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Construction and Immunogenicity Study of a Veterinary Recombinant Influenza A (H1N1) Inactivated Vaccine

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Abstract: The recombinant virus with human H1N1 influenza virus HA, *NA* gene fragments and six gene fragments of the PR8 strain was successfully rescued and prepared in an recombinant (H1N1) influenza inactivated vaccine as an antigen. Researchers chose BALB/c mice as animal models to evaluate the vaccine's immunity. The HI test results show that the vaccine can induce a high level HI antibody for human influenza A virus (H1N1) and swine influenza virus (H1N1) in mice. The HI antibody can be induced 1 week after immunization. Researchers monitored for 8 weeks after the second immunization and high antibody levels remained. The mouse challenge protection test showed that the vaccine can effectively protect the attack of the human influenza A (H1N1) and swine influenza virus (H1N1). It provides technical reserves for preventing a pandemic H1N1 influenza in the future in swine or other mammals.

Key words: Influenza A (H1N1) virus, veterinary inactivated vaccine, recombinant, PR8 strain, immunity

INTRODUCTION

The new H1N1 influenza virus is different from the seasonal H1N1 influenza virus. The new H1N1 influenza virus contains DNA gene fragments of avian, swine and human flu (Chang *et al*., 2009; Garten *et al*., 2009; Smith *et al*., 2009). Researchers at Hong Kong University isolated four new H1N1 influenza viruses from swine in 2009-2010. It suggests that the virus is transmitted from person and then returns to the swine. At the same time, nearly 20 countries including the United States, Canada, Australia, Singapore and Cameroon found the new H1N1 influenza virus in swine. The new H1N1 influenza virus has undergone genetic recombination into a new strain (Njabo *et al*., 2012; Vijaykrishna *et al*., 2010). It shows that the gene recombination may occur within a pandemic virus and a swine influenza virus and a new influenza virus may appear. The epidemic of H1N1 influenza virus has variation characteristics of the antigenic and genetic characteristics, a unique genome structure is effectively spread from human to human and lacks effective monitoring of the swine flu virus (Ma *et al*., 2008). Therefore, the influenza virus potentially caused by the outbreak may be hidden in swine and cannot be detected in a timely manner by forecasting and early warning (Mukherjee *et al*., 2011). In this study, recombinant viruses with the human H1N1 influenza virus HA and *NA* gene fragments and six gene fragments of the PR8 strain were prepared as an inactivated vaccine virus. The success of the vaccine preparation provides technical reserves for preventing a pandemic H1N1 influenza in the future in swine or other mammals.

MATERIALS AND METHODS

Viruses: The 1057 strain was identified and isolated from human strains in Guangdong province in 2010 and named A/Guangdong/1057/2010. The 170 strain was isolated from the swine in Guangdong province in 2010 and named A/Swine/Guangdong/170/2010. These viruses were propagated in 10 days old embryonated chicken eggs for 2 days at 35°C and then stored at -80°C.

Adjuvants: The Montanide GEL adjuvant, one of SEPPIC company's products in France is a water-based immune adjuvant, a water soluble synthetic polymer of high molecular weight polyacrylic acid dispersions that contains a small amount of MONTANIDE™ adjuvant.

Animals: Researchers purchased 6-8 weeks old female BALB/c mice from the Laboratory Animal Center of Southern Medical University, Guangzhou, China. The mice were kept under Specific Pathogen Free (SPF) conditions.

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The Receptor Destroying Enzyme (RDEs): The enzyme was a gift from the Guangdong Provincial Disease Control and Prevention Center.

Construction of plasmids: The viral cDNAs were amplified by RT-PCR with primers containing BsmBI sites (primers are available upon request) and then digested with BsmBI and cloned into the BsmBI sites of the pDL vector (a bidirectional transcription vector, eight-plasmid reverse genetic systems). The resulting plasmids (pDL-1057-HA, -NA; pDL-PR8-PB2, -PB1, -PA, -NP, -M and -NS) was confirmed by sequencing (primers are available upon request). The plasmids for transfection were prepared by using the Perfectprep Plasmid Mini kit (Eppendorf, Hamburg, Germany).

Generation of recombinant viruses using reverse genetics: An eight plasmid reverse genetic system was used to generate wild-type and recombinant viruses. A monolayer of 293T cells with approximately 90% confluence in 23 six-well plates was transfected with 5 μg of the eight plasmids (0.6 μg/each plasmid) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 5 μg of plasmids and 10 μL of lipofectamine 2000 were mixed, incubated at room temperature for 30 min and then added to the cells. After 6 h incubation at 37°C, the mixture was replaced with DMEM containing 2% fetal bovine serum and 0.2 μg mL^{-1} TPCK-treated trypsin. The supernatant was harvested after 2 days incubation and 100 μL of supernatant was injected into an embryonated egg for virus propagation. The inoculated eggs were incubated for 3 days and the allantoic supernatant was collected and tested by hemagglutination assay. The rescued viruses were confirmed by sequencing of the whole viral genome.

Preparation of inactivated vaccine: The virus vaccine was propagated in the allantoic cavity of 10 days old SPF embryonating chicken eggs at 35°C. Allantoic fluids were harvested 72 h after inoculation. Virus titers were determined using HA assays. The vaccine was generated according to standard techniques (Subbarao *et al*., 2003). The seed virus was grown to a high titer in eggs and the virions were purified by centrifugation, inactivated with formalin and filtered to remove bacteria (Horimoto *et al*., 2007). Injectable fluid and stable vaccine was obtained by using MONTANIDE ™ gel and mixing the adjuvant and antigenic media under a low shear rate. The ratio of MONTANIDE™ gel 01 is 15%. The sterility testing, physical properties testing and mouse safety test were performed for the inactivated vaccines.

Immunization: Thirty mice were divided into two groups: M1 and M2 (n = 15). Each mouse in the M1 group was vaccinated i.n. with 0.2 mL influenza inactivated vaccine. The M2 group was the negative control group that was administered PBS. Total 4 weeks after the primary vaccination, the mice were boosted with a second dose of 0.2 mL influenza inactivated vaccine by the i.n. route.

Hemagglutination Inhibition (HI): Serum samples were taken pre-test, 0, 1, 2, 4, 6, 8, 10 and 12 weeks after the 1st vaccination. Serum was treated with receptor-destroying enzyme for 18 h at 37°C, heat-inactivated at 56°C for 50 min and tested by HI assay with 1% chicken erythrocytes (Mukherjee *et al*., 2011; Wood *et al*., 2012). The sera from mice that was administered PBS were used as negative controls. All assays were performed in triplicate and repeated 3 times. The reference viruses used for serologic testing were the 170 strain and 1057 strain.

Protection experiments: Protection experiments were performed by challenging mice with a nasal administration of the 10^5 EID_{50} 1057 strain and 170 strain, respectively 2 weeks after the last immunization. Mice were followed 9 days after the infection and weight loss was monitored. Mouth and rectal swabs were collected 3, 5, 7 and 9 days after the infection and a positive rate of the virus was detected.

RESULTS AND DISCUSSION

The recombinant virus with human H1N1 influenza virus HA, *NA* gene fragments and six gene fragments of the PR8 strain was successfully rescued and named r1057. And the r1057 strain as an antigen, an recombinant (H1N1) influenza inactivated vaccine was prepared. Researchers chose BALB/c mice as animal models to evaluate the vaccine's immunity. Sera were assayed for the presence of H1N1 influenza-specific antibodies using a HI assay. As shown in Fig. 1, the HI titers of the sera

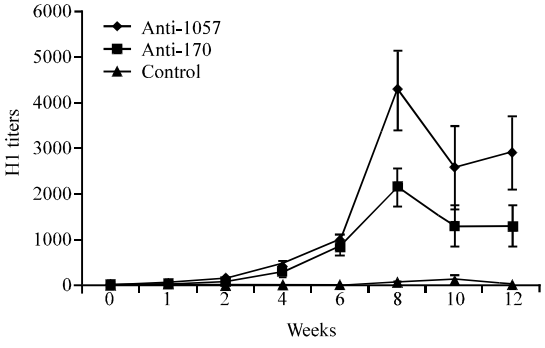

Fig. 1: HI antibody titers of mice

from mice immunized with influenza inactivated vaccine against 1057 strain reached 53±7, 1 week after the 1st vaccination, 136±29, 2 weeks after the 1st vaccination and 457±78, 4 weeks after the 1st vaccination. The antibody levels had a rapid increase after a second vaccination, reached 1006±105, 2 weeks after the 2nd vaccination (i.e., 6 weeks after the 1st vaccination) and 4267±853, 4 weeks after the 2nd vaccination and decreased to 2560±905, 6 weeks after the 2nd vaccination. The HI titers remained at 2880±805, 8 weeks after the 2nd vaccination. The HI titers of the sera from mice immunized with influenza inactivated vaccine against 170 strain reached 23±4, 1 week after the 1st vaccination, 84±22, 2 weeks after the 1st vaccination and 289±94, 4 weeks after the 1st vaccination. The antibody levels had a rapid increase after a 2nd vaccination, reached 829±189, 2 weeks after the 2nd vaccination (i.e., 6 weeks after the 1st vaccination), reached 2133±427, 4 weeks after the 2nd vaccination and decreased to 1280±453, 6 weeks after the 2nd vaccination. The HI titers remained at 1280±453, 8 weeks after the 2nd vaccination.

The protective characteristics of standard immunization were tested in an *in vivo* experiment. Researchers concentrated on the possibility that the vaccine could induce cross-protection against the 1057 and 170 strain. The challenge was made 2 weeks after the last immunization using the 1057 and 170 strain, respectively. The viral progression was monitored for 9 days. Because the 1057 and 170 strain are not lethal in mice, in order to determine whether the vaccination could elicit protection against cross-infection, researchers confirmed whether the mice would release the virus and monitored the mice for weight change. The results showed that the vaccinated mice resisted challenges with the H1N1 virus strains and did not isolate the virus. The virus isolation rate of the blank control group is: 66.7% challenged the 1057 strain and 75% challenged the 170 strain. In Fig. 2, vaccinated mice were resistant to challenges with the 1057 and 170 strain while the control animals that received the 1057 and 170 strain alone significant body weight loss starting on day 2 after virus inoculation. In contrast, all mice immunized with GelA vaccine had transient body weight loss on day 6 but the animals recovered over the following days.

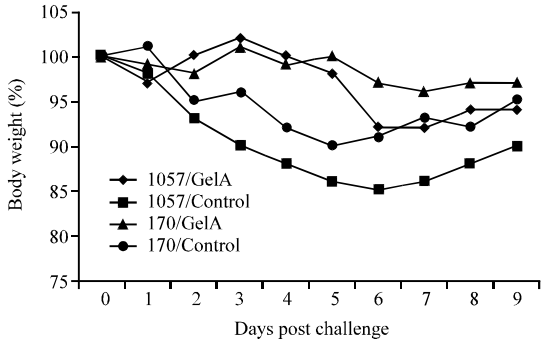

Fig. 2: Weight change in mice challenged with 1057 and 170 strain

CONCLUSION

The recombinant virus r1057 with human H1N1 influenza virus HA, *NA* gene fragments and six gene fragments of the PR8 strain was successfully rescued. The veterinary recombinant influenza A (H1N1) inactivated vaccine was successfully developed. Researchers chose mice as animal models and found the vaccine could elicit humoral immune responses against H1N1 influenza virus. The vaccine could induce cross protection against human influenza virus A (H1N1) and swine influenza virus (H1N1). It provides technical reserves to prevent an influenza virus A (H1N1) pandemic in swine or other mammals.

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