Protective immune responses in guinea pigs and swine induced by a suicidal DNA vaccine of the capsid gene of swine vesicular disease virus

Shi-Qi Sun, Xiang-Tao Liu, Hui-Chen Guo, Shuang-Hui Yin, You-Jun Shang, Xia Feng, Zai-Xin Liu and Qing-Ge Xie

Key Laboratory of Animal Virology of Ministry of Agriculture, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

A suicidal DNA vaccine based on a Semliki Forest virus (SFV) replicon was evaluated for the development of a vaccine against swine vesicular disease virus (SVDV). The 1BCD gene of SVDV was cloned and inserted into pSCA1, an SFV DNA-based replicon vector. The resultant plasmid, pSCA/1BCD, was transfected into BHK-21 cells and the antigenicity of the expressed protein was confirmed using an indirect immunofluorescence assay. Immunogenicity was studied in guinea pigs and swine. Animals were injected intramuscularly three times with pSCA/1BCD at regular intervals. Anti-SVDV antibodies were detected by ELISA, the lymphocyte proliferation response was tested by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide method and neutralizing antibodies were measured by microneutralization tests. The data showed that SVDV-specific antibodies, neutralizing antibodies and lymphocyte proliferation were induced in both guinea pigs and swine. Furthermore, after three successive vaccinations with pSCA/1BCD, half of the pigs were protected against challenge with SVDV. These results should encourage further work towards the development of a DNA vaccine against SVDV.

INTRODUCTION

Swine vesicular disease (SVD) is a highly contagious viral pig disease, characterized by the appearance of vesicles on the coronary bands, heels of the feet and less commonly on the snout and tongue. Due to the similarity of these lesions to those caused by foot-and-mouth-disease (FMD), SVD is subject to international controls and is listed by the World Organization for Animal Health. SVD was first identified in Italy in 1966 (Nardelli et al., 1968) and several more outbreaks have been reported subsequently in Europe and eastern Asia (Brocchi et al., 1997). However, in the recent past, reports of SVD have been limited to Portugal and Italy.

SVD vaccines have been developed previously to control the disease, both in monovalent form (Gourreau et al., 1975) and in combination with FMD (Mitev et al., 1978), and an SVD subunit vaccine has also been described, although it was not very efficacious (Jiménez-Clavero et al., 1998). Although the inactivated virus vaccines are effective in protecting against clinical signs, there has been little, if any, assessment made of their ability to reduce wild-type virus transmission and no effective vaccine is available commercially. Once introduced, SVD could be a difficult disease to eradicate and improved methods of control would be highly beneficial, including the development of safer and more effective vaccines to protect and control this disease.

Suicidal DNA vaccines, based on the alphavirus replicon, have emerged as an important strategy to enhance immunogenicity and to improve the biosafety of conventional DNA vaccines (Berglund et al., 1998; Leitner et al., 1999; Lundstrom, 2000). Unlike the conventional DNA vaccine construct in which heterologous gene expression is driven directly by the RNA polymerase II-dependent promoter, suicidal DNA vaccines based on the replicon of alphaviruses, including Sindbis virus (SINV) (Herweijer et al., 1995), Semliki Forest virus (SFV) (Liljestrom & Garoff, 1991) and Venezuelan equine encephalitis virus (Davis et al., 1989), constitute RNA self-amplifying replicons in eukaryotic cells (Morris-Downes et al., 2001). The plasmids include a full-length human cytomegalovirus (CMV) promoter-driven expression cassette and are able to produce their replicase complex following cytoplasmic transport of the corresponding RNA. The replicase produces a full-length RNA encoding itself, as well as an abundant subgenomic mRNA encoding the heterologous protein. The RNA self-amplifying property is of considerable interest with respect to vaccine biosafety: the vector replicates at the RNA level and not at the DNA level, so the rate of foreign DNA present in vivo and possessing ‘genome integration potential’ is controlled and does not increase following vaccination (contrary to some attenuated or recombinant vaccines). Furthermore, when a suicidal DNA vaccine is transfected...
into cells, it leads eventually to apoptosis of the transected cells (Kohno et al., 1998; Leitner et al., 2000), which is particularly important in alleviating the concerns of potential integration and cell transformation generated by the use of conventional DNA vaccines (Gurunathan et al., 2000; Lewis & Babiuk, 1999).

Several groups have demonstrated the ability of suicidal DNA vaccines to induce high-level humoral and cell-mediated immunity against a variety of antigens, with immunized animals developing more prominent immune responses than those receiving a conventional DNA vaccine encoding the same antigen (Berglund et al., 1998; Deshpande et al., 2002; Hariharan et al., 1998; Kirman et al., 2003; Leitner et al., 2000). In addition, it has been demonstrated that suicidal DNA vaccines could break immunological tolerance by activating innate antiviral pathways, in contrast to conventional DNA vaccines encoding the same antigen (Leitner et al., 2003). All of these advantages indicate that suicidal DNA vaccines are an attractive vaccine-delivery vehicle and an alternative strategy to conventional DNA vaccines.

The aim of this study was to assess the immunogenic properties and protection value of a suicidal DNA vaccine against SVD.

**METHODS**

**Cell culture and virus propagation.** BHK-21 cells and IBRS-2 cells were cultured routinely at 37°C in a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. SVD virus (SVDV) HK/70 (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China; Ye et al., 2005) was propagated in IBRS-2 cells cultured in DMEM. After 12 h of incubation at 37°C, when more than 80% of the cells showed cytopathic effect, the cells were subjected to three freeze–thaw cycles. The viral suspension was clarified by centrifugation at 800g for 10 min and stored at −70°C.

**Construction of recombinant vectors.** Viral RNA was extracted from the viral suspension using an RNasy mini kit (Qiagen). A first-strand synthesis reaction was then performed using random hexamers (TaKaRa Bio) to anneal and prime the viral RNA for reverse transcription with avian myeloblastosis virus reverse transcriptase in the presence of RNasin. The cDNA was used in a high-fidelity PCR with forward primer 5′-TAGCCGCCCACATG-GCTGCCCTCAATTCC-3′ and reverse primer 5′-GCTAGGGGCTGTTTTTTCATGG-3′. The primers were designed according to the sequence of the HK/70 strain (GenBank accession no. AY429470). The forward primer contained a Kozak sequence and initiation codon (ATG) for optimal initiation of translation and the reverse primer contained a stop codon (TAG) for correct termination, as indicated by the underlined nucleotides. Following gel purification, the PCR product was cloned into the dephosphorylated SmaI site of pSCA1, the SFV DNA-based replicon vector (DiCiccommo & Bremner, 1998; kindly provided by Dr Roderick Bremner, Vision Science Research Program, Canada) and the resultant plasmid was named pSCA/1BCD. The fidelity of the recombinant plasmid was confirmed by restriction digestion and sequence analysis. The expression plasmid was introduced into Escherichia coli DH5α and large-scale DNA production runs were performed using EndoFree Plasmid Mega kit columns (Qiagen).

**In vitro plasmid expression.** Expression of SVDV capsid protein from pSCA/1BCD, driven by the CMV promoter, was verified using an immunofluorescence assay (IFA). pSCA/1BCD was introduced into BHK-21 cells (in 35 mm wells) using Lipofectamine Plus reagent (Invitrogen). Two days after transfection, cells were analysed for expression of SVDV proteins. A monolayer of cells cultured on coverslips was fixed in cold 100% acetone (−20°C for 30 min). Samples were incubated with rabbit anti-SVDV serum (37°C for 30 min) in a humid box and then with fluorescein-conjugated goat anti-rabbit serum (Sigma) for 30 min at 37°C (Guo et al., 2005). Fluorescence was observed under a Leica microscope.

**Immunization and challenge.** Guinea pigs weighing 400–500 g were obtained from the Laboratory Animal Centre of Lanzhou Veterinary Research Institute, China. The DNA vaccine was prepared by diluting the purified plasmid DNA preparation to 1 mg ml⁻¹ in Dulbecco’s PBS (DPBS; Sigma). Groups of seven guinea pigs were inoculated three times at 3-week intervals with 200 µg pSCA/1BCD DNA vaccine for primary administration and with 300 µg pSCA/1BCD DNA vaccine for booster immunization. The diluted DNA was injected into the quadriceps muscle of both rear legs using a syringe and needle. Guinea pigs inoculated with the same amount of control pSCA1 DNA, without the insert, were used as controls. Serum was collected at weeks 0, 3, 6 and 9 post-immunization (p.i.).

Nine 2-month-old pigs were purchased from a conventional breeding/finishing farm. Six pigs were immunized intramuscularly with pSCA/1BCD and three with pSCA1 as controls. All pigs were immunized three times with a 2-week dose interval with 300 µg DNA vaccine for primary administration and 500 µg DNA vaccine for booster immunization. Three weeks after the final immunization, all pigs were challenged subcutaneously with a 10⁵ median mouse lethal dose of SVDV strain HK/70. All pigs were housed in an isolation facility and examined for 15 days after challenge.

**ELISA for SVDV-specific antibodies.** Serum samples from guinea pigs were evaluated by an indirect ELISA test using the recombinant VP1 protein of SVDV, produced in E. coli, as antigen. The VP1 protein was expressed in E. coli using the pGEX expression system (Amersham Pharmacia Biotech) and the recombinant product was purified by glutathione S-transferase agarose affinity chromatography. Ninety-six-well flat-bottomed plates (Nunc) were coated with recombinant VP1 protein in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking with 5% BSA in PBS, plates were incubated with duplicate twofold serial dilutions of test sera for 1 h at 37°C. Rabbit anti-guinea pig IgG peroxidase conjugate (Sigma) at a 1:2000 dilution was added for 1 h at 37°C, followed by the addition of the substrate 3',5',5'-tetramethylbenzidine. Absorbance was determined at 450 nm using a Bio-Rad microtitre plate reader.

SVDV-specific antibodies in pig serum were detected using a commercial competitive sandwich ELISA kit (Ceditest; Cedi-Diagnostics B.V.) following the manufacturer’s instructions.

**Serum neutralization assay.** Prior to testing, sera were incubated for 30 min at 56°C to inactivate complement. Sera were diluted twofold serially in 96-well microtitre plates, mixed with 200 TCID₅₀ SVDV strain HK/70 in a 100 µl volume and incubated for 1 h at 37°C. After incubation, 100 µl IBRS-2 cell suspension containing 10⁴ cells was added and plates were incubated for 3 days at 37°C in 5% CO₂. Thereafter, cells were examined for SVDV-specific
cytopathic effect and neutralization titres were calculated as \(-\log_{10}\) of the reciprocal of the highest dilution resulting in 50% neutralization.

**Lymphocyte proliferation assay.** Blood was collected from immunized animals in blood-collecting tubes containing heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Ficoll-Paque Plus (density 1.077; Amersham Biosciences) for 30 min at 18 °C. Mononuclear cells were collected from theuffy coat and centrifuged, and residual red blood cells were lysed by incubation in water for 1 min followed by the 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% FBS, 5×10⁻⁵ M 2-mercaptoethanol and penicillin/streptomycin). PBMCs were added to 96-well flat-bottomed plates at a concentration of 100 µl per well (2×10⁵ cells per well). Subsequently, 100 µl per well of medium with or without inactivated SVDV was added and mixed. Phytohaemagglutinin (50 µg ml⁻¹; Sigma) was used as a positive control. Each sample was tested in triplicate. The plates were incubated at 37 °C for 45 h in 5% CO₂ followed by incubation with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) for 3 h and then 10% SDS/0.01 M HCl was added to every well until the deposit was diluted. Absorbance was determined at 570 nm.

**Statistical analysis.** Data were analysed using Student’s t-test. P values of less than 0.05 were considered statistically significant.

## RESULTS

**Expression of pSCA/1BCD plasmid in BHK-21 cells**

In order to demonstrate expression of the SVDV capsid proteins, transfected BHK-21 cells were analysed by IFA. Cells transfected with pSCA/1BCD showed specific green fluorescence but the negative control, which was transfected with the same amount of pSCA1, without the insert, and non-transfected cells did not show any fluorescent emission (data not shown).

**Immunogenicity of suicidal plasmid pSCA/1BCD in guinea pigs**

To evaluate the immunogenicity of the recombinant replicon plasmid pSCA/1BCD, it was inoculated into guinea pigs as described in Methods. Blood was collected at week 0 (prior to vaccination), week 3 (21 days after the first vaccination), week 6 (21 days after the second vaccination) and week 9 (21 days after the third vaccination) to test for the presence of anti-VP1 antibody, neutralization antibody and lymphocyte proliferation.

Total anti-VP1 antibody response was determined by an indirect ELISA. The mean antibody level of the pSCA/1BCD-vaccinated group was significantly higher (0.01 < P < 0.05) than those of the pSCA1 and DPBS control groups (Fig. 1). After the booster immunization, the mean antibody level increased, but this was not statistically significant (P > 0.05). Serum samples were evaluated further in an SVDV serum neutralization test (SNT) (Table 1). The results were similar to the ELISA antibody responses.

Compared with the control group, the vaccinated group showed a significant lymphocyte proliferation response (P < 0.01) after the first vaccination, which increased after the booster vaccination (Fig. 2).

### Table 1. Neutralizing antibody titres of guinea pigs

Results were measured by SNT and are shown as \(-\log\) titre (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 0</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated SVDV</td>
<td>&lt;0.6</td>
<td>1.4±0.1</td>
<td>1.8±0.2</td>
<td>&gt;2.1</td>
</tr>
<tr>
<td>pSCA/1BCD</td>
<td>&lt;0.6</td>
<td>0.9±0.2</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>pSCA1</td>
<td>&lt;0.6</td>
<td>&lt;0.6</td>
<td>&lt;0.6</td>
<td>&lt;0.6</td>
</tr>
</tbody>
</table>

**Immunogenicity test in swine**

Following the positive results of the guinea pig experiment, further immunogenicity testing was carried out in swine.
Blood samples were collected before the primary inoculation (day 0), booster inoculations (days 14 and 28) and viral challenge (day 49). SVDV-specific antibodies were measured by ELISA and SNT, and lymphocyte proliferation was tested using the MTT method.

Total anti-SVDV antibody responses determined by a commercial competitive ELISA are presented in Table 2. Two out of six animals inoculated with suicidal DNA vaccine were seropositive on day 14 p.i. The antibody level was enhanced by booster immunization and an additional two animals were seropositive on day 28 p.i. (day 14 after the second immunization), whilst the remaining two animals were still seronegative after three immunizations. No animals seroconverted in the control group inoculated with plasmid pSCA1.

Induction of neutralizing antibody was similar to that observed for the ELISA antibody (Table 2). Briefly, neutralizing antibody was detected in four out of six immunized animals, whilst all of the control animals remained negative.

For the proliferative PBMC responses, the stimulation index of the immunized group was higher than that of the control group (Fig. 3), although this was not statistically significant ($P > 0.5$).

**DISCUSSION**

SVDV is an important pathogen of swine, causing vesicular lesions that are indistinguishable from those caused by FMDV, and against which there is currently no vaccine generally available. The development of safe and effective vaccines is increasingly important to the control of this disease. DNA vaccines have been shown to induce protective cellular and humoral immune responses and can overcome many problems associated with conventional vaccination; for example, the potential danger associated with the production and distribution of attenuated live vaccines. In

---

**Table 2.** SVDV-specific antibodies (% inhibition), neutralizing antibodies (−log titre) in pigs at various days p.i. and the time of appearance of SVD symptoms in pigs post-challenge (p.c.).

*NS, No clinical signs.*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Animal number</th>
<th>SVDV-specific antibodies (% inhibition)</th>
<th>Neutralizing antibody (−log titre)</th>
<th>Time of appearance of SVD symptoms (day p.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 days p.i.</td>
<td>14 days p.i.</td>
<td>28 days p.i.</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pSCA1</td>
<td>1</td>
<td>19.3</td>
<td>26.6</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.0</td>
<td>18.2</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.7</td>
<td>20.7</td>
<td>17.8</td>
</tr>
<tr>
<td>pSCA/1BCD</td>
<td>4</td>
<td>6.4</td>
<td>7.9</td>
<td>35.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>23.8</td>
<td>31.2</td>
<td>51.1*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>18.0</td>
<td>10.2</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12.7</td>
<td>55.4*</td>
<td>68.9*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.5</td>
<td>58.3*</td>
<td>80.8*</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>11.5</td>
<td>41.2</td>
<td>53.6*</td>
</tr>
</tbody>
</table>

*Inhibition $\geq 50\%$: SVDV-specific antibodies are present in the sample.

---

**Fig. 3.** Specific proliferation of PBMCs in immunized pigs presented as the mean stimulation index (SI). SI was calculated as the ratio of mean absorbance values of wells containing antigen-stimulated cells to mean absorbance values of wells containing only cells with medium.
addition, the proteins are synthesized, processed and
presented in cells, which is more similar to natural infection
than administration of conventional inactivated vaccines.
However, in all cases, the protection level is generally low
compared with that of attenuated or inactivated vaccines.
More recently, suicidal DNA vaccines have emerged as an
important strategy in enhancing immunogenicity and
improving the biosafety of conventional DNA vaccines
(Berglund et al., 1998; Leitner et al. 1999; Lundstrom, 2000).
The goal of this study was to construct a suicidal DNA
vaccine against SVDV and assess its immunogenic proper-
ties and ability to protect against challenge.

SVDV particles are composed of 60 copies each of the four
capsid proteins VP1–VP4, which enclose a single-stranded,
positive-sense RNA genome of about 7400 nt. Proteins VP1
(1D), VP2 (1B) and VP3 (1C) are exposed at the viral
surface, whereas VP4 (1A) is in intimate contact with the
RNA and thus not accessible from the outer shell surface in
the intact virions. It has been shown that both conforma-
tion-dependent neutralizing sites and linear epitopes are
located mainly in the outer capsid proteins (Borrego et al.,
2002a, b; Jiménez-Clavero et al., 2000; Kanno et al., 1995;
Nijhar et al., 1999; Rebel et al., 2000). Furthermore, a
recombinant bacterially expressed SVDV polypeptide, p1, is
able to induce an SVDV-specific cellular and humoral
immune response in pigs (Jiménez-Clavero et al., 1998).
For this reason, the 1BCD gene was chosen in this study.

It has been determined that alphavirus DNA-based expres-
sion vectors have broad potential application for genetic
immunization (Berglund et al., 1998; Dubensky et al., 1996)
and gene therapy (Herweijer et al., 1995). SINV and SFV
DNA-based vectors, tested with a variety of antigens in mice,
have elicited stronger protein expression and resulted in the
development of humoral and cellular immune responses at
higher levels than conventional promoter/reporter gene
plasmids (Berglund et al., 1998; Hariharan et al., 1998). The
DNA-based SFV expression vector used in this study was
described by DiCiccomo & Bremmer (1998), who demon-
strated that this DNA-based SFV vector could produce high
levels of protein rapidly.

In our study, the preliminary in vitro work indicated that the
recombinant plasmid pSCA/1BCD could express the SVDV
capsid protein faithfully, but the expression efficacy was not
evaluated fully. The in vivo study in guinea pigs and swine
showed that SVDV-specific immune responses were
induced when they were injected intramuscularly with
plasmid DNA. However, the humoral immunity level was
relatively low, with neutralizing antibody titres (−log titre)
of between 0.9 ± 0.2 and 1.2 ± 0.1 in guinea pigs and
between 0.7 and 1.4 in swine. The differences in stimulation
index of lymphocytes between the vaccinated and control
groups were not statistically significant in swine. Although
the reason for this is not clear, it may be related to the nature
of the antigen itself. Previous studies have shown that
different antigens can produce different results using
alphavirus expression systems. For instance, it has been
shown that the humoral immune responses induced by an
SFV-based DNA vaccine were relatively lower than those
obtained from the conventional DNA vaccine when the NS1
antigen was expressed (Vidalin et al., 2000), although this
result contrasts with other reports using other antigens or
other alphavirus expression systems (Berglund et al., 1998;
Hariharan et al., 1998; Leitner et al., 2000). Likewise, this
difference was not observed when the prME spike complex
antigen was expressed (Vidalin et al., 2000). Another
explanation may be related to the probability that the quantity
of DNA taken up by muscle cells was low because of the large
size of the plasmid (∼13.8 kb), resulting in the production of
only a small quantity of antigen. Alternatively, the reverse may
be true: when a large amount of replicon-based DNA was
transfected into muscle cells, the replicase may have produced
large amounts of double-stranded RNA, which rapidly
induced apoptosis, thus terminating the expression and
processing of viral antigen. This latter hypothesis is supported
indirectly by data reported by Kim et al. (2004), who showed
that suicidal DNA vaccine potency could be enhanced by
delaying suicidal DNA-induced cell death.
ACKNOWLEDGEMENTS

This work was supported by grants from The National Basic Research Program of China (973) no. 2005CB523201 and ‘863’ Program no. 2003AA241110. We are grateful to Dr Roderick Brenner (Vision Science Research Program, Canada) for kind providing pSCA1 plasmid and Dr Trevor Drew, Jason Hang and Professor Chaqing Qiu for critically reading the manuscript. We also thank Hong Tian, Jinyan Wu, Zhenli Gong, Haixue Zheng and Xinguo Dai for helping in animal experiments and Mrs Kelan Chai for animal care.

REFERENCES


