

CHENG Anchun, WANG Mingshu, CHEN Xiwen, XINI Nigen, DOU Wenbo, LI Xuemei, LIU Wumei, WANG Gang, ZHANG Pingying

Cellular immune responses of BALB/c mice induced by intramuscular injection of PRRSV-ORF5 DNA vaccine with different doses

© Higher Education Press and Springer-Verlag 2007

Abstract BALB/c mice were immunized with 50 µg, 100 µg, 200 µg of pcDNA-PRRSV-ORF5 DNA vaccine respectively by intramuscular injection, with PBS and pcDNA3.1(+) as controls. Fluorescence activated cell Sorter (FACS) was used to detect the number of CD₄⁺ and CD₈⁺ T-lymphocytes. T-lymphocyte proliferation test was used to detect proliferation of the T-lymphocyte cells in peripheral blood lymphocytes of mice vaccinated with pcDNA-PRRSV-ORF5 DNA vaccine. The results showed that the difference in ConA response to T-lymphocytes in blood was highly significant between all experimental groups and the control group ($P < 0.01$). The number of CD₄⁺ T-lymphocytes in experimental groups was significantly higher than that of the control group 7 d after vaccination. The number of CD₈⁺ T-lymphocytes in the experimental groups was higher than that of the control group 28 d after vaccination. Mice immunized with a higher dose (200 µg) of DNA vaccine demonstrated higher cellular immune response than those immunized with a lower dose (100 µg, 50 µg) of DNA vaccine. The results demonstrated that pcDNA-PRRSV-ORF5 DNA vaccine

could induce a good cellular immune response which may be dose-dependent.

Keywords PRRSV, ORF5 DNA vaccine, cellular immune response, mice

1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease of swine that was first recognized in the United States in 1987 and in Europe in 1990 (Keffaber, 1989). PRRS has caused calamitous economic losses in the pig industries of the related countries (Goyal, 1993; Wensvoort, 1994). This disease, once called “mystery swine disease (MSD)” and “Blue-eared disease of pigs”, has already spread to the Asia-Pacific countries and regions in recent years. It is currently one of the more serious diseases causing economic losses in pig industries world wide (Shin et al., 1993). Due to factors such as air propagation, continued infection, sub-clinical infection, immunosuppression, etc., the prevention of PRRS faces difficulties. However, the appearance of genetic engineering vaccines and gene vaccines has brought new hope for the prevention and treatment of the PRRS. The gene vaccine (nucleic acid vaccine) is made from the carrier and the genetic fragment of the pathogen that codes for the protective immune response. The gene vaccine which enters the organism is not combined with the host chromosome, but it can express proteins bringing out various kinds of immune responses including humoral and cellular immune responses. Compared with routine recombinated vaccines, the gene vaccine is easy to structure and prepare, stable in quality, and can lead to induction of humoral and cellular immune responses via different routes (Yankauckas et al., 1993). In this study, BALB/c mice were immunized intramuscularly with pcDNA-PRRSV-ORF5 DNA vaccine of Sichuan isolate using different doses. The purpose of this study was to explore the pathway of the cellular immune response

Translated from *Chin J Vet Sci Mar*, 2006, 26(2): 111–114 [译自: 中国兽医学报]

CHENG Anchun (✉), WANG Mingshu (✉), CHEN Xiwen
Key Laboratory of Animal Disease and Human Health of Sichuan Province, Ya'an 625014, China
E-mail: chenganchun@vip.163.com

CHEN Xiwen, CHENG Anchun, WANG Mingshu, XINI Nigen, DOU Wenbo, LI Xuemei, LIU Wumei, WANG Gang, ZHANG Pingying
College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Yaan, Sichuan 625014, China

CHEN Xiwen
Institute of Applied Animal Technology, Mianyang Normal College, Mianyang 621000, China
Key Laboratory for Molecular Biology and Biopharmaceuticals, Mianyang Normal College, Mianyang, Sichuan 621000, China

The first three authors contribute equally to this work.

induced by intramuscular injection of different doses of the laboratory-made pcDNA-PRRSV-ORF5 DNA vaccine, and to provide scientific basis for clinical practice of the gene vaccine.

2 Materials and methods

2.1 Plasmid and vaccine

pcDNA3.1 (+) plasmid: invitrogen products; pcDNA-PRRSV-ORF5 including ORF5 gene, and the GP5 protein of ORF5 code, as an envelope glycoprotein, which was the most important protein in the immunity of PRRSV, were prepared in the authors' laboratory.

2.2 Experimental animals

100 three-week-old female BALB/c mice that each weighed from 18–22 g, with the serum anti-PRRSV antibody negative, were provided by the Laboratory Animal Centre of Sichuan University.

2.3 Main reagent

RPMI1640 culture (GIBCO BRL Company) was compounded with distilled water according to instructions in the manual, and filtered to sterilize fungi. ConA (Sigma) was mixed with RPMI1640 to a concentration of 1 mg/mL, and filtered to sterilize fungi. MTT (Sigma) for T-lymphocyte proliferation test was mixed with PBS to a concentration of 5 mg/mL, and filtered to sterilize fungi. Lymphocyte-separating medium was provided by Shanghai Hua Jing Biological High-technology Co., Ltd.. FITC anti-mouse CD4⁺ and CD8⁺ monoclonal antibody were provided by Bio Legend Company.

2.4 Main instruments

The main instruments used in the experiment were as follows: Fluorescence-Activated Cell Sorter, FACS, (ELITE ESP, Coulter Company); Gene gun (PDS 1000 / He System type, BIO-RAD); High Speed Frozen Centrifuge (AllegraTM21R type); Enzyme-linked Immune Detector (ELx800 type, Biotek Company); Benchtop Horizontal Centrifuge (BECKMAN COULTERTM Company, U.S.A.).

2.5 Experimental design

100 three-week-old female BALB/c mice were randomly divided into 5 groups, 20 for each group. Groups A–C were given intramuscular injections of pcDNA-PRRSV-ORF5 at concentrations of 200 µg, 100 µg, and 50 µg respectively. Group D mice were given intramuscular injections of 200 µg pcDNA3.1 (+) plasmid, and Group E mice were intramuscularly injected with 200 µg PBS. All test groups were given the

immune injections twice at a 15 d interval. Before immunization, BALB/c mice were injected with 0.5% procaine hydrochloride at 100 µL per mouse in the left and right quadriceps femoris for pretreatment, thus relaxing the muscles and improving the absorbing rate of the DNA. Immunization with the DNA was then done on the same site after 3 d.

2.6 Collection of the blood specimens

Blood samples were randomly collected by extirpating the eyeballs of 3 mice from each group on the 7th, 15th, 28th, 41st, 55th, 70th day after the first immunization, respectively. The cellular and plasma components were separated, and the lymphocytes obtained were centrifuged. These were later used to make the lymphocyte transformation tests and for the analyses of the changes in peripheral blood T-lymphocyte subpopulations of CD4⁺ and CD8⁺.

2.7 Lymphocyte transformation tests

2.7.1 Separation of peripheral blood lymphocytes

This was carried out based on the work of Jin (2002). A mixture of 0.5 mL blood coagulant with 0.5 mL Hank's liquid was gently added to 1 mL of the lymphocyte liquid. This was then centrifuged at 2 000 r/min for 15 min to carefully collect the intermediate layer lymphocytes. Hank's liquid was used to wash and precipitate the lymphocytes twice, and complete RPMI1640 culture (including 200 U/mL penicillin, 200 µg/mL streptomycin, 10% Calf Serum and 20 mmol/L Hepes) was used to dilute the cells and make a single cell suspension at 1×10^7 /mL.

2.7.2 Inducing culture of the lymphocytes

Based on the research done by Liu et al. (1999), 100 µL single-cell suspensions were placed in a 96-hole cultivating board with a ConA concentration of 25 µg/mL, with controls negative. Each sample was placed in 3 holes and cultivated in 5% CO₂ at 37°C for 68 h. After 10 µL MTT (5 mg/mL) was added per hole, each sample was continually cultivated for 4 h, and finally, two hours after 100 µL of 10% SDS at 0.04 mol/L HCl (acid SDS) was added per hole, the reaction was ended.

2.7.3 Detection and data processing

The values of OD_{490nm} determined by Enzyme-Linked Immune Detector were statistically processed using SPSS10.0 and Sigma-Plot 9.0.

2.8 T-lymphocyte subpopulation detection

2.8.1 Pretreatment of peripheral blood lymphocytes

0.1 mL blood coagulant was added to 8 mL RBC Lysis Buffer, keeping the buffer at room temperature for 10 min and

then centrifuging it at 1 500 r/min for 10 min. The supernatant was removed, evenly mixed with 5 mL PBS and then re-centrifuged at 1 500 r/min for 10 min.

2.8.2 Fluorescence Isothiocyanate FITC marked T-lymphocyte

FITC-marked monoclonal anti-mouse antibody (code: 111) at 0.1 mg/mL, was diluted 10 times (0.01 mg/mL). From this, 0.5 mL cell supernatant per tube was taken, to which 10 μ L (0.1 μ g) monoclonal antibody was added. Keeping the temperature at 4°C for an hour, 1 mL PBS buffer liquid was added and the resulting solution mixed evenly at a low speed. This was then centrifuged at a force of 200 \times g for 5 min, and after removing the supernatant, the bottom cells were suspended in 1 mL PBS and examined.

2.8.3 FACS measuring and data processing

3 000 cells were tested by FACS, and the data were statistically processed by using SPSS10.0 and Sigma-Plot 9.0 to get the mean and sedimentation rate.

3 Results

3.1 Clinical features of BALB/c mice after the vaccine inoculation

BALB/c mice showed no changes in vigor and appetite after being inoculated with the gene vaccine pcDNA-PRRSV-ORF5 in different doses. Examination of the organ samples taken from the mice in all test groups revealed no visible pathological changes in morphology.

3.2 Results of T-lymphocyte proliferation testing

After BALB/c mice were immunized with different doses of pcDNA-PRRSV-ORF5 DNA vaccine by intramuscular

injection, the values of OD₄₉₀ from groups A, B and C were seen to be higher than those in groups D and E beginning on the 7th day after immunization. They reached a peak on the 41st day, and then dropped gradually, with the difference between the test and control groups being extremely remarkable ($P < 0.01$) at this point. There was no remarkable difference within groups A, B, and C ($P > 0.05$), but in the course of the entire test, the value taken from Group A was noted to be slightly higher than those from groups B and C. Fig. 1 shows the result in details.

3.3 Dynamic changes in the peripheral blood T-lymphocyte subpopulation

3.3.1 The CD4⁺ in the peripheral blood of BALB/c mice after different inoculations

The quantities of CD4⁺ peripheral blood lymphocytes from groups A, B and C were higher than those from groups D and E beginning on the 7th day after immunization, up to the peak ($P < 0.01$) on the 55th day. There were no remarkable differences within groups A, B, and C ($P > 0.05$), but in the course of the entire test, the value taken from Group A was slightly higher than those from the other two groups. The detailed results are shown in Fig. 2.

3.3.2 The CD8⁺ T-lymphocytes in the peripheral blood of BALB/c mice after different inoculations

The quantities of CD8⁺ periphery blood T-lymphocytes from groups A, B, C, D and E all dropped gradually from the 7th day after immunity, reaching the lowest levels on the 15th day, then rose gradually, and later declined to some degree. Groups A, B, and C values were higher in contrast to groups D and E, and extremely notable on the 41st day ($P < 0.01$). However, these later approached the values of the control group rapidly. Fig. 3 shows the results in details.

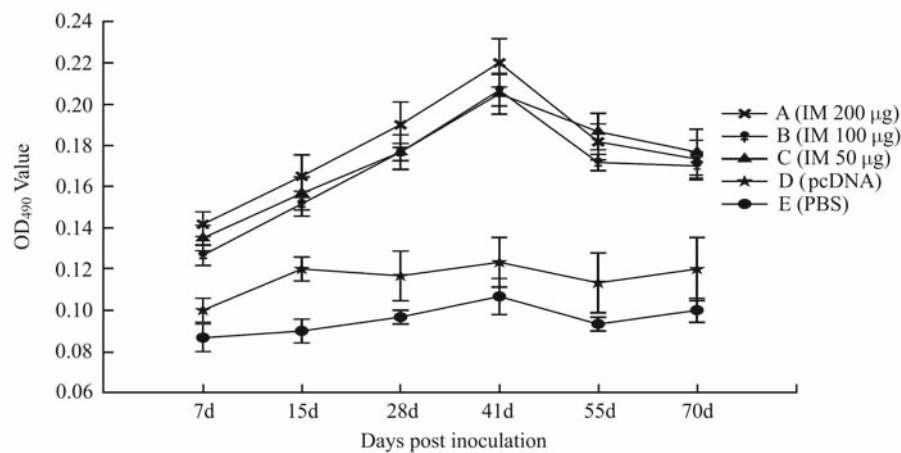


Fig. 1 Changes of T-lymphocyte proliferation test OD₄₉₀ value after inoculation at different doses in BALB/c mice

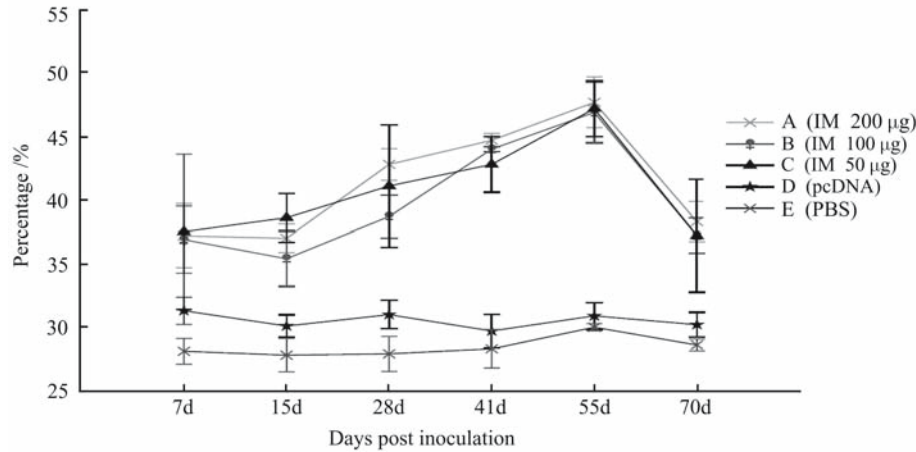


Fig. 2 Changes of CD₄⁺ in the peripheral blood of BALB/c mice after inoculation at different doses

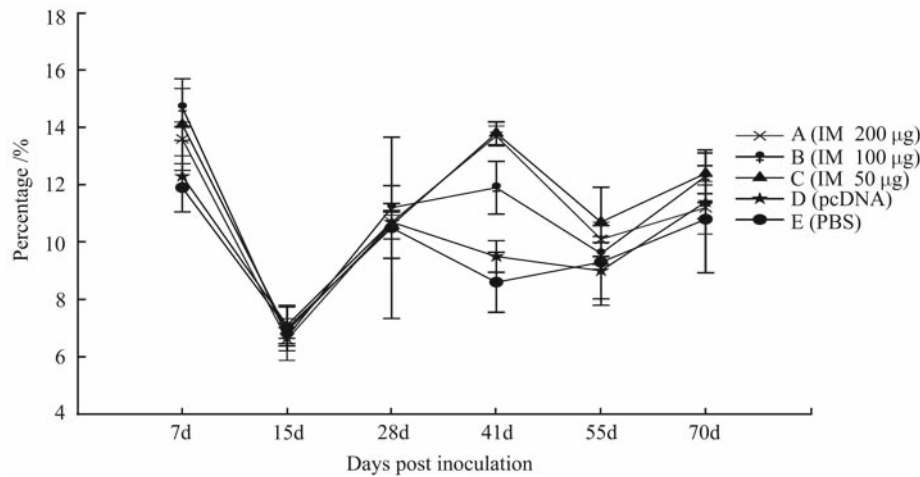


Fig. 3 Changes of CD₈⁺ in the peripheral blood of BALB/c mice after inoculation at different doses

4 Discussion

4.1 Lymphocyte proliferation test

There is a close relationship between an organism's immunogenicity and the corresponding response of T and B lymphocytes. Under the stimulation of antigenic and mitotic ConA and LPS, the efficiency of lymphocytes turning into lymphoblasts is an important index in judging immune function (Liu, 1989). ConA induces T-lymphocyte hyperplasia and LPS B lymphocyte hyperplasia. After BALB/c mice were immunized intramuscularly with different doses of pcDNA-PRRSV-ORF5 DNA vaccine, peripheral blood lymphocytes demonstrated obvious responsiveness to ConA, thus proving that the gene vaccine can induce a cellular immune response. Meanwhile, peripheral blood lymphocytes also manifested responsiveness to ConA after BALB/c mice were immunized with pcDNA3.1 (+). Therefore, induction of T-lymphocyte hyperplasia is not specific to ConA. The plasmid DNA contains immune stimulate array (ISS) with non-methylation

CpG as its central part. It can strengthen the immune effect, therefore, the plasmid of the gene vaccine itself can induce an organism to secrete immune cytokines and act as an adjuvant (Krieg et al., 1998).

4.2 Dynamic changes in the CD4⁺ T-lymphocyte in the peripheral blood of BALB/c mice

The CD4⁺ T lymphocyte is the central cell in immune response (Gong, 2002). Generally speaking, after a virus infects a cell, virus duplication can cause a reduction in the quantity of CD4⁺ T-lymphocytes. Changes in the CD4⁺ T-lymphocytes represent the disease process. Therefore, they could determine the degree to which the virus has impaired immunity during an infection. The percentage of circulating CD4⁺ T-lymphocytes is related to the severity of a pig's disease. If the CD4⁺ T-lymphocyte proportion is small, a pig may be seriously infected. Therefore, a change in CD4⁺ T-lymphocyte quantity could provide a powerful new basis for early diagnosis and prognosis, as well as seeking rational

preventive and curative measures for the disease (Shimizu et al., 1996).

It was reported that after SPF piglets were inoculated with the PRRS virus, CD4⁺ T-lymphocyte quantity was counted and observed to have dropped notably for at least 2 weeks continuously (Feng et al., 2003). However, this research showed the CD4⁺ T-lymphocyte quantity was apparently higher than that of any control group until the 70th day after BALB/c mice were immunized with pcDNA-PRRSV-ORF5 gene vaccine. The results demonstrated that pcDNA-PRRSV-ORF5 DNA vaccine could induce mice to produce a good cellular immune response.

4.3 Dynamic changes of the CD8⁺ in the peripheral blood of BALB/c mice

The main function of CD8⁺ restricted cell's toxic T cell (CTL) is to discern and kill or wound the viral-infected cells (Cohen, 1993). This research result showed that pcDNA-PRRSV-ORF5 DNA vaccine could induce mice to produce certain cellular immune responses that CD8⁺ T-lymphocytes initiate (CTL) for a relatively short period of about 2 weeks. Meanwhile, on the 15th day, the CD8⁺ T-lymphocyte quantity of both experimental groups and control groups dropped due to vaccine inoculation, indicating that the host would have decreased defensive capability during this period. The results showed that the host's CD8⁺ T-lymphocyte dropped and was easily infected in the early vaccine inoculation. It therefore points out that protection should be strengthened during early vaccine inoculation.

CD4⁺ and CD8⁺ T-lymphocytes play a very important role in gene vaccine immune response (Doolan et al., 1996; Manickan et al., 1995). From dynamic changes in CD4⁺ and CD8⁺ T-lymphocyte quantity in the experimental groups, it can be seen that the CD4⁺ T-lymphocyte quantity was apparently higher than that of the control group from the 7th to the 70th day after immunization. The CD8⁺ T-lymphocyte quantity of each experimental group was higher than that of control groups for a very short period on the 41st day after immunization. The results indicate that the pcDNA-PRRSV-ORF5 gene might mainly induce mice to produce good cellular immune responses that CD4⁺ T-lymphocyte induces after vaccine inoculation.

4.4 Effect of different doses of pcDNA-PRRSV-ORF5 DNA vaccine

Many researches have already verified that intramuscular injection can make the Th1 and Th2 auxiliary immune response occur, which is a better immune method (Zelphati et al., 1998). However, intramuscular injection demands a large enough dosage so the dose of the nucleic acid vaccine is an important factor in inducing an effective immune response—too little may not induce an effective immune response, and too much may result in wasting the vaccine. If a

high-efficient immune response is induced, there must be a nucleic acid vaccine with enough quantities. Xiong et al. (2003) reported that if a large dose of hepatitis virus surface antigen (HgsAg) nucleic acid vaccine was inoculated in mice at a time, it could produce a high level cellular immune response, while a small dose might make an obvious cellular immune response occur after strengthening the immunity. Davis et al. (1997) used 1 µg, and 10 µg DNA, and separately injected the target denatured muscle. Results showed that the induced CTL reacted equally to that using 10 µg, and 100 µg DNA, and the reaction of CTL after immunity was induced depended on the dose of DNA received. Zeng et al. (2003) achieved the same result. In our research, there was a trend of large doses inducing higher cellular immune responses than small doses. This indicates that the pcDNA-PRRSV-ORF5 gene vaccine has some dose-dependent characteristics on cellular immune response function.

There is no definite conclusion as to the dose of nucleic acid vaccine injection that can induce the ideal immune response. It mainly depends on the different injecting techniques and the injected subjects. As to mice, intramuscular injection requires DNA dosage to be 10–200 µg directly with the syringe, and if too little, gene expression is incomplete; if too much, gene expression may not reflect a dose-dependent character. Barry and Johnston (1997) reported that when the nucleoprotein gene plasmid DNA with influenza virus was directly injected into mice in different doses (10–250 µg), the protective immune response would occur, but 50 µg DNA led to the highest immune response. Immune response dropped to some extent when DNA dosage rose to 250 µg. He pointed out that when the nucleic acid vaccine and other non-peculiar DNAs were together injected, this required less than the necessary dose of vaccine alone, and the dose of DNA and absorption of DNA were involved in the pathway leading to the immune response, and the antigen required to produce the cellular immune response was less than that needed to produce the humoral immune response. Results demonstrated that pcDNA-PRRSV-ORF5 DNA vaccine could induce good cellular immune response in the range of 50–200 µg, and in a dose-dependent manner.

Acknowledgements This work was supported by Sichuan Bio-tech Project (No. 01NG018-03), the National State Key Program Project of Science and Technology (No. 2004BA901A03), Program for New Century Excellent Talents in University, Ministry of Education, China (No. NCET-04-0906).

References

- Barry M A, Johnston S A (1997). Biological features of genetic immunization. *Vaccine*, 15(8): 788–791
- Cohen J (1993). Naked DNA points way to vaccines. *Science*, 259(5102): 1691–1692
- Davis H L, Brazolot Millan C L, Mancini M, McCluskie M J, Hadchouel M, Comanita L, Tiollais P, Whalen R G, Michel M L (1997). DNA-based immunization against hepatitis B surface antigen (HBsAg) in normal and HBsAg-transgenic mice. *Vaccine*, 15(8): 849–852

- Doolan D L, Sedegah M, Hedstrom R C, Hobart P, Charoenvit Y, Hoffman S L (1996). Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. *J Exp Med*, 183(4): 1739–1746
- Feng W H, Tompkins M B, Xu J S, Zhang H X, McCaw M B (2003). Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol*, 94(1–2): 35–45
- Gong F L (2000). *Medical Immunology*. Beijing: Science Press, 6 (in Chinese)
- Goyal S M (1993). Porcine reproductive and respiratory syndrome. *J Vet Diagn Invest*, 5(4): 656–664
- Jin B Q (2002). *The Cell and Molecule Immunology Experimental Technique*. Xi'an: Publishing House of the Fourth Army Medical University, 61–65 (in Chinese)
- Keffaber K K (1989). Reproductive failure of unknown etiology. *Am Assoc Swine Pract Newsletter*, (1): 1–9
- Krieg A M, Yi A K, Schorr J, Davis, H L (1998). The role of CpG dinucleotides in DNA vaccines. *Trends Microbiol*, 6(1): 23–27
- Liu M, Ma H, Li B Q (1999). Investigation of detecting murine lymphocyte proliferate response by MTT assay. *Chinese Journal of Laboratory Animal Science*, 9(3):146–149 (in Chinese)
- Liu Y B (1989). *Animal's Immunology Experimental Technique*. Changchun: The Science Tech Publishing House of Jilin, 226–330 (in Chinese)
- Manickan E, Rouse R J, Wire W S, Rouse B T (1995). Genetic immunization against herpes simplex virus. Protection is mediated by CD4+ T-lymphocytes. *Journal of Immunology*, 155: 259–265
- Shimizu M, Yamada S, Kawashima K, Ohashi S, Shimizu S, Ogawa T (1996). Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. *Vet Immunol Immunopathol*, 50(1–2): 19–27
- Shin J H, Kang Y B, Kim Y J (1993). Sero-epidemiological studies on porcine reproductive and respiratory syndrome in Korea. I. Detection of indirect fluorescent antibodies. *RDA J Agri Sci*, 35: 572–576
- Wensvoort G (1994). Porcine epidemic abortion and respiratory syndrome. In: *Proc 13th Int Pig Vet Soci Congr*. Bangkok, Thailand: 1994, 11–14
- Xiong Y L, Liu H P, Zu P, Jia Y Z, Zhang Y J (2003). Modulatory function of high dose hepatitis B surface antigen vaccine to cellular immune responses in mice. *Chinese Journal of Pathophysiology*, 19(9): 1250–1252 (in Chinese)
- Yankauckas M A, Morrow J E, Parker S E, Abai A, Rhodes G H, Dwarki V J, Gromkowski S H (1993). Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene. *DNA Cell Biol*, (12): 771–776
- Zelphati O, Nguyen C, Ferrari M, Felgner J, Tsai Y, Felgner P L (1998). Stable and monodisperse lipoplex formulations for gene delivery. *Gene Ther*, 5(9): 1272–1282
- Zeng Y, Wang E H, Yi X R, Zhang Y J (2003). Research on immunore-sponse of mice by different dose recombinant (yeast) hepatitis B vaccine. *China Pharmacist*, 6(9): 532–534 (in Chinese)