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Antiviral Research 76 (2007) 59–67
Antiviral Research
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Immunogenicity of plasmids encoding P12A and 3C of FMDV and swine IL-18

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Received 3 March 2007; accepted 9 May 2007

Abstract

In this paper, two recombinant plasmids (pVIR-P12AIL18-3C and pVIR-P12A-3C) containing foot and mouth disease virus (FMDV) capsid polypeptide, 3C coding regions of O/NY00 and using/or not swine IL18 as a genetic adjuvant were constructed, and evaluated for their ability to induce humoral and cellular responses in mice and swine. In addition, the ability to protect swine against homologous virus challenge was examined. Mice and swine were given booster vaccination twice and once, respectively, and swine were challenged 10 days after the booster vaccination. Control groups were inoculated with pVAX1 or phosphate-buffered saline (PBS). All animals vaccinated with pVIR-P12AIL18-3C and pVIR-P12A-3C developed specific anti-FMDV ELISA antibody and neutralizing antibody and T lymphocyte proliferation and CTL cytotoxic activity was observed. In addition, we found that pVIR-P12AIL18-3C possessed stronger immunogenicity than pVIR-P12A-3C. The pVIR-P12AIL18-3C and pVIR-P12A-3C provided full protection in 3/4 and 2/4 swine from challenge with FMDV O/NY00, respectively. The results demonstrate the potential viability of a DNA vaccine in the control and prevention of FMDV infections.

Keywords: Foot and mouth disease virus; P12A; IL-18; DNA vaccine

1. Introduction

Foot and mouth disease (FMD) caused by foot and mouth disease virus (FMDV) is an infectious disease affecting cloven-hoofed animals, in particular swine, cattle, sheep, goats, and deer. This disease poses a serious threat for animal health and exacts an economic toll on the livestock industry. In countries where disease eradication has not been achieved, vaccination plays a crucial role in its control. Although inactivated virus vaccines effectively prevent FMD, their use is accompanied by dangerous residual potency problems, including incomplete viral inactivation or virus escape from vaccine-producing facilities (Sutmoller et al., 2003; Beck and Strohmaier, 1987; Doel, 2003). As a result, alternative approaches are being investigated, including construction of modified live-virus, subunit vaccines, synthetic peptides, naked DNA plasmids, and recombinant viruses (Mason et al., 1997; Grubman et al., 1993; Taboga et al., 1997; Beard et al., 1999; Moraes et al., 2002; Zheng et al., 2006).

DNA-based vaccination offers an attractive alternative to overcome the disadvantages of inactivated virus vaccine, however, it has been recognized that DNA vaccines alone often generate only weak immune responses. Various efforts have been made to enhance immune responses, usually involving the co-expression of cytokine genes (Kim et al., 1998; Svanholm et al., 1997; Sin et al., 1999; Xiang and Ertl, 1995; Park et al., 2006; Li et al., 2006), CpG motifs (Weeratna et al., 2000), BCG (Zhang et al., 2005) or co-stimulator genes (Agadjanyan et al., 1999) as "genetic adjuvants".

In this study, we chose the swine Interleukin-18 (IL18) as an adjuvant to construct a recombinant plasmids pVIR-P12AIL18-3C co-expressing the capsid (P12A), 3C protease genes of an FMDV field strain, O/NY00 (Genbank accession no. AY333431; Zheng et al., 2005) and swine IL-18. Subsequently, the ability of these recombinant plasmids to elicit an FMDV-specific immune

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doi:10.1016/j.antiviral.2007.05.003

response in mice was examined and the efficacy to provide challenge protection was examined in swine. The results indicate the recombinant plasmid pVIR-P12AIL18-3C may provide a potential vaccine affording protection to mammals against FMDV challenge.

2. Material and methods

2.1. Viruses and inactive vaccine

FMDV O/NY00 was preserved and passaged in BHK21 to obtain a titer of $1 \times 10^{6.4}$ $TCID_{50}$/0.01 ml by our labs.

2.2. Plasmids

Eukaryotic expression vector pVAX1 and pIRES1neo were purchased from Invitrogen Co. Ltd. (USA) and Clontech Co. Ltd. (USA). The pMD18-T cloning vecter was purchasd from TaKaRa Biotechnology (Dalina china) Co. Ltd. Recombinant plasmid pUTAL3CP1 (Zheng et al., 2006) encoding the capsid (P12A) and 3C protease genes of FMDV O/NY00, and another recombinant plasmid pUMT-IL18 (Zheng et al., 2003) encoding swine Interleukin-18 mature protein gene were previously constructed by our lab.

2.3. Animals

Twenty-four BALB/c female mice (4–5 weeks old) were provided by the Changchun Academy of Agricultural Science.

Forty-one large White cross-bred Landrace swine weighing 20–25 kg were used for the study.

2.4. Construction of the FMDV DNA vaccine

P12A gene fragment of FMDV O/NY00 had been amplified from pUTAL3CP1 using the plasmid pUTAL3CP1 and the P12A-specific primers:(sense: 5′-AGC CCG GGA GCA TGG ACA CTG GCA GCA TTA-3′, an ATG start codon, a Kozat box and an *Sma*I site were incorporated into the most upstream 5′ primer; antisense: 5′-CCG CCG TCT CCC AGG GTT GGA CTC GAC-3′), then been inserted into pMD18-T simple vector (TaKaRa Co. Ltd.) to acquire the recombinant plasmid pMD18-T-P12A. The plasmid pUMT-IL18 was digested with *Bam*HI to produce an IL18 fragment that was then inserted into the *Bam*HI site of pMD18-T-P12A to generate recombinant plasmid pMD18-T-P12A-IL-18.

For the construction of plasmid pIRES3C, 3C gene was amplified by PCR using the plasmid pUTAL3CP1 and the 3C specific primers primers (sense: 5′-GCC CCGGGAGTGGT-GCCCCTCCGACCG-3′, antisense: 5′-GCTCTAGACTCGT-GGTGTGGTTCAGGA-3′). The 3C PCR product was digested with *Sma*I and *Xba*I and subcloned into pIRES1neo at the *Sma*I and *Xba*I sites to replace the Neo^r gene of pIRES1neo. The construct, designated as pIRES3C, was confirmed by restriction digestions with *Sma*I and *Xba*I. Subsequently, the recombinant plasmid pIRES3C was digested with *Nru*I and *Xba*I and subcloned into the EcoRV and *Xba*I sites of pVAX1 to construct pVIR-3C. At last the P12A-IL18 was excised with *Sma*I and cloned into the multiple cloning sites under the promoter CMV of pVIR-3C, and the recombinant plasmid pVIR-P12AIL18-3C was obtained. The recombinant plasmid pVIR-P12A-3C was constructed in the same way (Fig. 1).

2.5. Transfection

Five hundred thousand HeLa cells were seeded onto coverslips on six-well plates and incubated at 37 °C in a CO_2 incubator until the cells were 50–80% confluent. The following day, 10 μg of plasmid DNA in 100 μl of reduced-serum MEM was mixed with 6 μl of Lipofectamine™ reagent (Invitrogen, USA) in 100 μl of reduced-serum MEM. The mixture was then incubated at room temperature for at least 30 min before it was diluted into 800 μl reduced-serum MEM and then added to the cells. After incubation for 5 h at 37 °C in a humidified incubator, 1 ml of medium containing 5% fetal calf serum was added to each well.

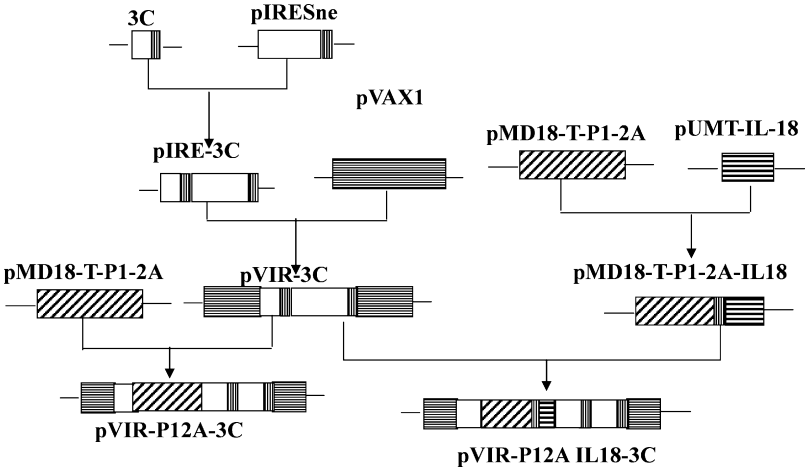

Fig. 1. Schematic representation of construction of pVIR-P12A-3C and pVIR-P12AIL18-3C.

2.6. Expression confirmation of pVIR-P12A-3C and pVIR-P12AIL18-3C in HeLa cells

2.6.1. Indirect immunofluorescence assay (IFA)

The expression of the pVIR-P12A-3C or pVIR-P12AIL18-3C protein was confirmed by indirect immunofluorescence assay (IFA). Two days after transfection, the medium was aspirated; the transfected cells were washed three times with 1× PBS, then fixed with a mixture of methanol and acetone. The cells were then immersed in 1% BSA blocking buffer for 2 h to avoid non-specific binding. Anti-FMDV positive sera from a rabbit vaccinated with inactivated virus vaccine were incubated for 1 h at room temperature. After three 5 min washes with 1× PBS, 100 μl of a fluorescein-conjugated affinity-purified anti-rabbit IgG (Invitrogen, USA) diluted in 0.025% Evans Blue in PBS was added per coverslip and incubated in the dark at room temperature for 2 h. After 5 min washes with 1× PBS, we examined the coverslips using a fluorescence microscope (Olympus, Japan).

2.6.2. Western blotting analysis of the cells transfected with the recombinant plasmid

The expression of the pVIR-P12A3C and pVIR-P12AIL18-3C were confirmed by Western blotting. Three days after transfection, pVIR-P12A3C and pVIR-P12AIL18-3C were harvested. The total cellular lysates were prepared with lysis buffer (10 mM Tris–Cl pH 7.4, 1 mM$MgCl_2$, 0.5% NP40, 20 μg/ml DNaseI) and electrophoresised through an SDS-10% polyacrylamide gel. Then the proteins were transferred onto nitrocellulose membrane (Bio-Rad). Membranes were incubated with anit-FMDV VP1 monoclonal antibody and rabbit anti-porcinal IL-18 antibody (provided by Genetic Engineering Laboratory, Academy of Military Medical Science), respectively, at a dilution of 1:500 and 1:50. Bound antibody was detected by incubation of the membrane with goat anti-mouse IgG antibodies and goat anti-rabbit IgG antibodies labeled by the alkaline phosphatase (Santa Cruz Biotechnology) at a dilution of 1:2000, respectively, followed by visualizing with NBT/BCIP substrates (Promega).

2.7. Preparation of DNA plasmids

All plasmids for DNA immunizations were grown in Escherichia coli DH5α^{TM} strain (Invitrogen), and large-scale preparation of the plasmid DNA was carried out by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

2.8. Vaccines and vaccination

2.8.1. Experiment 1

Twenty-four BALB/c mice were randomly divided into four groups. Animals in group 1.1 and 1.2 were inoculated with 100 μg of plasmid pVIR-P12A3C and pVIR-P12AIL18-3C dissolved in 100 μl PBS, respectively. Group 1.3 and 1.4 were negative controls inoculated with 100 μl PBS or 100 μg of pVAX1 vector dissolved in 100 μl PBS, respectively. All of the mice were intramuscularly immunized three times at 2-week intervals. Sera were collected by tail bleed on days 0, 10, 21, 28, and 38 after primary immunization. Antiserum was prepared from blood samples and stored at −20 °C. Ten days after the last immunization, 6 mice from each group were sacrificed, blood samples were collected from the ophthalmic venous plexus, and spleen cells were cultured in 10% fetal calf serum/RPMI-1640.

2.8.2. Experiment 2

Sixteen healthy swine weighing between 20 and 25 kg each were divided into 4 groups (four swine per group). Animals in groups 2.1 and 2.2 were vaccinated with 500 μg of pVIR-P12AIL18-3C and pVIR-P12A-3C in 1 ml of PBS, respectively. Groups 2.3 and 2.4 were negative controls injected with 1 ml PBS or 500 μg of pVAX1 vector in 1 ml of PBS, respectively. All groups were boosted with equivalent doses at 21 dpi. All vaccines were administered by intramuscular (IM) inoculation. The serum was collected from each swine on days 0, 7, 14, 21 and 28 to detect specific antibodies against FMDV.

2.9. Evaluation of the humoral immune response to FMDV

Indirect enzyme linked immunosorbent assays (I-ELISA) were performed on mouse and swine serum to measure the amount of anti-FMDV antibodies present in each sample. Briefly, 96-well flat-bottomed plates (Costar, USA) were coated with FMDVO/NY00 inactivated by formaldehyde in 0.1 M carbonate/bicarbonate buffer, pH 9.6, overnight at 4 °C. After being blocked with 5% BSA–PBS, plates were incubated with duplicate 20-fold dilutions of test sera for 1 h at 37 °C. Peroxidase-conjugated anti-mouse IgG (or anti-swine IgG) conjugate (Sigma, USA) at 1:4000 dilution was then added for 1 h at 37 °C, followed by the substrate (10 mg OPD + 20 ml 0.015% hydrogen peroxide in phosphate/citrate buffer). The optical density (OD) of the ELISA plate was read at 492 nm.

Serum samples from all swine were analyzed for neutralizing antibody titers by using a neutralization assay with a monolayer of BHK-21 cells (Yu and Cui, 1997). Sera were inactivated at 56 °C for 30 min, and 50 μl of each sample or control serum was added to the well at the end of each row of a 96-well tissue culture plate, and then diluted in a twofold serial dilution across the plates. Then 50 μl of 100 $TCID_{50}$ FMDV O/NY00 was added to each well, and the plate was vortexed for 1 min. After incubation at 37 °C for 90 min, 100 μl of 10^6 cells/ml BHK-21 cell in Eagle's MEM containing 8% fetal bovine serum were added to each well. Endpoint titers were determined after 72 h incubation at 37 °C with 5% CO_2 and expressed as the reciprocal of the final serum dilution that resulted in the neutralization of the virus activity by 50%.

2.10. CTL assay

After the mice were killed (at 10 days after the third inoculation), the splenocytes were sterilely isolated as described by Jiang et al. (2005) and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μg/ml Concanavalin A (Sigma), and 10 U/ml of IL-2 and cultured *in vitro* as the effector cells. A modified P815 cell, which stably displayed VP1 pro-

tein of FMDV, was previously constructed by transfecting with a mammalian expression plasmid pDisplay-VP1 (data not shown). The stimulator cells were harvested and treated with 25 μg/ml Mitomycin C at 37 °C in 5% CO2 for 2 h. Then, the cells were pelleted and washed four to five times with RPMI 1640 medium. The effector cells (4×10^7 cells) were incubated with stimulator cells at an effector–stimulator ratio of 10:1 for 3 days at 37 °C in 5% CO_2. To measure the specific lysis of these target cells, the lactate dehydrogenase (LDH) release assay was preformed according to protocol. In 96-well round-bottom plates, target cells were incubated with effector cells at various effector–target ratios for 4 h in phenol red-free RPMI 1640 containing 3% fetal calf serum. The supernatant (100 μl per well) was then transferred to 96-well plates, and lysis was determined by measuring LDH release using a Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). Supernatant absorbance values were recorded at 490 nm on an ELISA microplate reader. The percentage of specific lysis of P815 target cells for a given effector cell sample was calculated by the following formula: specific lysis = (OD of experimental LDH release − OD of effector cell spontaneous LDH release − OD of target cell spontaneous LDH release)/(OD of maximum target LDH release − OD of target spontaneous LDH release) × 100%. All determinations were performed in triplicate.

2.11. T lymphocyte proliferation assay

Blood was collected from immunized swine in heparin 14 days, 28 days after first immunization as described (Garcia-Valcarcel et al., 1996). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in Ficoll-Hypaque density 1.110 (Tian Jin Yang Biological Manufacture Co. Ltd.) for 20 min at 18 °C mononuclear cells were collected from the buffy coat, centrifuged and residual red blood cells were lysed by 1 min incubation in water followed by addition of 2× Eagle's solution. After two washes in PBS the cells were resuspended in complete medium (RPMI-1640 supplemented with 25 mM HEPES, 2 mM glutamine, 10% swine serum (Sigma), 5×10^{-5} M B2-mercaptoethanol and penicillin–streptomycin). Proliferation assays were carried out in triplicate cultures of 200 μl (2×10^6 ml^{-1} cells) in 96-well U-bottomed plates (NUNC, Gibco). PBMC were stimulated with 1:100 live virus. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 for 45 h. Then the proliferation responses were detected by MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (5 mg/ml, Sigma) method and the stimulation index (SI) was calculated. The stimulation index was determined from the formula: stimulation index (SI) = experimental OD/negative OD. To assure that cells were healthy, 10 μg/ml ConA was used as a polyclonal stimulator for positive control.

2.12. Determination of ID_{50} of type O FMDV in swine

To determine the ID_{50} of type O FMDV in swine, 25 FMDV sero-negative swine were randomly divided into five groups (five each group). The stock FMDV was diluted at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} with 0.85% NaCl. Each swine was injected i.m. 3 ml of the diluted FMDV solution. Clinical symptom (i.e. blister formation on foot, mouth, and nose and body temperature over 40 °C) was observed until 21 days after viral challenge. The swine were diagnosed as FMD positive in the presence of the above clinical symptoms. The ID_{50} was calculated to be 1×10^{-5}/3 ml.

2.13. Challenge of animals

Thirty-one days after primary immunization the swine were challenged with one thousand 50% infecting dose (ID_{50}) of FMDV (serotype O) and examined for protection against FMD in the following 14 days according to the appearance of FMD symptoms, such as an increase in body temperature (above 41 °C) and the appearance of blisters on the mouth or hooves.

2.14. Statistical analyses

Differences between groups were analyzed by the Student's *t*-test using SPSS software. Results were expressed as the mean ± standard deviation (S.D.).

3. Results

3.1. Expression analysis of pVIR-P12A3C and pVIR-P12AIL18-3C in HeLa cells

The expression of the pVIR-P12A3C or pVIR-P12AIL18-3C was confirmed by an indirect immunofluorescence assay. Transfected cells were reacted with anti-FMDV positive sera and fluorescein-conjugated anti-rabbit IgG (Invitrogen, USA). As anticipated, we see the cells transfected with pVIR-P12A3C and pVIR-P12AIL18-3C displayed fluorescence, whereas the negative controls did not (Fig. 2).

Furthermore, in order to demonstrate the appropriate expression of the FMDV capsid precursor P12A and processing to capsid proteins VP1, transfected cells were harvested and the cellular lysates were prepared 3 days post-transfected. The P1, VP1 and IL18 proteins were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. The proteins were probed respectively by anti-FMDV VP1 monoclonal antibody and rabbit anti-IL-18 antibody, goat anti-mouse antibody and goat anti-rabbit antibody conjugated with alkaline phosphatase. The product of the expected size (P1: 80 kDa and VP1: 23 kDa) was detected by western blotting in extracts of infected cells with pVIR-P12A-3C and pVIR-P12AIL18-3C. Control cells, transfected cells with pVIR-3C, did not show P12A and VP1 expression (Fig. 3A). Furthermore, one band with molecular weights of 18 kDa was detected in transfected cells with pVIR-P12AIL18-3C, demonstrating that IL-18 was expressed. Transfected cells with pVIR-P12A-3C did not show IL18 expression (Fig. 3B).

3.2. Antibody responses

Serum antibody titers against type O FMDV were measured by indirect ELISA. Specific anti-FMDV antibody of

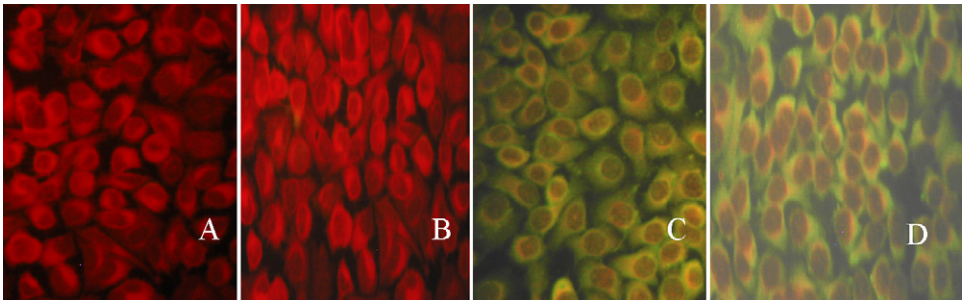

Fig. 2. Fluorescent photos of HeLa cells transfected by plasmids DNA (stained by azovan blue). (A) HeLa cell; (B) pVAX1; (C) pVIR-P12A-3C; (D) pVIR-P12AIL18-3C.

plasmids pVIR-P12A-3C and pVIR-P12AIL18-3C immunizing mice appeared at 10, 21, 28, 38 day after the first immunization and rose quickly after booster immunization. Interestingly, the highest antibody titres were found among all the groups with pVIR-P12AIL18-3C. The antibody titers from both groups of the pVIR-P12A-3C and pVIR-P12AIL18-3C were statistically much higher than control groups ($P < 0.05$) (Fig. 4a), and we saw a statistical difference between pVIR-P12A-3C and pVIR-P12AIL18-3C 10 day after the first immunization ($P < 0.05$).

Similar to the antibody response seen in mice, specific antibody titers of pVIR-P12A-3C and pVIR-P12AIL18-3C vaccinated swine increased rapidly at the second week after the first vaccination, then continued to increase after the second vaccination. Antibody titers were not statistically different between the two groups until the second week after the first vaccination ($P < 0.05$) (Fig. 4b). There is significant difference ($P < 0.01$) compared to control groups.

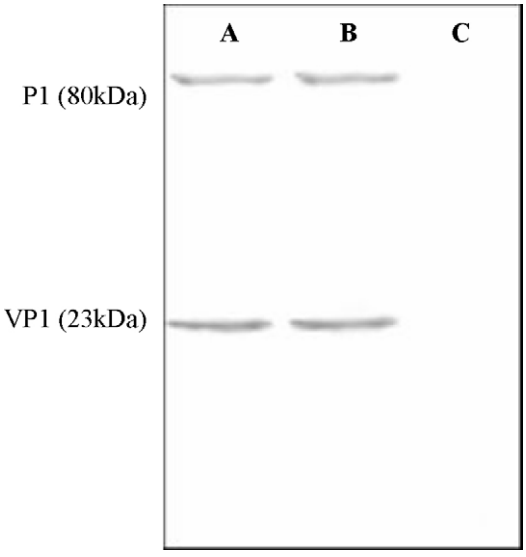

Fig. 3. Western blotting analysis of target protein and IL-18 expressed in HeLa cells transfected by pVIR-P12A-3C and pVIR-P12AIL18-3C. (Panel A) Western blotting analysis of P1 proteins expressed in HeLa cells: lane 1, HeLa cells transfected by pVIR-P12A-3C; lane 2, HeLa cells transfected by pVIR-P12AIL18-3C; lane3, HeLa cells transfected by pVIR-3C. Arrow at the left indicates the position of VP1 protein and P1 polypeptide. (Panel B) Western blotting analysis of IL-18 expressed in HeLa cells: lane 1, protein marker; lane 2, HeLa cells transfected by pVIR-P12AIL18-3C; lane 3: HeLa cells transfected by pVIR-P12A-3C. Arrow at the right indicates the position of IL-18.

3.3. Neutralizing antibody response against FMDV in vaccinated swine and challenge results of swine with FMDV O/NY00

pVIR-P12A-3C and pVIR-P12AIL18-3C vaccinated groups developed neutralizing antibody against FMDV and the tendency of the antibody titers were different among all groups (Table 1). The positive (groups 2.1 and 2.2) and negative con-

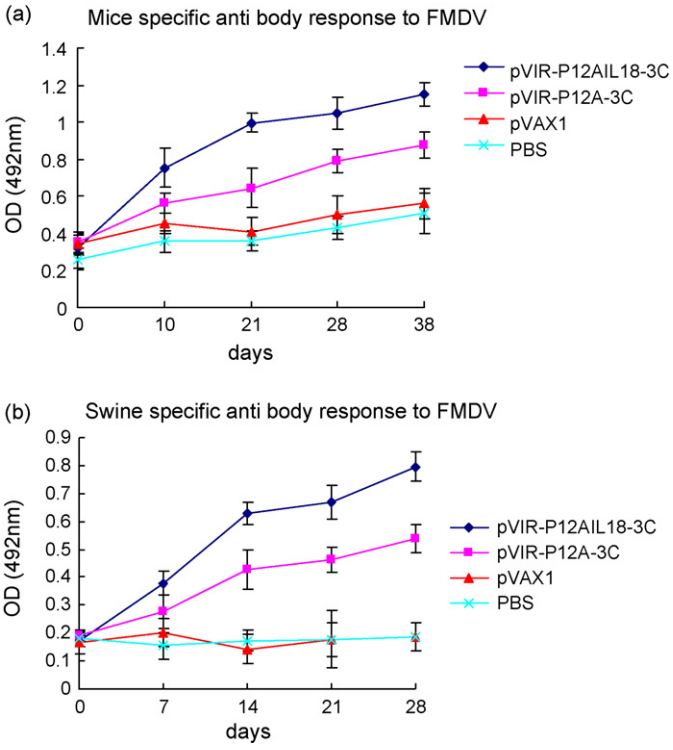

Fig. 4. (a) Mice-specific antibody response to FMDV after DNA vaccination. Mice were intramuscularly immunized three times at 2-week intervals with 200 μg plasmid pVIR-P12AIL18-3C, pVIR-P12A-3C, pVAX1 and PBS, respectively, each time. Total antibody (anti-viral whole protein) was detected by ELISA at a dilution of 1:50 in triplicate for all sera collected 10, 21, 28 and 38 days after first immunization. The results are presented as mean ± standard deviations (S.D.) of A492 of six individuals (a). (b) Swine were intramuscularly immunized at 3-week intervals with 500 μg plasmid pVIR-P12AIL18-3C, pVIR-P12A-3C, pVAX1 and PBS, respectively, each time. Total antibody (anti-viral whole protein) was detected by ELISA at a dilution of 1:200 in triplicate for all sera collected weekly before and after first immunization. The results are presented as mean ± standard deviations (S.D.) of A492 of six individuals (b).

Table 1
Titres of neutralizing antibodies and protection results

Gruop	Neutralizing antibody titer[a]		Protection[b]	Severity of sysmptoms	Time of showed symptoms
	Day 0	Day 28			
pVIR-P12AIL18-3C (rate of protection: 75% (3/4))					
Group 2.1-1	0.6	1.74	Total	None	No
Group 2.1-2	0.6	1.05	Total	None	No
Group 2.1-3	0.6	1.47	Total	None	No
Group 2.1-4	0.6	0.9	None	Sever	9
pVIR-P12A-3C (rate of protection: 50% (2/4))					
Group 2.2-1	0.6	1.69	Total	None	No
Group 2.2-2	0.6	0.90	None	Sever	7
Group 2.2-3	0.6	1.0	Total	None	No
Group 2.2-4	0.6	0.75	None	Sever	3
pVAX1 (rate of protection: 0% (0/4))					
Group 2.3-1	0.6	0.75	None	Sever	4
Group 2.3-2	0.6	0.6	None	Sever	3
Group 2.3-3	0.6	0.6	None	Sever	3
Group 2.3-4	0.6	0.75	None	Sever	3
PBS (Rate of protection: 0% (0/4))					
Group 2.4-1	0.6	0.6	None	Sever	3
Group 2.4-2	0.6	0.6	None	Sever	3
Group 2.4-3	0.6	0.6	None	Sever	2
Group 2.4-4	0.6	0.75	None	Sever	3

Neutralizing antibody was measured; serum samples were taken on days 0 and 28 before the challenge with live FMDV. Severity of symptoms was based on daily monitoring, completed on day 14 post-challenge, and is scored as: none, no lesions and no temperature or 1 day with temperature above 40 °C and no lesions; severe, lesions on all four feet, gums and snout, 1 day with temperature above 40 °C.

[a] Log 10 reciprocal antibody titers to neutralize 100 $TCID_{50}$ of homologous FMDV in 50% of the wells.

[b] All of swine were intramuscular injection challenged with 1000 ID_{50} of live virus at the neck region 10 days after the second immunization. All swine were kept in separated open-topped crates within one house. After a 14-day observation, the swine were examined and eventually destroyed. Rate of protection (%) = number of swine infected/number of swine challenged.

trol groups (groups 2.3 and 2.4) followed expectations, yielding protection or disease, respectively (Table 1). The negative control animals that were not protected developed fever, lameness and vesicles in all four feet and in the snout within 4 days. Two animals of four animals vaccinated with plasmid pVIR-P12A-3C were totally protected, without any signs of FMD. Three animals of four animals vaccinated with plasmid pVIR-P12AIL18-3C were totally protected, and development of FMD lesions of another one was delayed, the blisters did not appear until 9 days post-challenge. Although these numbers are small, the result does encourage further exploration of the potential of swine IL-18 as an adjuvant in DNA vaccination against FMDV, particularly in view of the significant effect on anti-FMDV antibody production.

3.4. CTL activity of immunized mice

The cytotoxic activities of the inoculated mice were measured by non-radioactive LDH release assay and the specific lysis rates are shown in Fig. 5. Both the pVIR-P12A-3C and pVIR-P12AIL18-3C elicited specific CTL cytotoxic activities compared with pVAX1 and PBS-inoculated groups ($P < 0.01$). The specific lysis rate of the pVIR-P12AIL18-3C group was higher than that of pVIR-P12A-3C ($P < 0.05$) (Fig. 5).

3.5. T lymphocyte proliferation response of swine

Swine in the pVIR-P12A-3C and pVIR-P12AIL18-3C inoculated groups showed a strong specific T lymphocyte proliferation response (stimulated with live virus) and a relatively weak non-specific proliferation response (stimulated with ConA). In contrast, swine in the pVAX1 and PBS-inoculated group only developed a weak non-specific T lymphocyte proliferation

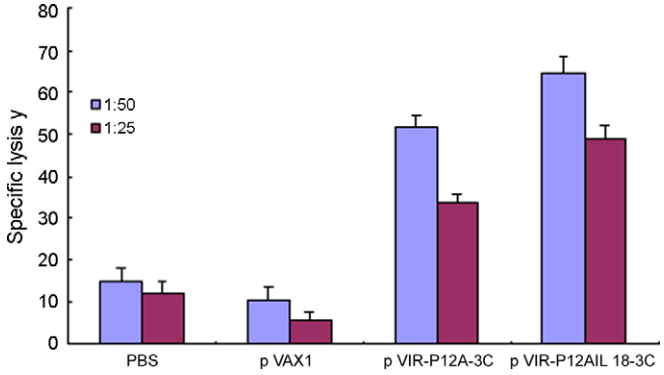

Fig. 5. Specific lysis of target cells by restimulated effector cells from the immunized mice. Effector cell-target cell ratios are indicated on the abscissa. The percent specific lysis are demonstrated on the vertical axis. This test was performed at 38 dpi.

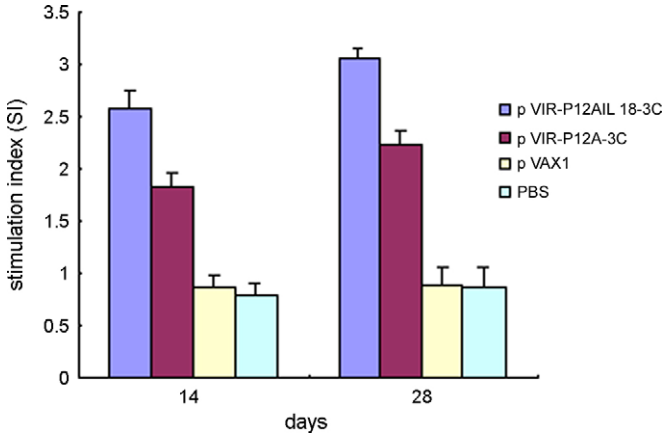

Fig. 6. T lymphocyte proliferation response of swine stimulated by live virus and ConA, respectively. The stimulation indexes (SI) are demonstrated on the vertical axis. This test was performed at day 14, day 28 after first vaccination, indicated on the abscissa.

response. Analysis of the response showed there was a difference ($P < 0.01$) between the pVIR-P12AIL18-3C group and all other groups (Fig. 6).

4. Discussion

Although it is generally accepted that protective immunity to FMDV is principally due to neutralizing antibody, the cellular immune response provides an essential regulatory role in the induction and expression of the serological response. Therefore, an appropriate cellular immune response is essential to resolve FMD (Francis et al., 1987; Collen et al., 1989; Sanz-Parra et al., 1998). With this in mind, DNA vaccines offer significant advantages over the traditional virus inactivated vaccines or recombinant proteins, as the antigen is expressed intracellularly in the immunized animal. As a result, the processing and presentation of the viral epitope occurs in a way that is similar to natural infection. Indeed DNA has been demonstrated to stimulate all effector branches of the immune response, including antibody production, T lymphocyte proliferation response and CTL cytotoxic activity (Sharma and Khuller, 2001; Berzofsky et al., 2001). However, DNA vaccination alone is limited in that it often generates only weak immune responses, particularly the cellular response, in the absence of suitable adjuvants. Adjuvants, such as the Freund's complete adjuvant (FCA) (Grubman et al., 1993), or QS21 (Taboga et al., 1997), are widely used in various vaccine formulations for the enhancement of immune responses. Some cytokines, for example, IL-1 (Park et al., 2006), IL-12 (Sin et al., 1999), IFN (Moraes et al., 2003), colony-stimulating factor and TNF, as immunomodulators, have been reported to be effective in animal models or clinical tests. Among the large array of cytokines, IL-18 was initially identified as a potent IFN-γ-inducing factor. IL-18 mRNA is expressed in a wide range of cells, including various types of immune competent cells and non-immune cells (Schijns, 2000; Pollock et al., 2003). Similar to IL-12, the dominant function of IL-18 is to facilitate Th1 immune responses. However, more recent studies showed that IL-18 may also promote Th-2 type responses and may play a role in antibody formation (Pollock et al., 2003). Many other studies have shown the positive effects of plasmid encoding IL-18 as a molecular adjuvant on DNA vaccinations (Billaut-Mulot et al., 2001; Dupr'e et al., 2001; Shi et al., 2006; Mingxiao et al., 2006). In this paper, we selected swine IL-18 as adjuvant to improve the immunogenic of DNA.

Our study showed that DNA vaccine pVIR-P12AIL18-3C co-expressing IL-18 could elicit higher levels of CTL activity, T lymphocyte proliferation response and antibody than DNA vaccine pVIR-P12A-3C ($P < 0.05$) without IL-18 in swine or mice. Furthermore, results of viral challenge also showed the pVIR-P12AIL18-3C provided more forceful protection than the pVIR-P12A-3C. And compared with control groups, the appearance of FMD symptoms in the pVIR-P12AIL18-3C group and pVIR-P12A-3C groups were delayed. The similar result of effect of a molecular adjuvant on genetically engineering vaccine also have been proved by Cedillo-Barron et al. (2001), Moraes et al. (2003), Zhang et al. (2005) and Shi et al. (2006, 2007). Li et al. used GM-CSF as a molecular adjuvant and found some enhancement of the antibody response in swine. Shi et al. (2006) use BoIFN-γ or rBoIL18 and rVP1 to co-inoculate to mice, and found the potent immune enhance effects of BoIFN-γ and rBoIL18. Moraes et al. (2003) used interferon alpha as a molecular adjuvant and found the swine inoculated with a combination of Ad5-pIFNalpha and Ad5-A24 and challenged 5 dpi were all completely protected from disease and developed a significant FMDV-specific neutralizing antibody response. Zhang et al. (2005) used Bacillus Calmette-Guerin (BCG) as a molecular adjuvant and found that codelivery of BCG-DNA with DNA vaccines against FMD, AjD and CSF can enhance the induction of antigen-specific, especially, cell-mediated immunity. Park et al. (2006) inoculated mice with the encoding plasmids which was constructed to express VP1/interleukin-1alpha (IL-1alpha) and precursorcapsid (P1) in combination with 2A (P1-2A)/IL-1alpha. The results show that although the immunized groups did not carry a high level of neutralizing antibodies, the plasmids encoding the VP1/IL-1alpha, and P1-2A/IL-1alpha fused genes were effective in inducing an enhanced immune response.

It has been demonstrated that baculovirus, recombinant fowlpox virus, vaccinia and adenovirus directed co-expression of precursor FMDV capsid antigen and 3C protease, results in assembly of virus-like particles (Lewis et al., 1991; Abrams et al., 1995; Mayr et al., 1999; Zheng et al., 2006). The mature FMDV capsid protein has both linear and conformational neutralizing epitopes (Jackson et al., 2003). Although general antibody and T cell responses can be elicited by the administration of recombinant virus or naked DNA-expressing capsid precursor genes without functional 3C protease, the levels of neutralizing response and protection were extremely low (Beard et al., 1999; Sanz-Parra et al., 1998). Therefore, the correct proteolysis by 3C, as well as folding and assembly of the FMDV capsid polypeptide may play a key role in protective immunity. To date, a number of groups (Benvenisti et al., 2001; Ward et al., 2001) have used naked DNA containing the P12A and 3C proteinase coding regions of FMDV as vaccine candidates. Tests in swine or mice revealed that this approach afforded only partial protection after multiple inoculations. Based on the above rea-

son, we constructed two DNA vaccine containing P12A gene and 3C gene. Furthermore, the 2A gene of FMDV 2A was introduced as a linker between IL-18 and P1 proteins to allow autonomous, intra-ribosomal, self-processing of polyproteins to assure P1 and IL-18 to be expressed in pVIR-P12AIL18-3C. Several publications in the past few years have shown the potential of this new co-expression strategy (Lorens et al., 2004; Szymczak et al., 2004; Felipe and Ryan, 2004).

In conclusion, results of immune response and viral challenge showed that the two DNA vaccine vaccines were able to elicit a strong humoral and cellular response and were capable of partially protecting swine from FMDV type O challenge. Moreover, the pVIR-P12AIL18-3C-inoculated group induced stronger immune responses and provided better protection than the pVIR-P12A-3C-vaccinated group. This indicates that IL-18 might effectively enhance humoral-mediated immunity and cell-mediated immune responses to DNA vaccination. Overall, these studies demonstrate that this vaccination strategy may be useful, and analysis of other protective FMDV antigens should be explored alone or in combination with those employed in the current study.

Acknowledgements

We thank P. Li and J.S. Zhang for the assistance of cell culture and reagent preparation. This work was supported by grants from National 863 Project of China (no. 2001AA213071) and Key Technologies R&D Program of Jilin Province (no. 20040202-1).

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