Article

Comparative Immunization in BALB/c Mice with Recombinant Replication-Defective Adenovirus Vector and DNA Plasmid Expressing a SARS-CoV Nucleocapsid Protein Gene

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In order to investigate immunogenicity in the induction of humoral and cellular immune responses, severe acute respiratory syndrome associated coronavirus (SARS-CoV)-N gene recombinant replication-defective adenoviral vector, rAd-N, was generated and immunized BALB/c mice in a pcDNA3.1-N prime-rAd-N boost regimen. After humoral and cellular immune response detection, different levels of SARS-CoV N protein specific antibodies and interferon-γ (IFN-γ) secretion are shown compared to controls. The humoral immune response was induced more effectively by the DNA priming and recombinant adenovirus boosting regimen. There is a significant difference between heterogeneous and homologous vaccinations. The heterogeneous combinations were all higher than those of the homologous combinations in the induction of anti-N antibody response. Among the three heterogeneous combinations, pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N induced the strongest antibody response. In the induction of IFN-γ production, the homologous combination of rAd-N/rAd-N/rAd-N/rAd-N was significantly stronger than that of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N, but was relatively weaker than the heterogeneous combination of pcDAN3.1-N/pcDAN3.1-N/pcDAN3.1-N/rAd-N. This combination was a most efficient immunization regimen in induction of SARS-CoV-N-specific (IFN-γ) secretion just as the antibody response. These results suggest that DNA immunization followed by recombinant adenovirus boosting could be used as a potential SARS-CoV vaccine. *Cellular & Molecular Immunology*. 2006;3(6):459-465.

Key Words: SARS-CoV, DNA vaccine, nucleocapsid protein, adenovirus vector

Introduction

SARS-CoV, a new type of coronavirus, which is the causative agent of severe acute respiratory syndrome, contains five major open reading frames encoding the replicase polyprotein, the spike (S), the envelope (E), membrane (M) glycoprotein and the nucleocapsid protein (N) in the same order and of approximately the same size as those of the other coronavirus (1, 2). According to the prediction of Rota et al., N protein of SARS-CoV is a highly charged basic protein of 423 amino acids (1,269 base pairs) with seven successive hydrophobic residues near the middle of the protein. Amino acid sequence homology between SARS-CoV and other coronavirus is low, and this is a possible cause for the difference of pathogensis (2). Wang et al. reported that > 94% of the SARS patients were positive for N protein-specific antibodies. Antibodies against SARS-CoV N protein appear at early stage of infection (3). Thus N protein attracted our attention. As a typical virus infection of a cell, SARS-CoV binds to host cells *via* a specific SARS receptor (angiotension converting enzyme, ACE2). Following entry, the virus uncoats and nucleic acid is released. During this course, host defense system involving B and T cells is stimulated, in which generation of neutralization antibody and specific CTL is critical for preventing re-infection and vaccination design.

SARS-CoV vaccine should be an effective method for preventing SARS-CoV infection. A successful vaccine should be able to induce proper humoral and/or cellular immune response. DNA vaccination has been used to express antigens *in vivo* for the generation of both humoral and cellular immune responses, and is a promising therapy, especially for life-threatening diseases. Several different

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Received Nov 2, 2006. Accepted Dec 18, 2006.

research reports on DNA vaccines of SARS-CoV have been recommended, such as S gene recombinant DNA vaccine (4-6), N gene recombinant DNA vaccine (7-11). These genes are delivered and expressed by different plasmids or viral vectors and elicit different levels of humoral and cellular immune responses. DNA vaccine has been shown to possess several important advantages, including the ability to induce CTLs and antibodies through the class I and class II antigen-processing pathways, but its efficacy does not meet our expectations (12, 13). Different methods have been tried to increase the effectiveness of genetic immunization. Priming with a DNA vaccine can augment the efficacy of vaccines based on recombinant viral vectors. Thus, in this experiment, we constructed SARS-CoV N gene recombinant adenovirus vector and immunized BALB/c mice in order to investigate which is more effective in induction of homoral and cellular immune response among five combinations of SARS-CoV N gene recombinant adenovirus and DNA vaccine.

Materials and Methods

Cells and plasmids

SP2/0 cells and Vero E6 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% heat-inactivated fetal bovine serum (FBS, Sijiqing, Hangzhou, China). Human embryonal kidney 293A cells purchased from Invitrogen Corporation (San Diego, Calif., USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS. The LacZ gene recombinant adenoviral expression vector was purchased from Invitrogen Corporation. The plasmids of pcDNA3.1 and pcDNA3.1-N, the recombinant N protein and rabbit antisera (IgG polyclonal antibody anti-SARS-CoV-N protein) were kindly provided by Prof. Minsheng Zhu.

Construction of recombinant transfer vector containing N gene of SARS-CoV

According to the standard sequence of SARS-CoV N gene sequenced from AY304495 strain in GenBank, we designed the following primers to amplify N gene from the plasmid, pcDNA3.1-N, to construct recombinant adenoviral vector. PN1: 5'-CACCAT GTC TGA TAA TGG ACC CCA ATC AA-3'; PN2: 5'-TTA TGC CTG AGT TGA ATC AGC AGA A-3'.

In order to contruct Directional TOPO Cloning transfer vector in the desired orientation, CACC sequences were incorporated into the upstream primer at its 5' end. The final optimized conditions of PCR were consisted of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. One DNA strand of a 1,273-base pair product was observed by using a 1.5% agarose gel electrophoresis and purified for generation of pENTR/D-TOPO Cloning Vector. After the N genes with CACC sequence at their 5' ends were cloned into pENTR/D-TOPO Vector directionally, recombinant transfer vector DNA plasmid was constructed successfully. The plasmids were transformed into TOPO 10 chemically competent *E. coli* and incubated overnight at 37°C. Subsequently, the positive clones of N gene recombinant transfer vector DNA plasmids were amplified by PN1 and PN2 primers and sequenced, respectively.

Construction and identification of N gene recombinant adenoviral DNA plasmid

By using LR Clonase™ (Invitrogen, USA) as catalysis, the positive and purified clones of N gene recombinant transfer vector were mixed with adenoviral expression vector DNA to generate the N gene recombinant adenoviral DNA plasmid. Then, the plasmids were transformed into TOPO 10 chemically competent *E. coli* and incubated on LB plates containing 100 μg/ml ampicillin at 37°C overnight. Subsequently, we selected putative positive clones to extract and purify for PCR amplification and electrophoresis detection, respectively. In the meantime, those putative positive clones were cultrued on LB plates containing 30 μg/ml chloramphenicol because true expression clones would be ampicillin-resistant and chloramphenicol-sensitive.

Transfection of HEK293A cell line for package of recombinant adenovirus, rAd-N

On the day before transfection, the 293A cells were trysinized and counted, plating them at 5×10^5 cells per well in a 6-well plate. Two milliliters of normal growth medium, DMEM, containing 10% FBS were plated in per well. Those 293A cells plated 24 hours before they were transfected with Pac I-digested adenovirus plasmid DNA complexes by using Lipofectamine 2000™ (Invitrogen, USA). In the meantime, commercially available LacZ gene recombinant adenoviral DNA (rAd-LacZ) was transfected into 293A cells as experimental negative control. Culture medium was replaced with refresh complete culture medium every 2-3 days until visible regions of cytopathic effect (CPE) were observed. When approximately 50-70% of CPE was observed, adenovirus-containing cells and media were harvested.

Preparation and titering of adenoviral stocks

After the 293A cells were prepared at 80-90% confluency in 6-well plates incubated at 37°C in a CO_2 incubator for 24 h, 100 μl of crude adenoviral stocks were added to the cells per well. The adenovirus-containing cells were harvested when 50-70% of the cells had rounded up and were floating. Purified stocks were obtained by standard two-step CsCl gradient banding and the isolated virus was dialyzed in three steps to a final formation in phosphate-buffered saline (PBS)-5% sucrose and kept at -70°C. The End-Piont Dilution Assay was utilized to detect the titers of the two recombinant adenovirus in this study.

Western blot for expression of recombinant N protein

Vero E6 cells which were susceptible to SARS-CoV infection were infected by using rAd-N in 6-well plates in the concentration of 100 of multiplicity of infection (MOI-viral

Table 1. Procedure of the immunization experiment with the pcDNA3.1-N prime-rAd-N boost regimen and the time of collection of samples

Groups	Prime/0 week	First boost/2 weeks	Second boost/4 weeks	Third boost/6 weeks	Collecting samples/8 weeks
1	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N	blood and spleens
2	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N	rAd-N	blood and spleens
3	pcDNA3.1-N	pcDNA3.1-N	rAd-N	rAd-N	blood and spleens
4	pcDNA3.1-N	rAd-N	rAd-N	rAd-N	blood and spleens
5	rAd-N	rAd-N	rAd-N	rAd-N	blood and spleens
6	pcDNA3.1	pcDNA3.1	pcDNA3.1	pcDNA3.1	blood and spleens
7	rAD-LacZ	rAD-LacZ	rAD-LacZ	rAD-LacZ	blood and spleens

BALB/c mice were divided into 7 groups, each with 5 mice. After four times immunization, all mice were sacrificed at week 8 and sera and splenocytes were prepared.

particles/cell). After having cultured for 72 hours, the infected and normal control Vero E6 cells were harvested and frozen at -70°C. Some of them were prepared for Western blot. The plasmid constructs of pcDNA3.1-N and pcDNA3.1 were transfected into SP2/0 cells derived from BALB/c mouse plasmacytoma using the Lipofectamine 2000™ reagent (Invitrogen). The cells were cultured in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% heat-inactivated FBS, selected in the medium containing G418 (Promega, Madison, Wisc., USA) for 2 weeks. G418-resistant stable clones were screened by Western blot analysis. Protein samples were fractionated on 5-12% SDS-PAGE and transferred to PVDF membrane using protein transfer apparatus (Bio-Rad, USA). The membrane was blocked for 1 h with 5% skimmed milk in TBS buffer (50 mM Tris base, 150 mM NaCl, pH 7.5) containing 0.5% Triton X-100. Following the blocking the membranes were probed with rabbit IgG antibodies against the N protein. Goat anti-rabbit HRP-conjugated antibody (Jingmei Biotech, China) was added. The results were finally revealed by using the sensitive substrate of DAB kit (Wuhan Boster, China) for Western blot detection.

Mice and immunization experiment

Six-eight-week-old female BALB/c mice were purchased from Shanghai Experiment Animal Center (Shanghai, China) and divided into 7 groups. Each group contained 5 mice. For DNA immunization, mice were injected intramuscularly (*i.m.*) *via* quadricep muscles with 100 μg of pcDNA3.1-N or pcDNA3.1 plasmid DNA dissolved in 100 μl sterile PBS (100 μg/100 μl). For recombinant adenovirus immunization, mice were injected intraperitoneally (*i.p.*) with 1.55×10^9 or 2.4×10^8 plaque-forming units of rAd-N or rAd-LacZ diluted in 0.5 ml sterile PBS. Each immunization was at 2-week intervals. The detailed immunization procedure and the time of collecting samples are shown in Table 1.

Detection for SARS-CoV-N-specific IgG antibody

The BALB/c mice were sacrificed by cervical dislocation after their blood had been collected from the retro-orbital plexus using a capillary tube at week 8. Blood was collected and incubated for 4 h at 37°C, centrifuged for 10 min at 8,000 × g, and the supernatants were transferred into new sterile tubes. For inactivation, sera were incubated for 30 min at 55°C and stored at -20°C. The antibody activity of these sera was determined by enzyme-linked immunosorbent assay (ELISA). The ELISA kit for the diagnosis of SARS-CoV infection was purchased from Beijing Huada Aijier Corp. (Beijing, China). In this kit, 96-well plates were coated with inactivated SARS-CoV and the kit was used according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm with 600 nm for a reference wavelength. The antibody titers were expressed relatively as the values of $OD_{450\ nm}$.

IFN-γ assay

Spleens were removed from mice that had been sacrificed by cervical dislocation. Splenocytes separated from individual mice of each group were used for interferon-γ (IFN-γ) detections. After washing three times with PBS (pH 7.4), the splenocytes were resuspended at a final concentration of 2×10^6 cells/ml cultured in 12-well plates, and of 5×10^5 cells/well cultured in flat-bottom 96-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/ml L-glutamine, 50 μmol mercaptoethanol and 20 IU/ml of interleukin-2 (Promega) for detection. The suspensions were restimulated with recombinant N protein (5 μg/ml) for 72 h in 5% CO_2 at 37°C as experimental groups. Splenocytes stimulated with phytohemagglutinin (PHA, Guangzhou, China) at a concentration of 10 μg/ml were used as positive controls, and those without stimulation were used as negative controls. Each culture condition was assessed in triplicate. For the IFN-γ measurement, culture supernatants were harvested after 72 h of culture and stored at -70°C until assayed. IFN-γ levels in culture supernatants were measured by using mouse IFN-γ ELISA kit (Jingmei, China).

Results

Construction of N gene recombinant adenovius

Kanamycin-resistant positive clones of N gene recombinant

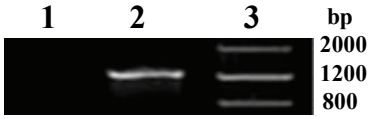

Figure 1. PCR for identification of SARS-CoV-N gene recombinant transfer vector plasmid. Lane 1, amplification of empty transfer vector for negative control; Lane 2, amplification of N gene recombinant transfer vector; Lane 3, DNA marker.

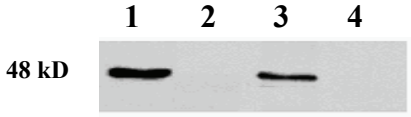

Figure 3. Western blot analysis for N protein expression. Lane 1, lysates of SP2/0 cells transfected with pcDNA3.1-N; Lane 2, lysates of SP2/0 cells transfected with pcDNA3.1 as negative control; Lane 3, lysates of Vero cells infected with rAd-N; Lane 4, lysates of Vero cells infected with rAd-LacZ as negative control.

transfer vector plasmids were amplified as templates by using PN1-PN2 primer pairs. Approximately 1,273 bp long DNA band was observed by using 1.5% agarose gel electrophoresis (Figure 1). The sequencing results of two putative clones among them were the same as N gene sequence of AY304495 in GenBank. Positive clones of N gene recombinant transfer vector DNA plasmid were mixed with adenoviral expression vector DNA to generate the N gene recombinant adenoviral DNA plasmid through homologous recombination. After recombination, positive clones were identified and purified for the next step of digestion with *Pac* I. These digested plasmid clones and *LacZ* gene recombinant adenovirus plasmids (digested with *Pac* I) were transfected 293A cells by using Lipofectamine 2000^{TM}. When approximately 50-70% of CPE was observed (Figure 2), adenovirus-containing cells and media were harvested and the titer was measured by the End-Point Dilution Assay. The titers of the two recombinant adenoviral stocks were calculated as 3.1×10^9 pfu/ml and 4.8×10^8 pfu/ml, respectively.

Expression of the SARS-CoV N protein in pcDNA3.1-N-transfected cells and rAd-N-infected cells

Whether the recombinant adenovirus, rAd-N, could express the SARS-CoV-N protein in Vero cells was examined. Vero cells infected with rAd-N or rAd-LacZ were lysed and subjected to Western blotting analysis. A 48-kD band corresponding to the SARS-CoV-N protein was clearly detected in rAd-N-infected cells, but not observed in rAd-LacZ-infected Vero cells, as demonstrated in Figure 3. SP2/0 cells transfected with pcDNA3.1-N and pcDNA3.1 were selected in medium containing G418 for 2 weeks.

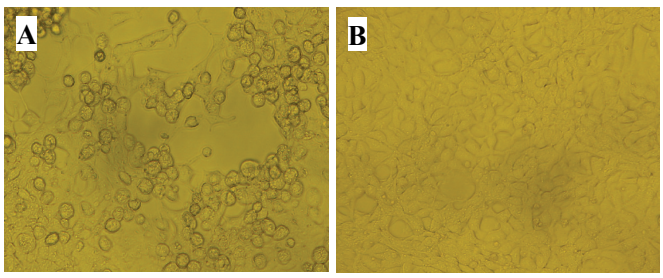

Figure 2. Observation of CPE. (A) CPE was observed in recombinant adenovirus-containing 293A cells. (B) Normal 293A cells were cultured as experimental control.

G418-resistant stable clones were screened by Western blot assay. A 48-kD band corresponding to the SARS-CoV-N protein was observed in the lane of SP2/0 cells transfected with pcDNA3.1-N, but not in the lane of pcDNA3.1-transfected cells as shown in Figure 3.

ELISA assay of the anti-SARS-CoV-N IgG antibody

SARS-CoV-N-specific antibody response was assessed using the standard ELISA kit for diagnosis of SARS-CoV infection. When the sera from immunized mice were separated at week 8, the mean antibody levels of the different immunized groups in the induction of an N-protein-specific antibody response were detected, and the results were shown in Figure 4. Among those 7 groups, the maximum 1.258 ± 0.231 was induced by a combination of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N at week 8. The heterogeneous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N, pcDNA3.1-N/pcDNA3.1-N/rAd-N/rAd-N, and pcDNA3.1-N/rAd-N/rAd-N/rAd-N were more efficient in the induction of SARS-CoV-N-specific antibody response than the two homologous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N and rAd-N/rAd-N/rAd-N/rAd-N at week 8. Among all humoral immune response experiments, the combination of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N induced the highest SARS-CoV-N specific antibody response after 8 weeks in BALB/c mice.

IFN-γ secretion assay

To analyze the cellular immune response, splenocytes restimulated with recombinant N protein, PHA, and without stimulation were used as positive or negative experimental control groups, respectively, for the IFN-γ assay. In the culture, the secretion of IFN-γ was detected using a standard ELISA, and the results showed that the mice immunized with the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N combination induced the maximum (186.35 μg/ml) SARS-CoV-N-specific IFN-γ. The other combinations induced relatively less amounts (45.68, 145.90, 123.44, 146.87, < 17, and < 17 μg/ml) of IFN-γ specific to SARS-CoV-N protein (Figure 5).

Discussion

Genetic immunization with DNA vaccines induces both antibody and cytotoxic T lymphocytes (CTLs) because they are expressed through the class I and class II antigen-

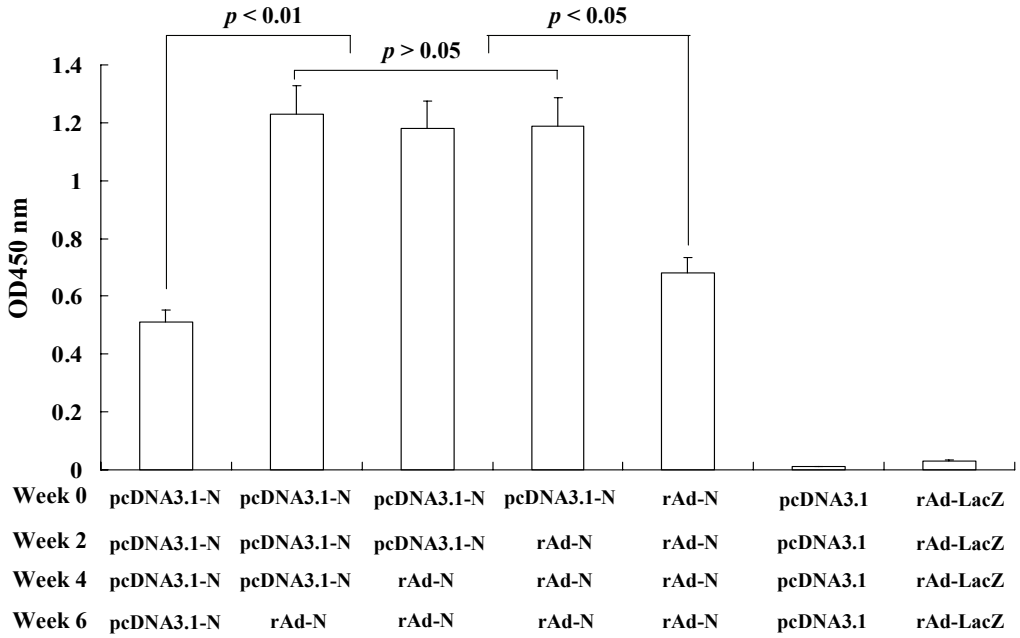

Figure 4. ELISA assay of SARS-CoV N-protein specific IgG antibody. Mice were immunized with the combinations as shown in Table 1, respectively, in prime-triple boosts immunization at 100 μg per mouse of pcDNA3.1-N or pcDNA3.1 and rAd-N were immunized at 1.55×10^9 pfu and rAd-LacZ at 2.4×10^8 pfu per mouse at two-week intervals. ELISA was used to measure IgG antibody titers in sera at a dilution of 1:100 of individual mouse and data are expressed as mean ± SD based on the OD values of 450 nm of five mice in each group.

processing pathway (14). Therefore, DNA vaccine approaches have been applied to generate protective immunity to various pathogens (15-20), but to date the strength of the immune response induced by DNA vaccines has been relatively weak compared with conventional vaccines. Different methods have been tried to increase the effectiveness of DNA immunization. Priming with a DNA vaccine can augment the efficacy of vaccines based on recombinant viral vectors. The rationale behind this strategy is that DNA priming elicits low-level but persistent immunity followed by strong boosting with virus encoding the same recombinant antigen as the DNA encodes (21). The ability of the prime-boost regimen to induce a higher response than the DNA vaccine or virus vaccine alone may relate to the ability of recombinant adenovirus to interfere with the maturation of infected dendritic cells, preserving efficient Ag presentation to CD8 T cells (22). So far, the most successful DNA immunization is likely to be a consecutive immunization involving priming with plasmid DNA and boosting with recombinant virus (23-25). The advantage of such recombinant viral vaccines is their high efficacy in generating humoral and cellular immune responses. A number of different viruses, such as adenovirus, vaccinia, rabies virus, canarypox virus, simian immunodeficiency virus and murine leukemia virus, have been used to construct recombinant viral vaccines (26-28). Among these, the replication-defective recombinant adenovirus is viewed to be a favorable choice as a viral vector vaccine, since it appears both to be safe and to induce strong humoral and cellular antigen-specific immune responses (29-31).

In this study, we tried to investigate which combination induced enhanced humoral and cellular immune responses after mice had been immunized with pcDNA3.1-N and rAd-N in different regimen. These experiments demonstrated that the DNA vaccine (pcDNA3.1-N) prime-adenovirus vector (rAd-N) boost regimen greatly enhanced the induction of a humoral and cellular immune response. The two homologous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N and rAd-N/rAd-N/rAd-N/rAd-N were relatively weaker than the heterogeneous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N, pcDNA3.1-N/pcDNA3.1-N/rAd-N/rAd-N, and pcDNA3.1-N/rAd-N/rAd-N/rAd-N in the induction of SARS-CoV-N-specific antibody response at week 8. The combination of DNA vaccine was not more efficient than that of recombinant virus vaccine in the induction of humoral and cellular immune response. The regimen of rAd-N/rAd-N/rAd-N/rAd-N was not stronger than any of the heterogeneous combinations in the induction of antibody production. It is likely that the repeated immunizations with recombinant adenovirus vectors may induce sufficient vector immunity to interfere with the presentation of the transgene upon subsequent boosts (32). In the induction of IFN-γ production, the homologous combination of rAd-N/rAd-N/rAd-N/rAd-N was significantly stronger than that of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N, but was relatively weaker than the heterogeneous combination of pcDAN3.1-N/pcDAN3.1-N/pcDAN3.1-N/rAd-N. Unfortunately, we were not able to detect the activities of pcDNA3.1-N prime-rAd-N boosts in

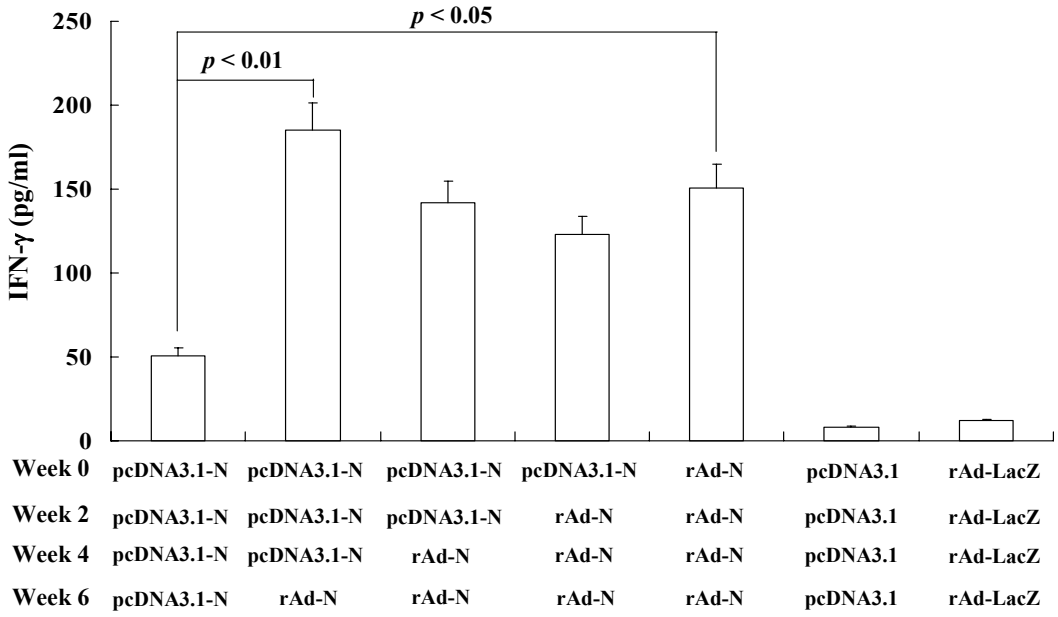

Figure 5. ELISA assay of IFN-γ secretion. IFN-γ levels were measured in splenocyte culture supernatants from single mouse of each immunized group by using standard ELISA assay. Levels of IFN-γ were showed as the mean ± SD of five mice in each group.

the induction of humoral and cellular immune responses at weeks 0, 2, 4 and 6, and lack the other data of antibody or IFN-γ production after the first, second and third immunization.

In summary, among all combinations of pcDNA3.1-N prime-rAd-N boost regimen, pcDAN3.1-N/pcDAN3.1-N/pcDAN3.1-N/rAd-N induced the strongest humoral and cellular immune response, such as antibody, IFN-γ production. These data should aid in the design of effective vaccine to prevent the spread of this new emerging pathogen.

Acknowledgement

This work was supported by the Education Department Foundation of Jiangsu Province, China (No.JH03-054).

References

1. Rota PA, Oberste MS, Monroe SS, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science. 2003;300:1394-1399.
2. Butter YSN, Khattra J, Asano JK, et al. The genome sequence of the SARS-associated coronavirus. Science. 2003;300:1399-1404.
3. Wang J, Wen J, Li J, et al. Assessment of immunoreactive synthetic peptides from the structural proteins of severe acute respiratory syndrome coronavirus. Clin Chem. 2003;49:1989-1996.
4. Zhou T, Wang D, Luo T, et al. An exposed domain in the severe acute respiratory syndrome coronavirus spike protein induces neutralizing antibodies. J Virol. 2004;78:7217-7226.
5. Bisht H, Roberts A, Vogel L, et al. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc Natl Acad Sci U S A. 2004;101:6641-6646.
6. He Y, Zhou Y, Liu Z, et al. Receptor-binding domain of SARSCoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. Biochem Biophys Res Commun. 2004;324:773-781.
7. Che XY, Hao W, Qiu LW. Antibody response of patients with severe acute respiratory syndrome (SARS) to nucleocapsid antigen of SARS-associated coronavirus. Di Yi Jun Yi Da Xue Xue Bao. 2003;23:637-639.
8. Kim TW, Lee JH, Hung CF, et al. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. J Virol. 2004;78:4638-4645.
9. Gao W, Tamin A, Soloff A, et al. Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet. 2003;362:1895-1896.
10. Wang ZJ, Yuan M, Matsumot UR, Hengge YF, Chang ZH. Immune response with DNA vaccines encoded different gene fragment of severe acute respiratory syndrome coronavirus in BALB/c mice. Biochem Biophys Res Commun. 2005;327:130-135.
11. Zhu MS, Pan Y, Chen HQ, et al. Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine. Immunol Lett. 2004;92:237-243.
12. Robinson HL, Pertmer TM. DNA vaccines for viral infections: basic studies and applications. Adv Virus Res. 2000;55:1-74.
13. Matsui M, Moriya O, Akatsuka T. Enhanced induction of hepatitis C virus-specific cytotoxic T lymphocytes and protective efficacy in mice by DNA vaccination followed by adenovirus boosting in combination with the interleukin-12 expression plasmid. Vaccine. 2003;21:1629-1639.
14. Corr M, Lee DJ, Carson DA, Tighe H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. J Exp Med. 1996;184:1555-1560.
15. Rodriguez F, Whitton JL. Enhancing DNA immunization.

Viralogy. 2000;268:233-238.
16. Lewis PJ, van Drunen Littel-van, den Hurk S, Babiuk LA. Altering the cellular location of an antigen expressed by a DNA-based vaccine modulates the immune response. J Virol. 1999:73:10214-10223.
17. Leitner WW, Hwang LN, DeVeer MJ, et al. α virus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. Nat Med. 2003;9:33-39.
18. Perkus ME, Tartaglia J, Paoletti E. Poxvirusbased vaccine candidates for cancer, AIDS, and other infectious diseases. J Leukoc Biol. 1995;58:1-8.
19. Tartaglia J, Pincus S, Paoletti E. Poxvirusbased vectors as vaccine candidates. Crit Rev Immunol. 1990;10:13-21.
20. Kuriyama S, Tominaga K, Kikukawa M, et al. Inhibitory effects of human sera on adenovirusmediated gene transfer into rat liver. Anticancer Res. 1998;18:2345-2351.
21. Berzofsky JA, Ahlers JD, Belyakov IM. Strategies for designing and optimizing new generation vaccines. Nat Rev Immunol. 2001;1:209-219.
22. Barouch DH, Santra S, Kuroda MJ, et al. Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. J Virol. 2001;75:5151-5158.
23. Amara RR, Villinger FJD, Altman SL, et al. Control of a mucosal challenge and revention of AIDS by a multiprotein DNA/MVA vaccine. Science. 2001;292:69-74.
24. Yang ZY, Wyatt LS, Kong WP, Moodie Z, Moss B, Nabel GJ. Overcoming immunity to a viral vaccine by DNA priming before vector boosting. J Virol. 2003;77:799-803.
25. Schneider J, Gilbert SC, Blanchard TJ. Enhanced immunogenicity for $CD8^+$ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nat Med. 1998;4:397-402.
26. Tartaglia J, Pincus S, Paoletti E. Poxvirus-based vectors as vaccine candidates. Crit Rev Immunol. 1990;10:13-21.
27. Kuriyama S, Tominaga K, Kikukawa M, et al. Inhibitory effects of human sera on adenovirus ediated gene transfer into rat liver. Anticancer Res. 1998;18:2345-2351.
28. Berzofsky JA, Ahlers JD, Belyakov IM. Strategies for designing and optimizing new generation vaccines. Nat Rev Immunol. 2001;1:209-219.
29. Casimiro DR, Chen L, Fu TM, et al. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J Virol. 2003;77:6305-6314.
30. Xiang Z, Gao G, Reyes-Sandoval A, et al. Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. J Virol. 2002;76:2667-2678.
31. Xiang ZQ, Yang Y, Wilson JM, Ertl HC. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. Virology. 1996;219:220-231.
32. Murata K, Garcia-Sastre A, Tsuji M, et al. Characterization of *in vivo* primary and secondary $CD8^+$ T cell responses induced by recombinant influenza and vaccinia viruses. Cell Immunol. 1996;173:96-107.