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MDCK cell-cultured influenza virus vaccine protects mice from lethal challenge with different influenza viruses

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Abstract Influenza epidemics are major health concern worldwide. Vaccination is the major strategy to protect the general population from a pandemic. Currently, most influenza vaccines are manufactured using chicken embroynated eggs, but this manufacturing method has potential limitations, and cell-based vaccines offer a number of advantages over the traditional method. We reported here using the scalable bioreactor to produce pandemic influenza virus vaccine in a Madin–Darby canine kidney cell culture system. In the 7.5-L bioreactor, the cell concentration reached to 3.2×10^6 cells/mL and the highest virus titers of 256 HAU/50 μL and 1×10^7 TCID50/mL. The HA concentration was found to be 11.2 μg/mL. The vaccines produced by the cell-cultured system induced neutralization antibodies, cross-reactive T-cell responses, and were protective in a mouse model against different lethal influenza virus challenge. These data indicate that microcarrier-based cell-cultured influenza virus vaccine manufacture system in scalable bioreactor could be used to produce effective pandemic influenza virus vaccines.

Keyword MDCK · Bioreactor · Influenza virus · Vaccine

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Introduction

Influenza epidemics continue to impose a significant impact on the world's population, resulting in human suffering and economic burden (Carrat and Flahault 2007). Vaccination is the major strategy to protect the general population from an influenza pandemic (Cox et al. 2009; Genzel and Reichl 2009). Most current seasonal influenza vaccines are manufactured using chicken embroynated eggs, but this manufacturing method has potential limitations, including the lack of reliable supplies of high-quality eggs, the presence of adventitious agents in eggs, and the cultivation of influenza virus in eggs can lead to the selection of variants in the hemagglutinin (Govorkova et al. 1996; Hu et al. 2011). Furthermore, the surge in the demand of a pandemic vaccine would require the switch from the seasonal vaccine production to pandemic vaccine manufacturing processes that currently are the bottleneck and are not expected to meet the global vaccination demand (Hu et al. 2011). These difficulties prompted the evaluation of mammalian cell culture-based vaccine production systems as alternatives to egg-based production (Genzel and Reichl 2009; Hu et al. 2011).

Cell-based vaccines offer a number of advantages over the traditional method: (a) cell lines are fully characterized and in compliance with regulatory guidelines; (b) the culture media are chemically defined and give consistent results for cell growth and virus propagation (Hu et al. 2011; Tree et al. 2001). There are two regulatory-approved continuous cell lines being used for influenza vaccine production: Madin–Darby canine kidney (MDCK) cells and Vero (African green monkey kidney) cells (Kistner et al. 1998; Genzel et al. 2004; Hu et al. 2008; Kistner et al. 2007; Audsley and Tannock 2008). Cell-derived influenza vaccines are capable of providing equivalent or even better protection in animal

models and humans than those obtained from egg-derived vaccines (Nerome et al. 1999; Tree et al. 2001; Kistner et al. 2007).

Currently, seasonal influenza vaccines comprise two influenza A strains: H1N1 and H3N2 and one influenza B strain (Genzel and Reichl 2009). But it is not possible to predict which strain will cause the next pandemic, an ideal influenza vaccine, which could elicit an immune response that protects the host from infection with a broad range of influenza viruses, is urgently needed.

In this study, we describe a well-defined manufacturing process for influenza vaccine production in a microcarrier cell culture bioreactor system for MDCK cell propagation. High virus yield was acquired; the antigenicity analysis and immunogenicity study in mice have shown that MDCK cell-based vaccine protected the mice against lethal challenge of different strains of influenza virus.

Materials and methods

Virus, cells, and medium

Clinical isolated human influenza virus strain A/Beijing/501/2009 (H1N1) (BJ501) was propagated in eggs, and then amplified to generate virus stocks in MDCK cells. Influenza virus strain A/Porto Rico/8/1934 (H1N1) (PR8) was amplified in eggs and titered in MDCK cells.

The MDCK (ATCC CCL-34) cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin G, and 100 μl/mL streptomycin sulfate. Master and working cell banks were established following the guidelines of the Pharmacopoeia of the PRC, 2010, part 3 and are being tested to fulfill the requirements for continuous cell lines used for manufacture of biological products. For virus propagation in MDCK cells, DMEM (without FBS) supplemented with 2 μg/mL of tosyl phenylalanyl chloromethyl ketone (TPCK)–trypsin (Sigma) was used as culture medium.

Virus titration

Hemagglutination (HA) titrations were performed in 96-well V-bottom plates. Fifty microliters of phosphate-buffered saline (PBS) was added to every well on the plates, and then 50-μL culture supernatants were added to the first well in each row to be tested. After mixing the contents of the first well by pipetting up and down, 50 μL was pipetted from the first well and placed it in the second well, and continued to make twofold dilutions of the virus suspension across the entire row, and 50 μL was discarded after the last row. Then, 50 μL 1% chicken red blood cells were added to each well. The plates were incubated for 30 min at room temperature (RT) and the HAs were determined visually.

Virus titers were measured using the 50% tissue culture infectious doses (TCID50) assay on MDCK cells. MDCK cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well and incubated at 37°C overnight. The virus culture supernatants were diluted tenfold serially. The MDCK cells were inoculated in quaternity with 200 μL diluted virus sample. After 3 days of incubation at 37°C, the HA assay was performed for the detection of cell infection. The TCID50 was determined via the Reed and Muench method (1938).

Cells and viruses culture in bioreactor

The cultures were performed in 7.5 L bioreactor (NBS) with working volume of 4 L. Cytodex 3 microcarriers (GE Healthcare) were pretreated according to the manufacturer's instruction. The seeding density was with 5×10^5 cells/mL. The agitation speed of the bioreactor was set at 65 rpm. During cell growth, pH, dissolved oxygen, and temperature were maintained at 7.4, 40% air saturation and 37°C, respectively. Microcarriers were sampled to count cell density daily. When the cells were grown confluent, the medium was exchanged to virus culture medium and the BJ501 virus was added to infect cells at a multiplicity of infection (MOI) of 0.001. Samples were taken to determine viral titer daily.

Purification of vaccine antigens

The virus culture medium was collected and centrifuged to remove cellular debris. The supernatant was then concentrated by an ultrafiltration using 300 K membrane (PERMEATE). The concentrated solution was purified using the sucrose density gradient zonal centrifugation (CP-70 ME, Hitachi). The fraction between 30% and 60% sucrose density was collected and ultracentrifuged to remove sucrose content. The purified virus was monitored by electron microscopy.

The purified viruses were inactivated with 0.01% formalin and split with 0.25% Triton X-100. Virus inactivation was confirmed on MDCK cells.

The HA antigen protein concentrations were measured using the single radial immunodiffusion (SRID) assay. The standard HA antigen (lot 09/146) and antiserum (lot 09/152) were purchased from the NIBSC, UK. The standard antiserum was used at 15 μL/mL agarose, as recommended by NIBSC.

Immunogenicity and protection efficiency assay in mice

All animal experiments were conducted according to the guidelines of the Chinese Animal Care for Laboratory

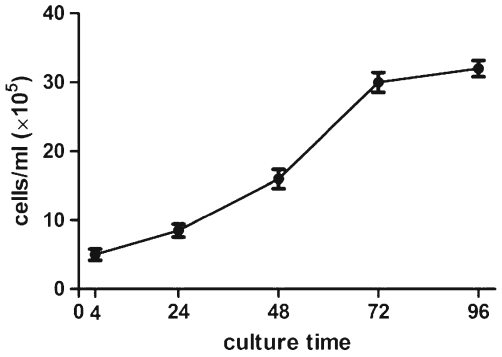

Fig. 1 Cell growth curve in a 7.5-L bioreactor. Mean and standard deviations of three repeat assays are shown

Animals, and the protocols were approved by the Animal Care and Use Committee of Beijing Institute of Microbiology and Epidemiology. Six-week-old female Balb/C mice were bred and maintained at the Institute of Jingfeng Medical Animal Laboratory and immunized with two doses of vaccine antigen at a 2-week interval intramuscularly with 15 μg virus antigen (based on HA content). Sera were collected at days 0, 14, 21, and 28.

Two weeks after the second immunization, mice were intranasally challenged with 3×10^5 TCID50 of the BJ501 or 2.5×10^6 TCID50 of the PR8, respectively. Body weights and survival were monitored daily over a period of 14 days.

Serum antibody titers and estimation of IgG subclass

Antigen-specific IgG and isotype (IgG1, IgG2a, and IgG2b) levels in the sera were determined by indirect ELISA. Ninety-six-well plates were coated with inactivated BJ501 viruses overnight at 4°C. After washing and blocking, serial dilutions of antisera were added in triplicates and incubated for 2 h at 37°C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or its isotypes (BETHYL) were incubated for 1 h at 37°C as second antibody. The 3, 3′, 5, 5′-tetramethyben-zidine was used as a substrate to estimate the enzymatic activity. The reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 405 nm using Microplate Reader (Bio-Rad). The end-point titer of the individual sera samples was considered positive if the absorbance was two-fold higher than the background. The means ± SD of antibody titers were calculated for each group at each time point.

ELISPOT assays

Spleens from immunized mice or control were removed and single cell suspensions were prepared. Single cell suspensions of lymphocytes were resuspended at 1×10^6 cells/200 μL in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. The cells were cultured in triplicate and plated in enzyme-linked immunosorbent spot (ELISPOT) plates (BD Pharmingen) that have been

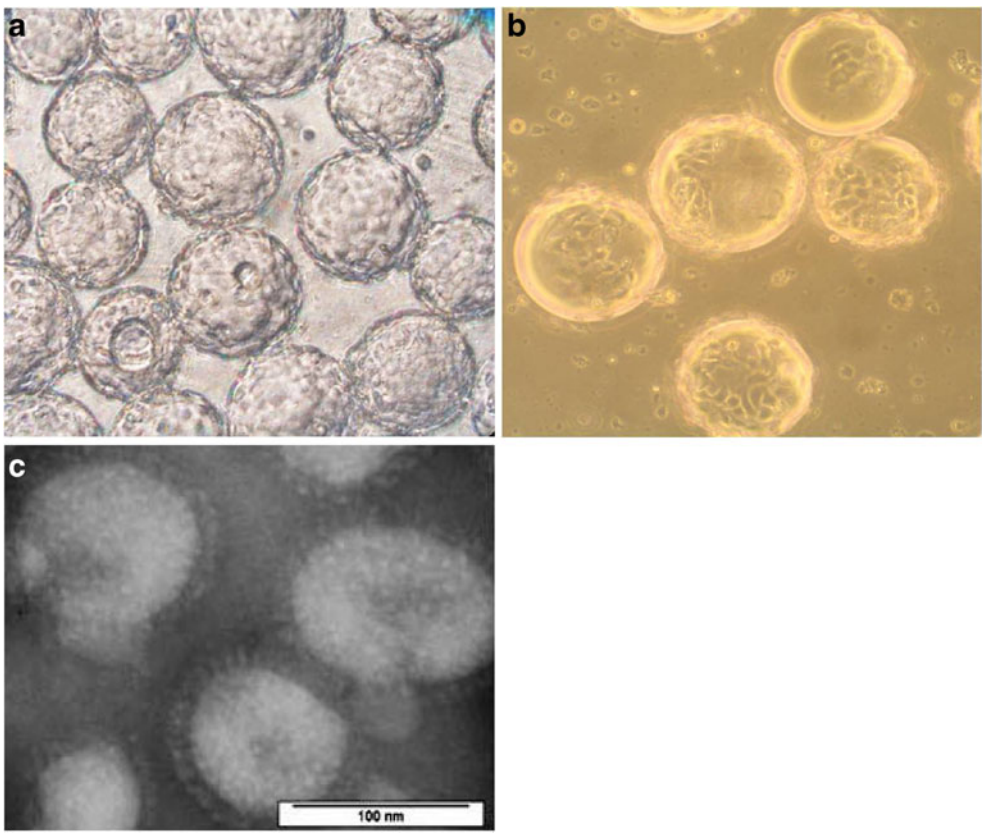

Fig. 2 Cells and viruses cultured on microcarriers in bioreactor. **a** MDCK cells cultured on microcarriers; **b** CPE of the MDCK cells cultured on microcarriers postinfection with influenza virus; **c** Purified virus particles cultured by cell culture system observed under electron microscopy (×135,000)

previously coated with IL-4 or IFN-γ capture antibody (BD Pharmingen) overnight at 4°C, and then stimulated with inactivated BJ501 virus at an MOI of 1. The spot-forming cells were detected by addition of biotinylated IL-4- or IFN-γ-detective antibody followed by the addition of streptavidin–HRP and development with AEC substrate solution. Wells containing no antigen or 10 μg PMA were used as negative and positive control, respectively. Spots were counted using AID Immunospot (Cellular Technology Ltd.).

Hemagglutination inhibition assay

Receptor-destroying enzyme-treated sera were serially twofold diluted with PBS in V-bottom 96-well plates, then incubated with 4 HA units of the BJ501 and PR8 viral antigen, respectively, for 30 min at room temperature, followed by the addition of 1% chicken erythrocytes and incubation for 40 min at RT. The highest dilution of the sera that inhibited hemagglutination was considered the hemagglutination inhibition (HI) titer.

Microneutralization assay

MDCK cells were seeded at 2×10^4 cells/well in 96-well plates and cultured to confluency at 37°C. Heat-inactived sera were diluted with DMEM at 1:50, then serially twofold diluted, followed by mixing with 100 TCID50/50 μL of the BJ501, PR8 viruses for 1 h at 37°C, respectively. The mixtures were added to MDCK cells in triplicate. The cells were incubated in the presence of TPCK-treated trypsin at 37°C and 72 h postinfection. The supernatants were tested for virus growth by hemagglutination assay. Neutralizing titers were determined via the Reed and Muench method.

Statistical analysis

Data were analyzed with the SPSS software (version 11); P values <0.05 were the criterion for statistical significance.

Results

Cells and viruses growth on microcarriers in bioreactor

Six grams per liter of cytodex 3 microcarriers was used to culture the MDCK cells in 7.5-L bioreactor with the seeding ration of 15 cells/microcarrier. As shown in Fig. 1, the cells could grow to high density after 3 days inoculation. Cell density increased from an initial concentration of 5×10^5 cells/mL to 3.2×10^6 cells/mL. As a nutrition indicator, the glucose concentration was determined at an interval of 6 h. The result indicated that the glucose concentration of the medium decreased from 4 to 1 g/L after ~12-h culture (data not show). So, the culture medium was changed at an interval of 12 h to avoid the depletion of nutrients.

When the cells were grown confluently (about 72 h), the medium was exchanged to virus culture medium and the BJ501 virus was added to infect cells at an MOI of 0.001. Samples were taken daily to observe the cytopathic effect (CPE) and determine viral titers. The BJ501 virus caused significant CPE in MDCK cell-confluent microcarriers (Fig. 2). The culture medium was collected at day 3 postinfection, the virus titers reached 1×10^7 TCID50/mL, the HAU peaked at 256 HAU/50 μL, and the HA concentration was found to be 11.2 μg/mL, which was determined by the SRID assay.

Humoral immune responses in mice

The collected culture medium was concentrated, and the viruses were sucrose gradient-purified. After sucrose removal,

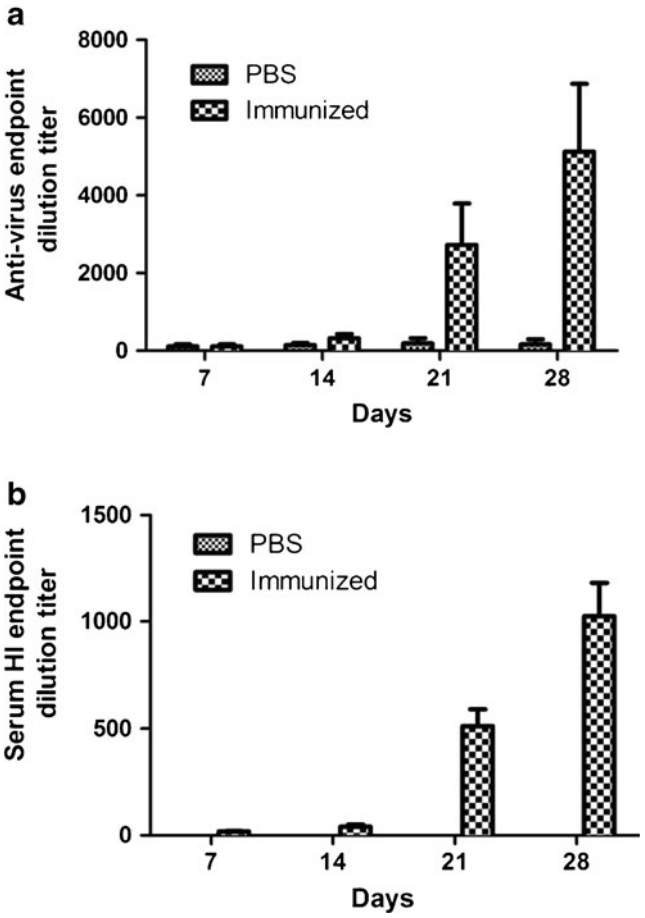

Fig. 3 Humoral immune responses generated by cell-cultured BJ501 influenza vaccine. **a** Serum IgG antibody response against the BJ501 antigen in mice. **b** Serum hemagglutination inhibition titer against the BJ501 in mice. Mice were immunized and sera were collected at days 7, 14, 21, and 28 post-immunization

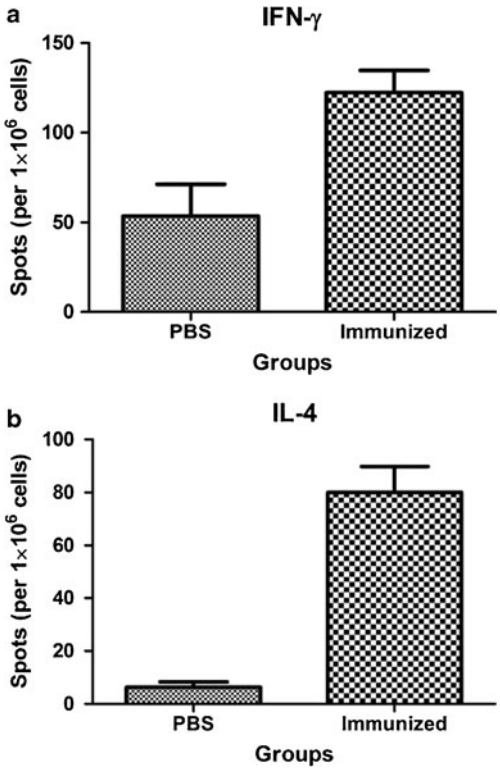

Fig. 4 The level of IFN-γ (**a**) and IL-4 (**b**) spot-forming cells in spleens of Balb/c mice as determined by ELISPOT assays. Mice were immunized and splenocytes were isolated and stimulated with inactivated the BJ501 virus. *Bars* represent means ± S.D. of spot counts in triplicate wells

the purified viruses were formalin-inactivated and Triton X-100 was splitted. The HA concentration was determined using the SRID, and 15 μg of HA/mouse was injected intramuscularly to test their ability to induce immune responses.

Indirect ELISA was used to detect the IgG antibodies in serum using plates coated with the purified BJ501 virus. As shown in Fig. 3a, compared to the control group (PBS group), vaccination with the cell-cultured vaccine induced significantly strong antibody responses, especially after boosted on day 14. Furthermore, the HI assays indicated 100% seroprotection rates against the BJ501 since 2 weeks after the prime immunization (40 HI titer is considered as the level of seroprotection) (Fig. 3b), but the HI titers against the PR8 were lower than 1:10 (data not shown). The microneutralization assays indicated that after the second immunization, on day 28, the sera microneutralization titers against the BJ501 reached a high titer of 1:400, but the titers against the PR8 were very low (<1:50).

T helper cell responses in mice

Balb/c mice immunized twice (days 0 and 14) were used to investigate the T helper cell responses. Splenocytes were prepared on day 21 and stimulated with the whole BJ501 viral antigens in vitro. The number of INF-γ- and IL-4-producing cells was determined by ELISPOT assays. The results indicated a mixed Th1 (INF-γ) and Th2 (IL-4) response was observed (Fig. 4).

Protection studies in mice

Balb/c mice were used for challenge and protection studies. At 2 weeks after the boost immunization, all animals were challenged with either the BJ501 or PR8 as described in the

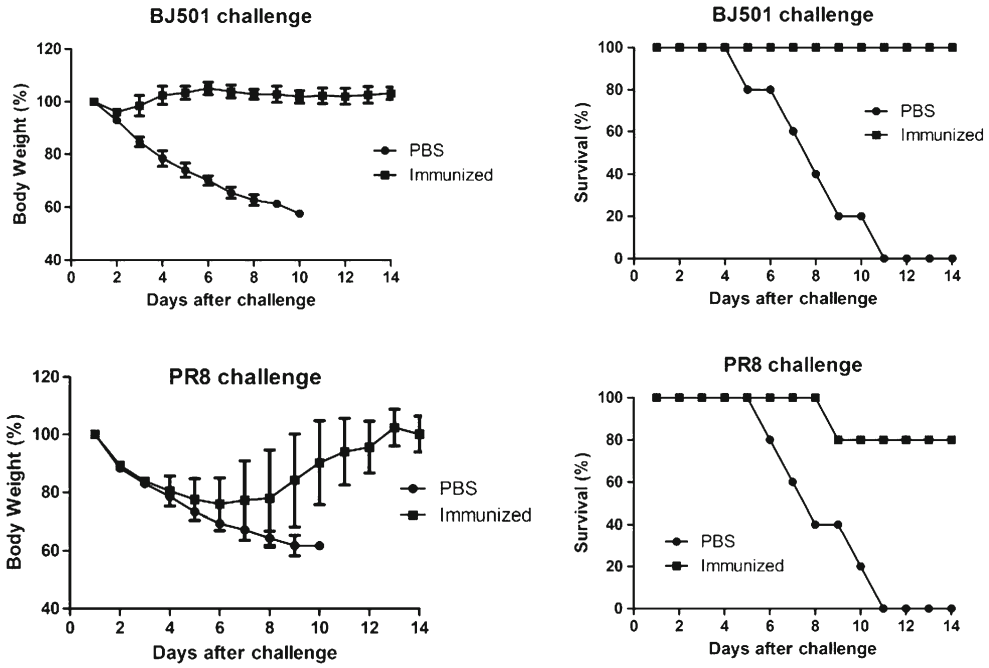

Fig. 5 Body weight changes and survival rates of mice after different lethal influenza virus challenge. **a** Body weight changes of mice after challenge with the BJ501; **b** Survival rates of mice after challenge with the BJ501; **c** Body weight changes of mice after challenge with the PR8; **d** Survival rates of mice after challenge with the PR8. Each point represents the mean of five mice on each day

"Materials and Methods" section. The control mice that received PBS showed significant body weight loss from day 2 after virus inoculation in both challenge tests and died on day 5–6 after challenge (Fig. 5a, c). Challenge with the BJ501 resulted in full protection against lethal infection by the immunization of the cell-cultured influenza virus vaccine (Fig. 5b), and challenge with the PR8 resulted in a transient body weight loss at day 7, recovery on the following 7 days, and an 80% protection (Fig. 5d). The results indicated that the cell-cultured influenza virus vaccine is effective to protect mice against lethal influenza virus challenge.

Discussion

Influenza virus vaccine supply is a challenge to the world, especially when pandemic vaccines are needed (Genzel and Reichl 2009). The egg-based technology has a long history of success of supplying seasonal influenza vaccines, but the lengthy time window required for vaccine manufacturing and the egg supply during a pandemic compel to take other available options into account (Hu et al. 2008; Genzel and Reichl 2009). The cell culture-based influenza virus vaccine production has been attractive in recent years, and several cell lines, such as Vero and MDCK cells, have been approved to produce influenza vaccines (Hu et al. 2011; Liu et al. 2009). Here, we describe the production and immune evaluation of the cell-cultured pandemic influenza virus vaccine.

MDCK cells are the most suitable substrate for producing influenza virus vaccines (Minor et al. 2009; Genzel et al. 2006a; Genzel et al. 2006b; Tree et al. 2001; Genzel et al. 2004; Liu et al. 2009; Genzel and Reichl 2009; Hu et al. 2008). In the present study, MDCK cells were used to propagate pandemic H1N1 influenza virus. In a 7.5-L scale, the MDCK cells were grown to a peak density of 3.2×10^6 cells/mL on cytodex 3 microcarriers, the virus titers reached 1×10^7 $TCID_{50}$/mL, the HAU peaked at 256 HAU/50 μL, and the HA concentration was found to be 11.2 μg/mL. Compared to other reported human influenza virus production on MDCK cells (Tree et al. 2001; Genzel et al. 2004; Hu et al. 2011), our results are lower. This may depend on the influenza virus strain used to propagate.

After concentration and purification, the cell-cultured influenza vaccine efficacy was evaluated. Our results indicated that the cell-cultured influenza vaccine has high immunogenicity and the titers of IgG, HI, and microneutralization against the BJ501 reached high level at 2 weeks after boost. But the corresponding titers against another H1N1 influenza virus, the PR8, are very low.

Besides the functional antibody, the T-cell responses are important in vaccine protection, especially in preventing severe disease and death (Kistner et al. 2007; Doherty et al. 2006). In the present study, the split cell-cultured influenza vaccine induced Th-1 responses effectively. This may be due to responses to the presence of the full set of virion proteins and may play important roles in vaccine protection, especially in protection against different virus stain challenge.

The virus challenge assays were used to evaluate the cross-protection potential in Balb/c mice. The immunization with the splitted cell-cultured influenza vaccine provided effective protection against lethal challenge with the BJ501 and the PR8 viruses, respectively. The vaccine-induced Th-1 immune responses may contribute to the protection against the PR8 virus, whereas the HI and microneutralization titers against the PR8 are very low. This is consistent with other reports that the vaccines composed with full set of virion proteins provide effective protective immune responses (Bodewes et al. 2011; Easterbrook et al. 2011). In summary, the present study demonstrates that microcarrier-based cell-cultured influenza virus vaccine manufacture system in a scalable bioreactor could be used to produce effective pandemic influenza virus vaccines.

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