mice were injected with 200 μg of empty vector. All of the animals received booster immunizations after 2 and 4 weeks, respectively. Sera were collected by retro-orbital puncture at two-week intervals after primary vaccination, inactivated at 56 °C for 30 min, and then kept at −80 °C prior to use. The procedures used in the handling and care of the animals were approved by the Animal Ethical Committee of the Second Military Medical University, Shanghai, China.

2.5. ELISA

Antibody titers were determined using endpoint ELISAs as described previously (Wang et al., 2008). ELISA Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 100 μl of 10 μg/ml *Galanthus nivalis* lectin (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) and incubated overnight at 4 °C. Then, plates were washed in PBS containing 0.05% Tween-20 (PBST), and non-specific binding sites were saturated with blocking buffer [PBST containing 3% bovine serum albumin (BSA)]. HA expressing HEK 293T cells were lysed, and 100 μl of lysate was added to each well. The plates were incubated for 2 h at room temperature (RT) and then washed extensively. Two-fold serial dilutions of each mouse serum sample were added to the wells (100 μl/well), and the plates were incubated for 40 min at RT. The plates were then washed and horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1:2000) was added to the wells. After a 40-min incubation at RT, the plates were washed and the color was developed using 3,3′,5,5′-tetramethylbenzidine as the substrate. The absorbance at 450 and 630 nm was measured. The cutoff value was established as the two-fold mean signal of the serum from control mice immunized with vector alone.

2.6. Preparation of 2009 H1N1 virus

Wild-type 2009 H1N1 virus was isolated from a confirmed case, grown and propagated in MDCK cells, and stored at −80 °C. All experiments with this isolate were performed in biosafety level 3 (BSL-3) laboratories at the Center of Disease Control and Prevention of Fujian Province, China. The Reed–Muench formula was used to calculate the 50% cell culture-infected dose ($TCID_{50}$) (Reed and Muench, 1938).

2.7. Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assays were performed according to standard methods (Kendal et al., 1982). Briefly, 25 μl of 2009 H1N1 virus with an HA titer of 4 HA units was mixed with 25 μl of two-fold dilutions of the receptor destroying enzyme (RDE)-treated serum in PBS in V-bottom 96-well plates. After 30-min incubation at RT, 50 μl of 0.5% chicken erythrocytes were added to each mixture and incubated at 4 °C until a positive hemagglutination reaction developed in the non-serum-containing control wells. The inhibition of hemagglutination at the highest serum dilution was considered as the HI titer of the serum.

2.8. Micro-neutralization assay

Confluent MDCK cells in a 96-well plate (1×10^4 cells/well) were infected with 1×10^3 $TCID_{50}$ viruses per well in medium containing 2 mg/L TPCK-trypsin and serially diluted immune serum, and then incubated for 2 h at 35 °C. After washing, the cells were cultured in medium without TPCK-trypsin to prevent re-entry of viruses. At 24 h post-infection, the cells were detached using 2 mM EDTA in PBS, washed in PBS buffer containing 2% FBS and 0.05% NaN_3, and incubated with anti-sera from the vaccinated subject (diluted 1:100 in the same buffer). Samples were incubated for 40 min at RT. Cells were then washed twice with PBS and incubated with FITC-conjugated rabbit anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA). Fluorescent cells were quantified by flow cytometry using a Cell Lab Quanta SC instrument (Beckman Coulter), and the number of focus-forming units per milliliter was calculated. The titers of immune serum that caused 90% inhibition of infectivity (IC_{90}) were determined.