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H5N1 influenza marker vaccine for serological differentiation between vaccinated and infected chickens

Chengjun Li, Jihui Ping, Bo Jing, Guohua Deng, Yongping Jiang, Yanbing Li, Guobin Tian, Kangzhen Yu, Zhigao Bu, Hualan Chen *

Harbin Veterinary Research Institute, CAAS, 427 Maduan Street, Harbin, Heilongjiang 150001, People's Republic of China

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ABSTRACT

Using plasmid-based reverse genetics, we generated a molecularly altered virus, H5N1/PR8-5B19, containing modified HA and NA genes from A/Goose/Guangdong/1/96 (GS/GD/1/96). In the H5N1/PR8-5B19 virus, the HA cleavage site was modified to resemble that of low-pathogenic avian strains and a portion of the NA stalk region was replaced by the immunodominant 5B19 epitope of the S2 glycoprotein of murine hepatitis virus (MHV). H5N1/PR8-5B19 is not lethal to embryonated eggs or chickens. Chickens immunized with the H5N1/PR8-5B19 inactivated vaccine produced high levels of HI antibody and a measurable antibody response against the MHV 5B19 epitope, and were fully protected against subsequent challenge with different highly pathogenic H5N1 avian influenza viruses. H5N1/PR8-5B19 is therefore an attractive marker vaccine candidate, eliciting a strong, protective antibody response and enabling serological discrimination between vaccinated and wild-type virus-infected chickens.

Highly pathogenic avian influenza (HPAI) is an extremely contagious and aggressive disease that causes rapid systemic illness and deaths in susceptible birds. A systemic program of vaccination has been employed to eradicate HPAI in Mexico, Pakistan, and Italy [1–3], in China and Vietnam [4]. The use of vaccination is gaining support as cost-conscious and effective alternative to culling practices for the control of H5N1 influenza in poultry.

The current commercially used inactivated vaccine generated by reverse genetics is safe and effective, providing complete protection from H5N1 highly pathogenic influenza viruses [4]. However, this vaccine does not allow for the serological distinction between vaccinated chickens and animals that have been infected by wild-type viruses. A marker vaccine is defined as one which can be used in conjunction with a diagnostic test to differentiate a vaccinated animal from a carried animal [5]. A genetically marked H5N1 influenza vaccine which could readily be distinguished from wild-type strains would therefore be of great value in the eradication plan, allowing for vaccination programs that would not interfere with the serological surveillance of circulating influenza viruses in the wild.

In the present study, we generated an attenuated H5N1 influenza marker vaccine seed virus, denoted H5N1/PR8-5B19, which derives its internal genes from A/Puerto Rico/8/34 (PR8) and modified H5 HA and N1 NA genes from the first highly pathogenic avian influenza virus isolated in China, GS/GD/1/96. H5N1/PR8-5B19 expresses the foreign 5B19 epitope of the S2 glycoprotein of the murine hepatitis virus (MHV). The inactivated vaccine produced from this virus elicited strong antibody responses to influenza virus and to the MHV 5B19 epitope, and provided complete protection to H5N1 HPAIV challenge. H5N1/PR8-5B19 is the first H5N1 vaccine candidate with the desired properties of efficient replication in eggs, safety of use in birds, and the ability to serologically discriminate between infected and vaccinated chickens.

Materials and methods

Modified HA and NA plasmid constructions. We deleted the multiple basic amino acids at the cleavage site of the HA gene of A/Goose/Guangdong/1/96 (GS/GD/1/96) as described previously [6,7]. The modified HA gene was then subcloned into SapI-digested pBD as described previously [8], yielding pBD-MutHA. The modified HA was confirmed by sequence analysis.

The NA gene of GS/GD/1/96 was amplified by RT-PCR from viral RNA and cloned into SapI-digested pBD. As shown in Fig. 1A, to replace the 20 amino acids between position 49 and 68 in the NA stalk region of GS/GD/1/96 with the immunodominant 5B19 epitope of S2 glycoprotein of the murine hepatitis virus, SPLLGCIGSTCAEDGN, the modified NA cDNA construct, pBD-NA-5B19, was prepared by PCR using pBD-NA as the template and two primer pairs, F1/R1, (5′-CTAGCCTCGAGAATTCACGCGTGGTAC-3′/5′-GCCGATGCATCCCAGCAGGGGACTTGGTTCAGCTTGGTGTTGAT TCCCTG-3′) and F2/R2, (5′-GGGATGCATCGGCTCCACCTGTGCCGAG GACGGCAACATCAGCAATACCAATTTTCTTACTG-3′/5′-CTGCTCGAAG

* Corresponding author. Fax: +86 451 82733132.
E-mail address: hlchen1@yahoo.com (H. Chen).

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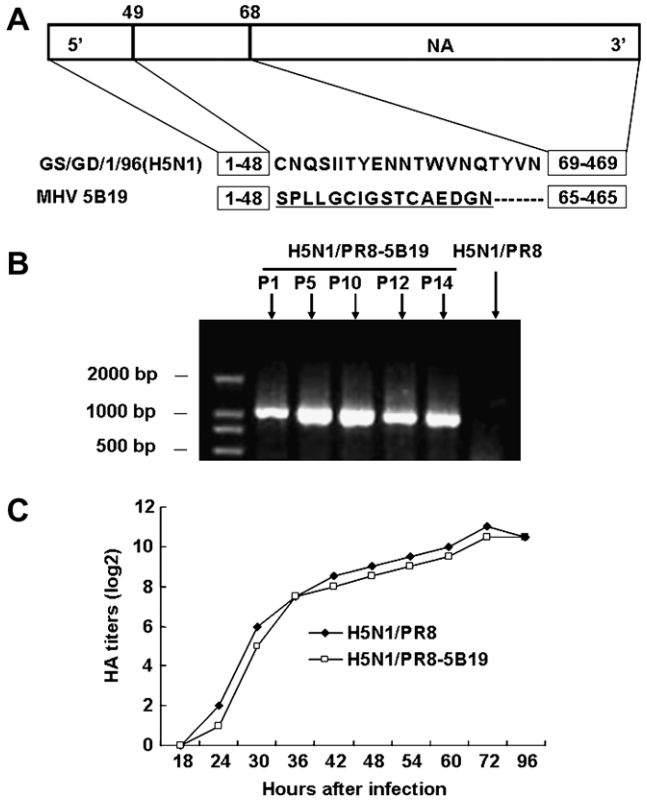

Fig. 1. Characterization of the recombinant H5N1/PR8-5B19 virus. (A) Schematic diagram of the recombinant NA-5B19 gene of GS/GD/1/96 (H5N1) in which the 20 amino acids between positions 49 and 68 has been replaced with the 16 amino acids of the 5B19 epitope of the S2 glycoprotein of the murine hepatitis virus. (B) RT-PCR analysis of the NA-5B19 gene of rescued H5N1/PR8-5B19 virus after different passage in embryonated eggs. RT-PCR amplification was carried out on allantoic fluid infected with H5N1/PR8-5B19 virus (passages 1, 5, 10, 12, and 14) or H5N1/PR8 virus with primer pair Marker 174U24/Marker 1200L25. The expected length of the RT-PCR product is 1051 bp. On the left the positions and sizes of marker DNA fragments are indicated. (C) Replication kinetics of rescued H5N1 viruses.

CGGCCGCCCGGGTCGACT-3′). The resulting 306-nt fragment of F1-R1 was digested with EcoRI and NsiI, and the resulting 1553-nt PCR fragment of F2-R2 was digested with NsiI and NotI. The fragments were then ligated into EcoRI and NotI sites of pBD in a trimolecular ligation reaction, yielding the pBD-NA-5B19. The presence of the introduced 5B19 sequence was confirmed by sequencing.

Rescue of reassortant viruses. The H5N1/PR8 and H5N1/PR8-5B19 reassortant virus was generated by plasmid-based reverse genetics as described previously [9–11], using plasmids bearing the modified HA and NA gene of the GS/GD/1/96 in the background of PR8 internal genes.

RT-PCR and sequence analysis. The H5N1/PR8-5B19 virus was serially passaged 14 times in embryonated SPF eggs to determine the stability of the introduced modifications in the virus. The allantoic fluid of passages 1, 5, 10, 12, and 14 was collected for RNA isolation with RNeasy Mini Kit (QIAGEN, Valencia, CA) and was reverse-transcribed with a 12-nucleotide primer (5′-AGCAAAAG-CAGG-3′) with the M-MLV reverse transcriptase (Invitrogen) for 1 h at 37 °C. PCR amplification was performed by using NA-5B19 specific primer pair (primer sequences available on request). The H5N1/PR8 virus was also subjected to the same RT-PCR reaction as a negative control.

Neuraminidase assay. The H5N1/PR8-5B19 and H5N1/PR8 viruses were diluted in PBS to $10^{8.0}$ EID_{50}/ml. Serial dilutions in PBS were then prepared of both viruses in 0.5 $\log_{10}$ (1:3.16) steps: undiluted, $10^{-0.5}$, 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3}, $10^{-3.5}$. NA activity was measured by a colorimetric enzymatic assay that is quantified by a spectrophotometer at 549 nm. An NA activity curve was constructed by plotting NA activity (OD value at 549 nm) versus the virus dilution. One unit of NA activity was defined as that dilution of virus that gave an OD reading at 549 nm of 0.5 under standard conditions.

Replication of H5N1/PR8-5B19 in embryonated eggs. To investigate the possible effects of the introduced 5B19 epitope in the NA gene on the efficiency of virus propagation, 10-day-old embryonated SPF chicken eggs were inoculated with $10^{3.0}$ EID_{50}/egg of H5N1/PR8-5B19 or H5N1/PR8 virus and incubated at 37 °C. The allantoic fluid from each group of 10 eggs was collected at 18, 24, 30, 36, 42, 48, 54, 60, 72, and 96 h after infection, and the average HA titers were determined with 0.5% chicken erythrocytes. The inoculated eggs were candled to check for death at each time point to determine the pathogenicity of the viruses on the embryo.

Pathotyping in chickens. To determine the pathogenicity of the H5N1/PR8-5B19 virus, the intravenous pathogenicity index (IVPI) was tested according to the recommendation of the Office International Des Épizooties [12]. Ten additional chickens were inoculated intranasally (i.n.) with 0.1 ml $10^{6.0}$ EID_{50} of H5N1/PR8-5B19 virus. Tracheal and cloacal swabs were collected for virus isolation on day 1, 3, 5, 7, and 9 post inoculation (p.i.). On day 21 p.i., all chickens were euthanized and bled, and sera were tested for evidence of seroconversion by HI test.

Immunization of chickens with the H5N1/PR8-5B19 inactivated vaccine. Formalin-inactivated vaccines were prepared from the H5N1/PR8 and H5N1/PR8-5B19 viruses as described previously [13]. To determine the presence of antibodies specific for the introduced 5B19 epitope, 10 SPF chickens at the age of 6 weeks were immunized intramuscularly with 0.2 ml of H5N1/PR8-5B19 inactivated vaccine containing 1.3 μg HA protein. Serum samples were collected weekly until 12 weeks after the immunization. In a second experiment, 10 SPF chickens immunized with the H5N1/PR8-5B19 inactivated vaccine were given a booster dose at 2 weeks after the first vaccination. Serum samples were collected weekly until 12 weeks after the first immunization. As a control, two groups of ten 6-week-old chickens were vaccinated with H5N1/PR8 inactivated vaccine or inoculated intravenously with 10^6 EID_{50} of H5N1/PR8 virus, respectively. Serum samples were collected 5 weeks after the immunization or infection. Serum samples were tested by HI test to determine the level of seroconversion and by enzyme-linked immunosorbent assay (ELISA) to measure the specific antibody response against the 5B19 epitope.

ELISA. To determine whether chickens immunized with H5N1/PR8-5B19 inactivated vaccine had developed antibodies specific for the introduced MHV 5B19 epitope, the serum samples were analyzed by ELISA with the procedures as previously described [14]. Maxisorb microtiter plates (Nunc, Rochester, NY) were coated overnight at 4 °C with 1 μg of the 16-mer synthetic peptide of the MHV 5B19 epitope ($_{846}$SPLLGCIGSTCAEDGN$_{861}$) in coating buffer (Carbonate-buffer, pH 9.6). Unbound antigen was removed by washing with PBST solution (PBS, Tween 20). The wells were then blocked with 5% glutin (Sigma, St. Louis, MO). Serum samples diluted 100-fold in PBS were added to the blocked wells and incubated for 90 min at 37 °C in a humidified atmosphere. After three washes with PBST solution, 100 μl horseradish peroxidase-labeled donkey anti-chicken immunoglobulin (Jackson ImmunoResearch Labs, West Grove, PA) was added and incubated 90 min at 37 °C. The wells were washed and the antigen–antibody complexes were detected by the addition of 100 μl of *O*-phenylendiamine dihydrochloride (OPD) substrate. Color development was allowed to proceed for 10 min at ambient temperature and was stopped by adding 50 μl of 2 M sulfuric acid. Optical densities (OD) were measured with a microplate reader at 490 nm. The cutoff value was set three standard deviations above the average OD values of the ser-