Vet Res Commun (2010) 34:371–380
DOI 10.1007/s11259-010-9364-7

ORIGINAL ARTICLE

Immune responses in pigs induced by recombinant canine adenovirus 2 expressing the glycoprotein 5 of porcine reproductive and respiratory syndrome virus

J.-X. Zhou · J.-D. Xue · T. Yu · J.-B. Zhang · Y. Liu · N. Jiang · Y.-L. Li · R.-L. Hu

Accepted: 12 April 2010 / Published online: 30 April 2010
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Abstract To develop a new type vaccine for porcine reproductive and respiratory syndrome (PRRS) prevention by using canine adenovirus 2(CAV-2) as vector, the Glycoprotein 5(GP5) gene from PRRSV strain JL was amplified by RT-PCR, and the expression cassette of GP5 was constructed using the human cytomegalovirus (HCMV) promoter and the simian virus 40 (SV40) early mRNA polyadenylation signal. The expression cassette of Glycoprotein 5 was cloned into the CAV-2 genome in which E3 region had been partly deleted, and the recombinant virus (CAV-2-GP5) was obtained by transfecting the recombinant CAV-2-GP5 genome into MDCK cells together with Lipofectamine™ 2000. Immunization trial in pigs with the recombinant virus CAV-2-GP5 showed that CAV-2-GP5 could stimulate a specific immune response to PRRSV. Immune response to the GP5 and PRRSV was confirmed by ELISA, neutralization test and lymphocyte proliferative responses, and western blotting confirmed expression of GP5 by the vector in cells. These results indicated that CAV-2 may serve as a vector for development of PRRSV vaccine in pigs, and the CAV-2-GP5 might be a candidate vaccine to be tested for preventing PRRSV infection.

J.-X. Zhou · J.-D. Xue · Y. Liu · N. Jiang · R.-L. Hu (✉)
Laboratory of Epidemiology, Veterinary Research Institute, Academy of Military Medical Science,
1068 Qinglong Road, Changchun, Jilin 130062, People's Republic China
e-mail: ronglianghu@hotmail.com

J.-D. Xue
College of Animal Science and Technology, Inner Mongolia University for the Nationalities,
Tongliao, People's Republic China

J.-B. Zhang (✉)
Centre of experimental Animal, JiLin University, Changchun, People's Republic China
e-mail: ZJb515@163.com

J.-X. Zhou
College of Animal Science and Technology, JiLin Agriculture University, Changchun,
People's Republic China

T. Yu · Y.-L. Li
College of Life Science, JiLin Agriculture University, Changchun, People's Republic China

Keywords Porcine reproductive and respiratory syndrome virus · Glycoprotein 5 · Canine adenovirus vector · Pigs · Recombinant vaccine · Immunization

Introduction

Porcine reproductive and respiratory syndrome (PRRS), characterized by severe reproductive problems, such as poor farrowing rates, premature farrowings, and increased stillbirths in sows, and respiratory distress such as pneumonia in piglets and growing pigs, cause tremendously economic losses and is now considered to be one of the most important diseases in swine breeding industry (Wensvoort et al. 1992; Dea et al. 2000). The causative agent, PRRSV, is an enveloped, positive-strand RNA virus belonging to the family of *Arteriviridae* and the genus of *Okavirus* (Fauquet et al. 2005). The genome of PRRSV is approximately 15 kb in length and contains 9 open reading frames (ORFs) named ORF1a, ORF1b, ORF2a, ORF2b, ORFs 3–7 (Wu et al. 2001). According to the sequence data, ORF1a and ORF1b represent nearly 75% of the viral genome and encode functional proteins involved in virus replication. The ORFs 2–7 are postulated to encode structural proteins. Glycoprotein 5 (25 kDa envelope protein), encoded by ORF5, is essential in the virus particle and responsible for the development of neutralizing antibodies, antigenic variability, apoptosis and possibly antibody-dependent enhancement phenomena (Meulenberg et al. 1995; Mardassi et al. 1996; Pirzadeh and Dea 1997, 1998; Weiland et al. 1999; Yang et al. 2000). So GP5 has been considered a primary candidate antigen for vaccine development of PRRS.

Current commercial PRRSV vaccines, including inactivated and attenuated vaccine, are incompletely effective in protection against PRRSV (Lager and Mengeling 1997; Osorio et al. 1998). Inactivated vaccine cannot always provide full protective immunity in pig although it has high index of safety (Meng 2000; Nilubol et al. 2004). The attenuated vaccine is able to provide protection against PRRSV to a certain degree, but the intrinsic risk of reversion to virulence under natural condition or residual pathogenicity remains a concern (Madsen et al. 1998; Mengeling et al. 1999). The outbreaks of PRRS reported in Danish herds in causal association with the introduction of a modified live vaccine had illustrated this point (Madsen et al. 1998). Therefore, many experimental vaccine systems, such as DNA, recombinant pseudorabies virus (PRV) and recombinant baculovirus, expressing the major immunogenic protein(s) of PRRSV, have been developed recently and tested against PRRSV(Barfoed et al. 2004; Bastos et al. 2004; Jiang et al. 2006a, b; Gagnon et al. 2003; Kwang et al. 1999; Pirzadeh and Dea 1998; Plana et al. 1997). However, the immunity protection was not ideal, relatively weak and tardy antibody responses, especially neutralizing antibodies, have been induced. Hence, there remains a need for further safe vaccines with higher efficacy against PRRSV infection (Barfoed et al. 2004; Bastos et al. 2004; Jiang et al. 2006a, b; Gagnon et al. 2003; Kwang et al. 1999; Plana et al. 1997).

Live vaccines based on recombinant viruses represent a particularly promising avenue of vaccine research. Recombinant adenoviruses are one of the preferred viral vectors used in vaccine production. Canine adenovirus (CAV) belongs to the genus *Mastadenovirus*, family *Adenoviridae*, including canine adenovirus type (CAV-1) and type 2 (CAV-2). CAV-2 can infect dogs, vulpes, raccoon dog and pigs, having no or slightly clinical situation and inducing high titer antibody to canine adenovirus (Fischer et al. 2002). Therefore, CAV-2 may be modified to vector to produce recombinant vector that can be used as vaccines against canine and porcine diseases. In this paper, we constructed a live recombinant virus

vaccine expressing the glycoprotein 5 of PRRSV using CAV-2 as vector, investigated its characteristics in vitro-transfected cells and examined its immunogenicity in piglets, aiming to explore the possibility of this recombinant virus as a vaccine against PRRSV.

Materials and methods

Vaccines

The commercial PRRSV KV vaccine was Ch-1R strain (Harbin Weike Biotechnology Development Company, Harbin, China), batch No. 0702001, approval no. (2007) 080011063.

Virus, plasmid and cells

PRRSV was isolated from clinical specimens in our laboratory. Marc-145 and MDCK cells were cultured in DMEM (Gibco Life Technologies, Inc., Rockville, Maryland, USA) supplemented with 5% FBS, penicillin (100 units/ml) and streptomycin (100 mg/ml). Plasmid pPOLYII-CAV-2 containing the whole genome of canine adenovirus 2 was constructed by Zhang et al. It is an infectious plasmid that can produce canine adenovirus particles after being transfected into MDCK cells (Zhang et al. 2002).

RT-PCR for PRRSV GP5 gene

Based on the ORF5-7 gene sequence of S1 (GenBank accession no. AF090173), PCR primers were designed as following: P1:5′-GATATCATGTTGGAGAAATGCTTGACCGC-3′; P2:5′-GGATCCCTAAGGACGACCCCATTG TTCC-3′, which were included *Eco*RV and *BamH*I restriction enzyme sites at their 5′-terminal, respectively. Viral RNA was extracted from PRRSV infected MARC-145 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. PRRSV GP5 cDNA was synthesized by reverse transcription of Viral RNA according to the routine methods.

Plasmid constructs

The 4.8 kb *Kpn*Ifragment containing the E3 region from pPOLYII-CAV-2 was first cloned into pVAX1 (Invitrogen, Carlsbad, California, USA), forming pVAX-E3. The GP5 cDNA was cloned into pEGFP-C1 (Clontech Laboratories, Inc., San Jose, California, USA) at the restriction site of *Nhe*I and *BamH*I, forming pEGP5-C1. The *Ase*I/*Ssp*I fragment of pEGP5-C1 containing the glycoprotein GP5 cDNA expression cassette was filled-in and cloned into the *Ssp*I/*Ssp*I site and Klenow/dNTPs blunted pVAX-E3, forming pVAX-△E3-GP5. The 5.5 kb fragment of *Sal*Iand *Nru*I double digested pVAX-△E3-GP5, containing the deleted E3 into which the glycoprotein GP5 expression cassette was inserted, was cloned into pPOLYII-CAV-2 by replacing the fragment between the *Sal*I and *Nru*Isites, forming pPOLYII-CAV-△E3-GP5. This recombinant genome was prepared for transfection. The cloning steps and plasmid preparation were performed as described elsewhere (Sambrook et al. 1989).

Production of the recombinant virus

Recombinant genome pPOLYII-CAV-△E3-GP5 was transfected into MDCK cells using Lipofectamine 2000™ (Invitrogen) according to the protocol described. Briefly, 4 μg

purified recombinant genome dissolved in 300 μl DMEM and 25 μl Lipofectamine 2000™ dispersed in 300 μl DMEM were mixed up at room temperature for 20 min, supplemented with 1.4 ml DMEM and then overlaid onto the 80% confluent MDCK cells. After 12 hour of incubation, the medium was replaced by a complete DMEM containing 5% newborn calf serum. Transfected cells were cultured at 37°C and propagated for days until cytopathic effect (CPE) appeared.

Detection of GP5 expression from the recombinant virus

The recombinant virus infected cell lysates were separated on 10% SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane (Pall Corporation) probed with positive serum against PRRSV and detected using horseradish peroxidase-labeled goat anti-pig IgG antibody (Sigma) as described elsewhere (Burnette 1981).

Titration of the recombinant virus

The $TCID_{50}$ of the recombinant canine adenovirus was assayed on 96-well cell culture plate (Nalge Nunc International, Denmark) according to the protocol described elsewhere (Rosenbaum et al. 1970).

Immunization of pigs with the recombinant virus

Twenty-four piglets, which have not been infect with PRRSV (demonstrated by RT-PCR and ELISA), were purchased from the Changchun Animal Breeding Center for Medicine, Changchun, China, and randomly divided into four groups, six piglets each group. Groups 1 and 2 were intramuscularly injected only once with 1.5 ml recombinant virus CAV-2-GP5 ($10^{7.6}$ $TCID_{50}$) and CAV-2, respectively; group 3 were intramuscularly inoculated with 1 ml inactivated PRRSV vaccine; group 4 were injected with PBS as control. Blood was collected before inoculation and every week or at an interval of 2 weeks after the inoculation. Sera were separated for detection of specific antibody against PRRSV.

Titration of neutralizing antibodies

Serum neutralizing test to PRRSV were performed as previously described (Jiang et al. 2006a, b) Neutralizing activity was expressed as the highest serum dilution that completely prevented the replication of virus in cells. GP5-specific IgG antibody responses were detected by indirect ELISA based on the purified GP5 expressed from *E. coli*.

Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed using PBMCs of immunized piglets. Swine PBMCs were isolated and plated in 96-well flat-bottom plates at 100 μl/well (2×10^5 cells / well). Subsequently, 100 μl/well of medium with or without 20 μg/ml of PRRSV proteins (an extract of PRRSV-infected Marc-145 cells concentrated by ultracentrifugation at 80,000×*g* for 2 hr) was added and mixed. Concanavalin A (5 μg/ml; Sigma) was used as a positive control. Each PBMCs sample was plated in triplicate. The proliferative activity was measured according to the method described by Bounous et al. (Bounous et al. 1992).The stimulation index (SI) was calculated as the ratio of the average A_{570} value of wells

containing antigen-stimulated cells to the average A_{570} value of wells containing only cells with medium.

Results

Generation of recombinant CAV-2 expressing PRRSV GP5 protein

To generate recombinant canine adenovirus containing GP5 protein of PRRSV, the GP5 expression cassette, which was driven by the cytomegalovirus (CMV) immediate early (IE) promoter with the SV40 polyadenylation signals, was constructed. The expression cassette was subcloned into the plasmid pPolyII-CAV-2, which included the whole CAV-2 genome, producing a recombinant plasmid pPOLYII-CAV-△E3-GP5. Seven days after transfection of the recombinant genome into MDCK cells, typical CPE (grape-cluster-like cells) was observed under microscope. Adenovirus-like particles were observed under the electron microscope after negative staining of the supernatant of the cell culture with potassium phosphotungstate. The growth characteristic of the recombinant virus was similar to that of the canine adenovirus vaccine strain YCA18. The $TCID_{50}$ of the recombinant virus was $10^{7.8}$/0.1 ml and the recombinant virus was referred to as CAV-2-GP5. The identification of the genome from the recombinant virus by restriction endonuclease digestion confirmed that the GP5 cDNA and its expression cassette were included in the recombinant virus.

Expression of GP5 protein from the recombinant virus

The result of western blot analysis was shown in Fig. 1. The molecular weight of the full GP5 protein was estimated to be 25 kDa, which is consistent with the molecule derived from the PRRSV.

Immune responses in pigs to the recombinant virus

PRRSV specific neutralizing antibodies were detected at 4, 6, 8, 10 and 12 weeks post immunization (p.i.). As shown in Table 1, only two out of six piglets inoculated with CAV-

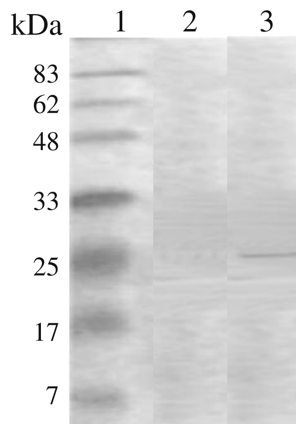

Fig. 1 Expression of GP5 protein from the recombinant virus in MDCK cells. The legend: Western blot analysis. Protein size marker (line 1), CAV-2 (lane 2), CAV-2GP5 (lane 3)

2-GP5 developed scarcely detectable neutralizing antibodies (the titer is 8) at 8 weeks p.i. but only one piglets produced at 10 and 12 weeks.

GP5 specific antibody responses, which were detected by indirect ELISA based on the purified GP5 expressed from *E. coli*, could be detectable at 2 weeks p.i. All piglets immunized with CAV-2-GP5 developed ELISA antibodies at 6 weeks p.i. and could persist for 6 weeks until the trials ended. But the PRRSV KV vaccine could produce higher antibodies titers than that of the recombinant CAV-2-GP5 (Fig. 2).

The lymphocyte proliferative responses were analyzed by in vitro stimulating the isolated PBMCs of immunized piglets at 4, 6 and 10 weeks p.i.. As shown in Fig. 3, vaccination with recombinant CAV-2-GP5 produced higher specific lymphocyte proliferative responses than that of CAV-2 ($P<0.05$), but lower than that of PRRSV KV vaccine ($P>0.05$).

Discussion

Despite the tremendous efforts in controlling PRRSV infections, the virus continues to plague the swine industry and damage pig production worldwide. In the prevention of PRRS, attenuated vaccines have the potential risk of virulence reversion and virulence scatter, so they are usually forbidden. Once the inactivated vaccine played a positive role to prevent PRRS, however, in recent years, PRRS is increasingly prevalent in China, and the inactivated vaccines have been unable to fully prevent the disease, sometimes even injecting vaccine will accelerate the infection by PRRSV (mainly because of antibody-dependent enhancement). Lack of safe and effective vaccine is the major barrier to control this disease. A lot of candidate engineering genetic vaccines have been studied and reported

Table 1 PRRSV-specific neutralizing antibodies in pigs immunized with the recombinant virus

Vaccine	Time *p.i.* (weeks)	Titers of neutralizing antibodies		
		<8	8	16
CAV-2	4	6*	0	0
	6	6	0	0
	8	6	0	0
	10	6	0	0
	12	6	0	0
CAV-2-GP5	4	6	0	0
	6	6	0	0
	8	4	2	0
	10	5	1	0
	12	5	1	0
PRRSV KV vaccine	4	6	0	0
	6	6	0	0
	8	3	1	2
	10	3	0	3
	12	3	1	2

* The number of pigs with the given antibody titer. In the trial, 6 pigs in each group were immunized

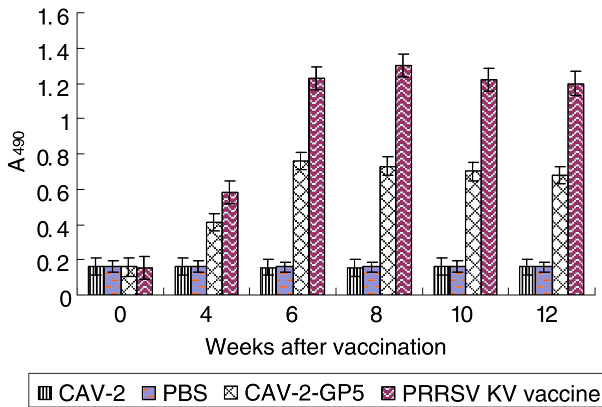

Fig. 2 Serum GP5 antibodies in pigs immunized with the recombinant virus. The legend: ELISA. The ordinate: A_{490}. The abscissa: weeks p.i.

at current years (Barfoed et al. 2004; Bastos et al. 2004; Jiang et al. 2006a, b; Gagnon et al. 2003; Kwang et al. 1999; Pirzadeh and Dea 1998; Plana et al. 1997), the adenovirus expression system has successfully been used to express many viral genes, human adenovirus 5 was also used as a vector for PRRSV recombinant vaccine and has been reported to induce immune response in mice(Jiang et al. 2006a, b). CAV-2 had been successfully used as a live vector expressing the glucoprotein of rabies and the VP1 protein of foot-and-mouth disease (Hu et al. 2006; Liu et al. 2006), it was used for expressing GP5 protein from PRRSV based on the following considerations: (i) CAV-2 can infect a wide variety of dividing or nondividing cells; (ii) Ad infections are ubiquitous without significant or severe clinical symptoms; (iii) they can be administered orally; (iv) Ads have their genome well characterized; (v) their genome rarely integrates into the host chromosome; (vi) techniques are well established for the construction of recombinant Ad vectors; and (vii) they have the ability to replicate at high titers in complementing cell lines. These characteristics, together with its relative ease of gene manipulation, virus production, and the high viral titer, make it valuable as a live vector for pig vaccine development.

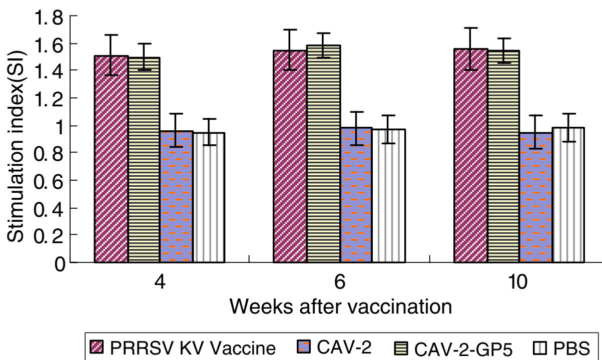

Fig. 3 Lymphocyte proliferative responses in immunized piglets after in vitro stimulation with purified PRRSV proteins. The ordinate: SI(A_{570}). The abscissa: weeks p.i.

In this trial, a recombinant CAV-2 expressing GP5 of PRRSV was developed, GP5 specific antibody responses could be detectable at 2 weeks p.i. and could persist for 12 weeks until the trials ended. But the PRRSV KV vaccine could produce higher antibodies titers than that of the recombinant CAV-2-GP5. Meanwhile, our data demonstrated that the recombinant CAV-2-GP5 could effectively induce neutralizing antibodies against PRRSV, but lower than that of the commercial killed PRRSV vaccine. One possible reason that CAV-2-GP5 induced low level neutralizing antibody titer might be that the GP5 protein was not correctly folded and the neutralizing epitopes of GP5 protein were not completely exposed. To find out the possible reason, a series of modified GP5 cDNA with its epitopes being exposed at the surface of a small vector protein is under design and cloning into the CAV-2 vector in our laboratory. For the other reason, we speculate the immunoadjuvant plays an important role in immunoresponse. Because using the recombinant virus dose not contain the immunoadjuvant, after injection, a large amount of recombinant virus is eliminated due to the immune clearance reaction in pigs, thus a little some survive, which substantially reduces the effective dose; meanwhile, the inactivated vaccine is protected by the adjuvant, thus virus antigens release slowly, which induced the higher titer of antibody against GP5.

Cell-mediated immunity plays an important role in protective immunity against PRRSV (Bautista and Molitor 1997), in the present study, specific lymphocyte proliferative responses were also observed in piglets immunized with recombinat adenovirus CAV-2-GP5. Of course, it should be necessary to evaluate further the immunogenicity of the recombinant adenoviruses and determine the ability to protect against the infection with virulent PRRSV.

Generally, developing a safe PRRSV recombinant vaccine is important for the control of PRRS worldwide. Our study contributes to further developing such a kind of vaccine. If the titer of antibody against GP5 protein can be further elevated by modification or recomposition of the epitopes on GP5, this kind of recombinant PRRS vaccine will play an effective role in preventing PRRS from spreading among porcine populations.

The objective of this paper is to explore the immunogenicity of recombinant virus, detect the titer of antibody induced by recombinant virus in pigs. Based on the results of this paper, we can understand whether it is necessary to construct the recombinant virus expressing other PRRSV structural protein, so we do not perform the virulence infection trial. Exactly, we have constructed the recombinant CAV-2 expressing M Protein (another structural protein of PRRSV), and the virulence infection trial will be conducted by immunizing pigs jointly with the two recombinant viruses expressing GP5 and M Protein. The relevant results will be reported in our next work.

Acknowledgments The authors thank Dr. Qiu Huaji for his help in detection of recombinant virus sera. This study was supported by the National Natural Science Foundation of China, Granted No. 30170704.

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