FULL PAPER *Virology*

Construction and Characterization of a Recombinant Canine Adenovirus Expressing GP5 and M proteins of Porcine Reproductive and Respiratory Syndrome Virus

Jinshun CAI[1,2], Yonghe MA[1], Jiaying LI[2], Changguo YAN[2], Rongliang HU[3] and Jiabao ZHANG[1]*

[1]*Laboratory Animal Center, Jilin University, Changchun, Jilin Province, 130062,* [2]*College of Agriculture, Yanbian University, Longjing, Jilin Province, 33400 and* [3]*Veterinary Institute, Academy of Military Medical Science, Changchun, Jilin Province, 13006, China*

(Received 12 February 2010/Accepted 17 March 2010/Published online in J-STAGE 12 May 2010)

ABSTRACT. The causative agent of porcine reproductive and respiratory syndrome (PRRS) is PRRS virus (PRRSV), which belongs to the family *Arteriviridae*. GP5/M protein complex of PRRSV binds to sialoadhesion expressed on the cells to infect the cells. In this study, we developed a canine adenovirus type 2 (CAV-2) recombinant, termed rCAV2-GP5/M, expressing GP5 and M proteins. To evaluate the immunogenicity of the recombinant virus, mice were inoculated subcutaneously with rCAV2-GP5/M, and specific antibodies against PRRSV in the sera were measured by enzyme-linked immunosorbent assay and the viral neutralization test. Two weeks post-immunization (w.p.i.), anti-PRRSV antibodies were detected in the sera, slightly increased by booster immunization at four w.p.i., and then gradually decreased. The viral neutralizing test showed that neutralizing antibodies were present in the sera collected at two w.p.i., increased by booster immunization, and reached the maximum titer at six w.p.i. Lymphocyte proliferation responding to PRRSV antigens was also observed from two w.p.i. Although further studies are needed to evaluate the usefulness of the recombinant virus to protect pigs from PPRSV, we succeeded in developing a candidate vaccine against PPRSV infection by using CAV-2 vector.

KEY WORDS: GP5, immunogenicity, M, porcine reproductive and respiratory syndrome virus, recombinant canine adenovirus.

J. Vet. Med. Sci. 72(8): 1035–1040, 2010

Porcine reproductive and respiratory syndrome (PRRS) was originally documented in the U.S.A. in 1980, and then rapidly spread to other pig-breeding countries and caused huge economical losses [8]. In China, PRRS was firstly recognized in northern China in 1995 and successively became epidemic in most pig farms within several years [7]. PRRS is characterized by late-term abortion, early farrowing, stillbirth and the birth of weak piglets, and is associated with the porcine respiratory disease complex in combination with secondary infections [3]. The causative agent of PRRS is PRRS virus (PRRSV), which belongs to the family *Arteriviridae*. Both GP5 and M proteins are the main structural proteins of PRRSV, and play important roles in viral pathogenecity and replication [14]. GP5 protein is 24.5–26 kDa in size, encoded by open reading frame (ORF) 5 of the viral genome, and induces neutralizing antibodies in pigs [1, 9, 21]. On the other hand, M protein is 18–19 kDa in size, encoded by ORF6, and contains neutralizing epitopes [4]. GP5 protein forms hetero-dimers with M protein in the endocytoplasmic reticulum that are packaged into viral particles [19]. Recently, Van Breedam *et al.* showed that the GP5/M glycoprotein complex directly interacts with a cellular receptor, sialoadhesion, to infect cells [20]. This interaction was dependent on the sialic acid binding capacity of sialoadhesion and on the presence of sialic acids on GP5 protein.

Canine adenovirus type 2 (CAV-2) infects a wide range of animals, including cats, dogs and pigs. The genome is 31 kilo base pairs (bp) in length and the E3 region is unnecessary for viral replication. A foreign gene can be inserted into the E3 region and CAV-2 is an excellent vector for expressing foreign antigens [11]. In this study, we utilized the CAV-2 virus to express GP5 and M proteins, intending to develop a vaccine candidate against PRRSV.

* CORRESPONDENCE TO: ZHANG, J., Laboratory Animal Center, Jilin University, 5333 Xi'an Road, Changchun, Jilin Province, 130062, China.
e-mail: zjb515@163.com

MATERIALS AND METHODS

Cells: Madin-Darby canine kidney (MDCK) cells [6] and MARC-145 cells (derived from rhesus monkey kidney) [12] were grown in minimum essential medium (MEM) (Invitrogen Corporation, Carlsbad, CA, U.S.A.) supplemented with 5–10% fetal bovine serum and antibiotics. These cells were cultured at 37°C with 5% CO_2 in air.

The amplification of GP5 and M genes from PRRSV strain CH1a: Primers GP51S and GP51R (Table 1) were designed according to the ORF5 sequence of strain CH-1a, which was isolated in China in 1996 [7]. *Eco*RV and *Bam*HI recognition sites were added to the ends of the primers, respectively. Primers M1S and M1R (Table 1) were designed according to the ORF6 sequence, and *Sma*I and *Xba*I sites were added to the ends of the primers, respectively.

GP5 and M genes of PRRSV were amplified by reverse transcription (RT)-polymerase chain reaction (PCR) using strain CH-1a [7] as a template. Viral RNA was extracted from PRRSV CH-1a-infected MARC-145 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA synthesized from viral RNA was subjected to PCR amplification. The PCR condition was as follows: one cycle of 94°C pre-denaturing for 3 min, 35

Table 1. Primers for amplifying PRRSV genes

Primers	Primer sequences	RE site introduced	PCR products
GP51S	5'-GAAGATATCTTCAGTATGTTGGGGAAATGCTTGACC- 3'	*Eco*RV	603 bp
GP51R	5'-TTTGGATCCGACGACCCCATTGTTCCGC- 3'	*Bam*HI	
M1S	5'-GTACCCGGGACCATGGGGTCGTCCTTAGATGACTTC- 3'	*Sma*I	525 bp
M1R	5'-GCGTCTAGATTTGGCATATTTGACAAGGTT- 3'	*Xba*I	

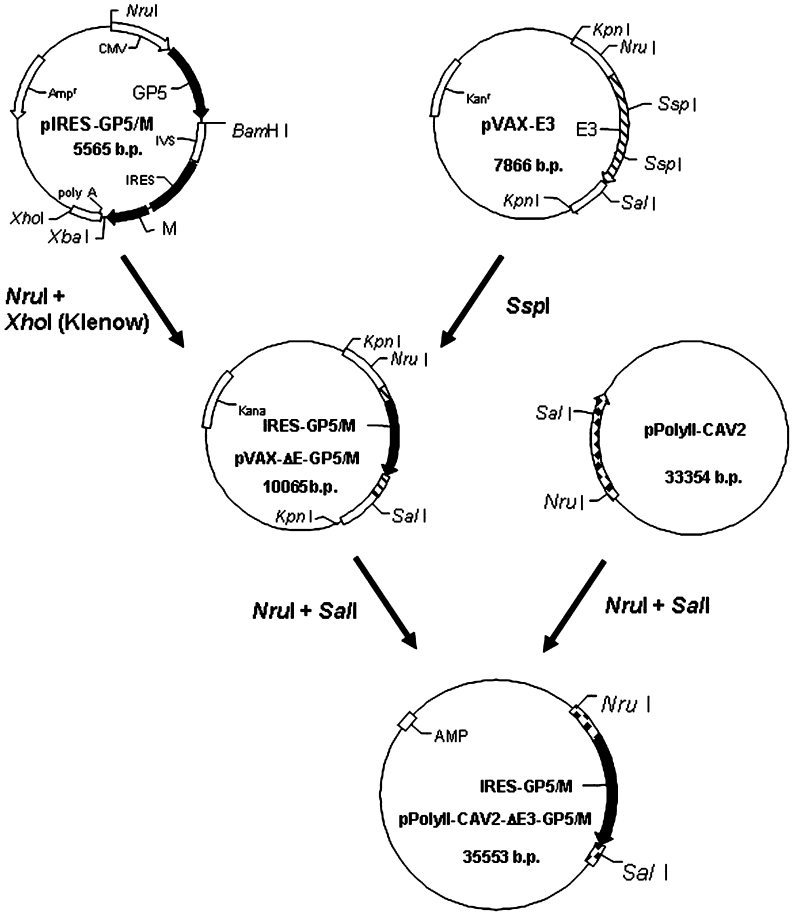

Fig. 1. Construction of recombinant adenovirus vector expressing GP5 and M proteins of PRRSV. Details are described in Materials and Methods.

cycles of 94°C denaturing for 45 sec, 62°C annealing for 45 sec, 72°C extension for 60 sec, and finally extension at 72°C for 10 min.

Construction of the recombinant viral genome: PCR amplified fragments of GP5 and M genes were digested with *Eco*RV and *Bam*HI, and *Sma*I and *Xba*I, respectively, and the digested fragments were cloned into pIRESneo to produce pIRES-GP5/M (Fig. 1). The pIRES-GP5/M was digested with *Xho*I and *Nru*I, and the expression cassette containing GP5 and M genes was recovered. This cassette was cloned into plasmid pVAX-E3 to produce pVAX-ΔE3-GP5/M (Fig. 1). Further, pVAX-ΔE3-GP5/M was digested with *Nru*I and *Sal*I, and the resulting fragment, ΔE3-GP5-M, was cloned into pPolyII-CAV2 digested with *Nru*I and *Sal*I. Consequently, the recombinant CAV-2 plasmid, termed pPolyII-CAV2-ΔE3-GP5/M, was obtained (Fig. 1).

Rescue of recombinant CAV-2 expressing GP5 and M proteins of PRRSV: Five micrograms of the recombinant CAV-2 plasmid pPolyII-CAV2-ΔE3-GP5/M, digested with *Asc*I and *Pme*I, were extracted and precipitated with ethanol. DNA was dissolved in MEM without serum and antibiotics, and then transfected into MDCK cells by Lipofectamine 2000^{TM} reagent (Invitrogen) according to the manufacturer's instructions. Cells were blind-passaged

until the cytopathic effect (CPE) appeared.

Detection of GP5 and M proteins by indirect immunofluorescent assay (IFA): Recombinant CAV-2, designated as rCAV2-GP5/M, was inoculated into MDCK cells and incubated for 24 hr. The culture medium was then discarded and washed twice with phosphate-buffered saline (PBS). For fixation, the infected cells were treated with cold ethanol for 30 min. After removal of the ethanol, the cells were washed with PBS. Fifty-fold diluted monoclonal antibodies against GP5 and M proteins, named 5D10 and 1A7, respectively [15, 16] were applied onto the cells and incubated at 37°C for 30 min. After washing with PBS six times, 100-fold diluted sheep anti-mouse-IgG conjugated with fluorescent isothiocyanate was applied onto the cells and incubated at 37°C for 30 min. After washing with PBS four times, the cells were observed under a fluorescent microscope.

Titration of rCAV2-GP5/M: The culture supernatant of MDCK cells infected with rCAV2-GP5/M showing CPE was recovered, filtrated through a 450 nm membrane filter and stored at –80°C in aliquots as stock virus until used. To titrate the stock virus, ten-fold serially diluted stock virus was inoculated into MDCK cells grown in a 96-multiwell plate. The plates were incubated at 37°C for five days and the presence of the virus was judged by CPE appearing in the wells. Virus titers were calculated using the method by Reed-Muench and expressed as 50% tissue culture infective dose ($TCID_{50}$)/m*l*.

Vaccination of mice with rCAV2-GP5/M: Fifty-four six-week-old Kunming inbred mice, purchased from the Experimental Animal Center, Changchun Biological Product Company (Changchun, China), were randomly divided into three groups, 18 in each group. Groups 1 and 2 were subcutaneously inoculated (0.5 m*l*/mouse) with rCAV2-GP5/M and wild-type (wt) CAV-2, respectively, and boosted 14 days after the initial vaccination. The third group was inoculated with PBS as a control. Sera were collected two, four, and six weeks after the booster and tested for the presence of neutralizing antibodies described in the following sections. Spleens from each group were pooled and analyzed for PRRSV-specific T-lymphocyte proliferation assay, as previously described [18]. To measure cell proliferation, we used a tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. The stimulation index (SI) was calculated as the ratio of average optical density at 570 nm (OD_{570}) value of wells containing PRRSV stimulated cells to the average OD_{570} value of wells containing only uninfected cell lysate with medium.

Enzyme-linked immunosorbent assay (ELISA): A commercial ELISA kit (HerdCheck PRRS; IDEXX Laboratories Inc., Westbrook, MA, U.S.A.) was used to determine PRRSV-specific antibody. Instead of using the anti-porcine IgG conjugated with peroxidase provided with the kit, a goat anti-mouse IgG conjugated with peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, U.S.A.) was used. The presence or absence of a specific antibody at a given dilution was determined using OD_{490} value rather than the sample-to-positive (S/P) ratio because the kit is designed and adjusted to detect swine IgG in sera.

Viral neutralization test: Two-fold serially diluted sera were mixed with an equal volume of 200 $TCID_{50}$ of PRRSV strain CH-1a. After incubation at 37°C for one hour, the mixtures were transferred to MARC-145 monolayers in quadruplicate in 96-well tissue culture plates. The plates were incubated and observed daily for up to five days for the appearance of CPE. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that completely inhibited the appearance of CPE.

RESULTS

Construction of GP5 and M expression cassette: GP5 and M genes of PRRSV strain CH-1a were amplified by RT-PCR and the amplicons were confirmed to be of the correct size (Fig. 2A). The structure of the recombinant plasmid, termed pIRES-GP5/M, harboring GP5 and M genes was verified by restriction enzyme (RE) digestion analysis using *Xho*I and *Nru*I (Fig. 2B). Furthermore, the sequences of GP5 and M genes and the junction regions of the inserted genes were verified by sequencing.

Production of rCAV2-GP5/M: The plasmid construct, pVAX-ΔE3-GP5/M, was confirmed by RE digestion analysis using *Sna*BI, *Kpn*I, and *Nru*I/*Sal*I (Fig. 2C). The recombinant virus, named rCAV2-GP5/M, was rescued by three consecutive blind passages on MDCK cells. The cells showed CPE characterized by cell rounding, enlargement, and grape-like clusters, as observed in cells infected with wt CAV-2. The structure of the recombinant viral genome was also confirmed by RE digestion analysis using *Pst*I, *Nru*I/*Sal*I, *Sac*I, *Sna*BI, and *Ssp*I. (Fig. 2D). The titer of the stock recombinant virus was $10^{5.6}$ $TCID_{50}$/m*l*.

MDCK cells inoculated with rCAV2-GP5/M were subjected to IFA using monoclonal antibodies against GP5 and M proteins of PRRSV (Fig. 3). Specific fluorescent signals against these proteins were observed in infected cells, demonstrating that both GP5 and M proteins were expressed in infected cells.

Immune responses induced by rCAV2-GP5/M in mice: Mice were inoculated subcutaneously with either rCAV2-GP5/M or wt CAV-2, and specific antibodies against PRRSV in the sera were measured by ELISA and the viral neutralization test. Two weeks post-immunization (w.p.i.), anti-PRRSV antibodies were detected in the sera, slightly increased by booster immunization at four w.p.i., and then gradually decreased (Fig. 4A). The viral neutralizing test showed that neutralizing antibodies were present in the sera collected at two w.p.i., increased by booster immunization, and reached the maximum titer at six w.p.i. (Fig. 4B).

PRRSV-specific T-lymphocyte proliferation was also observed from two w.p.i. and reached a peak at four w.p.i. after booster immunization, indicating that specific cellular responses were induced in the immunized mice efficiently (Fig. 5).

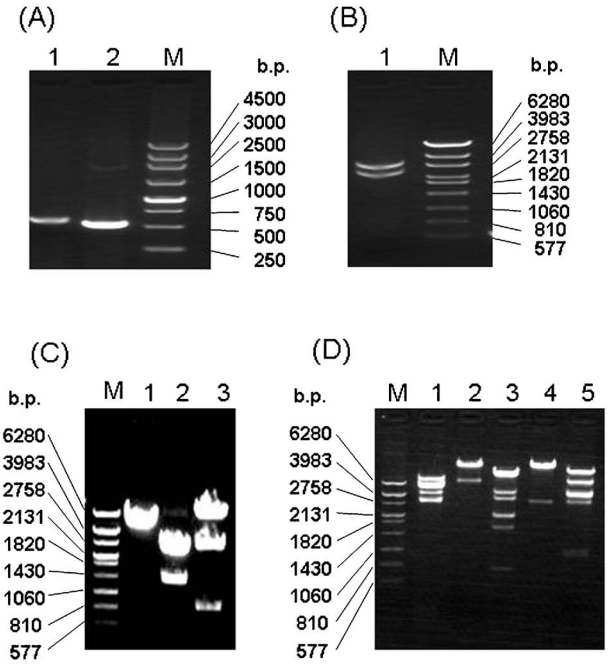

Fig. 2. (A) Amplification of GP5 and M genes of PRRSV by RT-PCR. cDNA synthesized from PRRSV viral genome was amplified using PCR primers for GP1 (lane 1) and M (lane 2) genes. Estimated sizes for amplified GP1 and M genes are 603 and 525 bp, respectively. (B) RE digestion analysis of pIRES-GP5/M. pIRES-GP5/M was digested with *Nru*I and *Xba*I (lane 1). Estimated sizes of the two fragments are 2,508 and 3,057 bp. (C) RE digestion analysis of the recombinant plasmid pVAX-ΔE3-GP5/M. pVAX-ΔE-GP5/M was digested with *Sna*bI (expected sizes are 5,301 and 4,764 bp) (lane 2), *Kpn*I (3,276, 2,906, 2,683 and 1,400 bp) (lane 3) and *Nru*I/*Sal*I (6,344, 2,721 and 1,000 bp). (D) RE digestion analysis of recombinant adenoviral DNA. rCAV2-GP5/M DNA was digested with *Pst*I (lane 1), *Nru*I/*Sal*I (lane 2), *Sac*I (lane 3), *Sna*bI (lane 4) and *Ssp*I (lane 5). DNA size makers are shown in lane M.

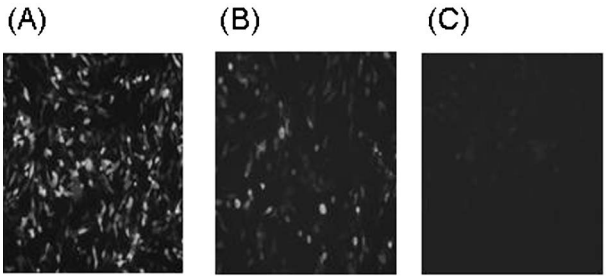

Fig. 3. Identification of the expression of GP5 and M proteins by IFA. MDCK cells were infected with rCAV2-GP5/M. Seventy-two hours after infection, the expressions of GP5 and M proteins were detected by IFA using anti-GP5 (A) and anti-M (B) monoclonal antibodies. Negative control without first antibody is shown in panel C.

DISCUSSION

In 1995, an outbreak of PRRS occurred in China and PRRSV was isolated from aborted fetuses. The isolate was designated as strain CH1a. [7]. To know the origin and genetic character of the PRRSV prevailing in China, Chou *et al.* determined the full nucleotide sequence of strain CH1a and compared the sequence with a reference strain isolated in the U.S.A. [5]. By phylogenetic analysis, strain CH1a was found to belong to the American type but not the European type [1]. Subsequently, several Chinese isolates (strains BJ-4, HUB1 and JXA1) of PRRSV were sequenced and also found to be the American type. In this study, we attempted to develop a recombinant vaccine to protect from the American type of PRRSV by using cDNA from strain CH1a.

To protect against PRRS, live attenuated and inactivated vaccines have been developed; however, the efficacy of the current vaccines is not satisfactory. Generally, live attenuated vaccines induce both humoral and cellular immunity in vaccinated animals, but current live attenuated vaccines protect pigs against only homologous or very similar strains of PRRSV due to the relatively high genetic variability of the virus. Live attenuated vaccines may restore virulence during replication in vaccinated animals. To overcome these drawbacks, many researchers are attempting to develop novel vaccines [2, 10, 17]. Notably, Jiang *et al.* expressed a fusion protein of GP5 and M proteins using a human adenovirus type 5 (HAV-5) vector [10]. When they inoculated the recombinant virus into human embryonic kidney 293 cells, these proteins were expressed and dimerized. When the recombinant virus was inoculated into pigs and mice, the animals produced a higher titer of neutralizing antibodies and lymphocyte proliferation responded to PPRSV antigens than animals inoculated with a HAV-5 recombinant expressing only GP5 protein [10].

CAV belongs to the group of mammalian adenoviruses, which includes CAV type 1 and 2. CAV-2 can infect dogs, foxes, raccoons and pigs with no or slight clinical signs and inducing a high titer antibody to CAV-2. Attenuated CAV-2 is a safe vaccine used for canine infectious laryngotracheitis. Using CAV-2 as a vector, recombinant vaccines expressing antigens of canine distemper virus and rabies virus have been constructed and induced protective immune responses in dogs and mink [13]. We thought that CAV-2 could be modified to be a recombinant vaccine to protect pigs from PPRSV.

In this study, we cloned GP5 and M genes into the multicloning site and downstream from the internal ribosomal entry site, respectively and then the expression cassette was cloned into E3-deleted CAV-2 vector. Recombinant CDV-2, named rCAV2-GP5/M, expressed both GP5 and M proteins efficiently in MDCK cells upon infection (Fig. 3). It was considered that both GP5 and M proteins were expressed in the cells in a more natural form than the fusion protein of GP5 and M proteins reported previously [10]. When rCAV2-GP5/M was inoculated into mice, immune

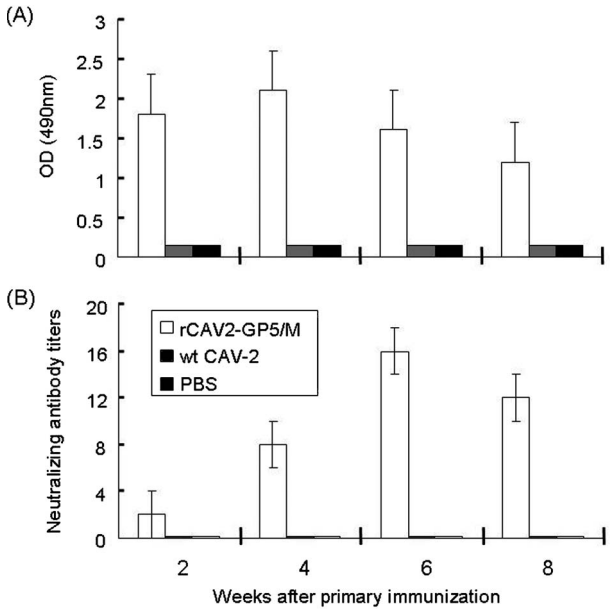

Fig. 4. (A) ELISA analysis of sera from mice immunized with rCAV2-GP5/M. Mice immunized with rCAV2-GP5/M compared to control mice immunized with wt CAV-2 or PBS are shown. Serum samples (n=6) were collected at various time-points and antibodies to PRRSV or wt CAV-2 were detected using a single dilution (1:100) ELISA. (B) Neutralizing antibodies against PRRSV were detected in mice immunized with rCAV2-GP5/M. Serum samples (n=6) were collected at various time-points and the titers of the neutralizing antibody were determined on MDCK cells. The titers of neutralizing antibodies were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Data are shown as the mean ± SE.

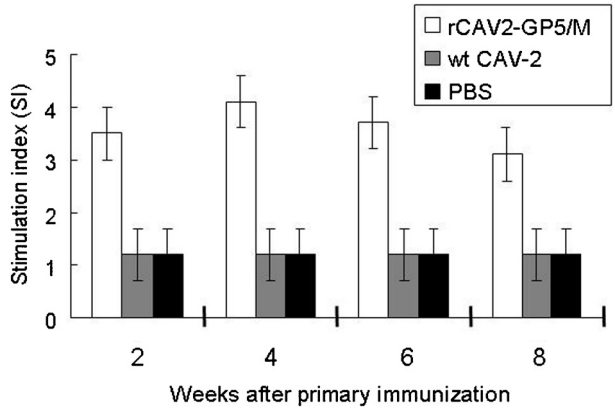

Fig. 5. Lymphocyte proliferative responses in mice immunized with rCAV2-GP5/M. Splenocytes samples (n=6) were collected at various time-points and were stimulated with PRRSV at a MOI of 1 in triplicate. After 66 hr of stimulation, MTS was added and the OD_{570} values were determined after a further 6 hr of inoculation. Error bars represent group standard deviations.

reactions were induced. The neutralizing antibody titers were relatively high and induced early (Fig. 4). In addition, the lymphocyte proliferation assay indicatesd that rCAV2-GP5/M induced cellular immunity in mice (Fig. 5). Both GP5 and M proteins may have formed hetero-dimers and both proteins contributed to induce immunity in mice. Although further studies are needed to evaluate the usefulness of recombinant virus to protect pigs from PPRSV, we succeeded in constructing a candidate vaccine against PPRSV infection by using CAV-2 vector.

ACKNOWLEDGMENT(S). This work was supported by the Fatal key projects of Jilin Province (09ZDGG008).

REFERENCES

1. Barfoed, A. M., Blixenkrone-Møller, M., Jensen, M. H., Bøtner, A. and Kamstrup, S. 2004. DNA vaccination of pigs with open reading frame 1–7 of PRRS virus. *Vaccine* **22**: 3628–3641.
2. Bastos, R. G., Dellagostin, O. A., Barletta, R. G., Doster, A. R., Nelson, E. and Osorio, F. A. 2002. Construction and immunogenicity of recombinant *Mycobacterium bovis* BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. *Vaccine* **21**: 21–29.
3. Bilodeau, R., Dea, S., Sauvageau, R. A. and Martineau, G. P. 1991. 'Porcine reproductive and respiratory syndrome' in Quebec. *Vet. Rec.* **129**: 102–103.
4. Cancel-Tirado, S. M., Evans, R. B. and Yoon, K. J. 2004. Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. *Vet. Immunol. Immunopathol.* **102**: 249–262.
5. Chou, H. J., Guo, B. Q., Dong, G. Z., Liu, W. X. and Yu, L. 1998. Genotyping of porcine reproductive and respiratory syndrome virus strain CH-1a isolated in China by RT-PCR and indirect immunofluorescence test. *Chin. J. Vet. Sci.* **18**: 118–121.
6. Gaush, C. R., Hard, W. L. and Smith, T. F. 1966. Characterization of an established line of canine kidney cells (MDCK). *Proc. Soc. Exp. Biol. Med.* **122**: 931–935.
7. Guo, B. Q., Chen, Z. S., Liu, W. X. and Cui, Y. Z. 1996. Isolation and identification of porcine reproductive and respiratory syndrome (PRRS) virus from aborted fetuses suspected of PRRS. *Chin. J. Anim. Poult. Infect. Dis.* **2**: 117–124.
8. Halbur, P. G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J. and Rathje, J. A. 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *J. Vet. Diagn. Invest.* **8**: 11–20.
9. Indik, S., Valícek, L., Klein, D. and Klánová, J. 2000. Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* **81**: 2497–2502.
10. Jiang, W., Jiang, P., Li, Y., Tang, J., Wang, X. and Ma, S. 2006. Recombinant adenovirus expressing GP5 and M fusion proteins of porcine reproductive and respiratory syndrome virus induce both humoral and cell-mediated immune responses in mice. *Vet. Immunol. Immunopathol.* **113**: 169–180.
11. Keriel, A., René, C., Galer, C., Zabner, J. and Kremer, E. J.

2006. Canine adenovirus vectors for lung-directed gene transfer: efficacy, immune response, and duration of transgene expression using helper-dependent vectors. *J. Virol.* **80**: 1487–1496.

12. Kim, H. S., Kwang, J., Yoon, I. J., Joo, H. S. and Frey, M. L. 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch. Virol.* **133**: 477–483.
13. Li, J., Faber, M., Papaneri, A., Faber, M. L., McGettigan, J. P., Schnell, M. J. and Dietzschold, B. 2006. A single immunization with a recombinant canine adenovirus expressing the rabies virus G protein confers protective immunity against rabies in mice. *Virology* **356**: 147–154.
14. Liu, G. Q., Cai, X. H., Chou, H. J., Guo, B. Q. and Dong, G. Z. Study advancement of porcine reproductive and respiratory syndrome virus. *Chin. J. Prev. Vet. Med.* **23**: 72–76.
15. Ma, S., Dai, J. J., Li, Y. F., Duan, S. Y. and Jang, P. 2008. Development and biological characterization of monoclonal antibody with neutralization activity against GP5 protein of porcine reproduction and respiratory syndrome virus. *J. Nanjing Agricultural Univ.* **31**: 72–76 (In Chinese).
16. Ma, S., Jang, P., Li, Y. F. and Duan, S. Y. 2006. Development of neutralization monoclonal antibody to M protein of porcine reproduction and respiratory syndrome virus by DNA vaccination. *J. China Immunol.* **22**: 929–932 (In Chinese).
17. Qiu, H. J., Tian, Z. J., Tong, G. Z., Zhou, Y. J., Ni, J. Q., Luo, Y. Z. and Cai, X. H. 2005. Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. *Vet. Immunol. Immunopathol.* **106**: 309–319.
18. Rompato, G., Ling, E., Chen, Z., Van Kruiningen, H. and Garmendia, A. E. 2006. Positive inductive effect of IL-2 on virus-specific cellular responses elicited by a PRRSV-ORF7 DNA vaccine in swine. *Vet. Immunol. Immunopathol.* **109**: 151–160.
19. Snijder, E. J., Dobbe, J. C. and Spaan, W. J. 2003. Heterodimerization of the two major envelope proteins is essential for arterivirus infectivity. *J. Virol.* **77**: 97–104.
20. Van Breedam, W., Van Gorp, H., Zhang, J. Q., Crocker, P. R., Delputte, P. L. and Nauwynck, H. J. 2010. The M/GP_5 glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. *PLoS Pathog.* **6**: e1000730.
21. Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K. K. and Weiland, F. 1999. Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. *Vet. Microbiol.* **66**: 171–186.