Comparison of immune responses against foot-and-mouth disease virus induced by fusion proteins using the swine IgG heavy chain constant region or β-galactosidase as a carrier of immunogenic epitopes

Guangjin Li,1, Weizao Chen, Weiyao Yan, Kai Zhao, Mingqiu Liu, Jun Zhang, Liang Fei, Quanxing Xu, Zutian Sheng, Yonggan Lu, Zhaoxin Zheng,*

aState Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, P.R.China
bInstitute of Animal Husbandry and Veterinary Medicine, Shanghai Academy of Agricultural Science, Shanghai 201106, P.R.China
cInstitute of Virology of Zhejiang Academy of Agricultural Science, Hangzhou 310021, P.R.China
dInstitute of Veterinary of Lanzhou, Lanzhou 730046, P.R.China

Received 10 April 2004; returned to author for revision 7 June 2004; accepted 22 July 2004
Available online 26 August 2004

Abstract

Previously, we demonstrated that a fusion protein (Gal-FMDV) consisting of β-galactosidase and an immunogenic peptide, amino acids (141–160)–(21–40)–(141–160), of foot-and-mouth disease virus (FMDV) VP1 protein induced protective immune responses in guinea pigs and swine. We now designed a new potential recombinant protein vaccine against FMDV in swine. The immunogenic peptide, amino acids (141–160)–(21–40)–(141–160) from the VP1 protein of serotype O FMDV, was fused to the carboxy terminus of a swine immunoglobulin G single heavy chain constant region and expressed in Escherichia coli. The expressed fusion protein (IgG-FMDV) was purified and emulsified with oil adjuvant. Vaccination twice at an interval of 3 weeks with the emulsified IgG-FMDV fusion protein induced an FMDV-specific spleen proliferative T-cell response in guinea pigs and elicited high levels of neutralizing antibody in guinea pigs and swine. All of the immunized animals were efficiently protected against FMDV challenge. There was no significant difference between IgG-FMDV and Gal-FMDV in eliciting immunity after vaccination twice in swine. However, when evaluating the efficacy of a single inoculation of the fusion proteins, we found that IgG-FMDV could elicit a protective immune response in swine, while Gal-FMDV only elicited a weak neutralizing activity and could not protect the swine against FMDV challenge. Our results suggest that the IgG-FMDV fusion protein is a promising vaccine candidate for FMD in swine.

© 2004 Elsevier Inc. All rights reserved.

Keywords: FMDV; IgG; Vaccine; Immune response

Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating viral disease of cloven-hoofed livestock. The causative agents of FMD are small icosahedral viruses of the Aphthovirus group within the Picornaviridae family (Belsham, 1993; Pereira, 1981). FMD virus (FMDV) exhibits a high potential for genetic and antigenic variation, which has led to the classification of seven serotypes: A, O, C, Asia I, SAT1, SAT2, and SAT3. The infectious particle is composed of 60 copies each of four structural proteins (VP1, VP2, VP3, and VP4) that enclose a single-stranded, positive sense RNA genome (Barteling and Vreeswijk, 1992; Baxt et al., 1984). VP1 carries important immunogenic sites recognized by host immune cells, including amino acids (aa) 141–160, a major B cell site, and aa21–40, a T-cell site (Baxt et al., 1984; Collen et al., 1991; Strohmaier et al., 1982).
Regular vaccination is an attractive control strategy for FMD, especially in endemic areas. Toward this aim, various vaccine-development approaches were undertaken. These include use of chemically inactivated viruses emulsified with adjuvant (Barteling and Vreeswijk, 1992; Sáiz et al., 2002), modified live-virus (Mason et al., 1997; McKenna et al., 1995), synthetic peptides (Bittle et al., 1982; Dimarchi et al., 1986; Wang et al., 2002), recombinant proteins (Broekhuijsen et al., 1987; Syred et al., 1987), recombinant virus (Berinstein et al., 2000; Chinsangaram et al., 2003; Mayr et al., 1999; Wu et al., 2003), and DNA vaccines (Cedillo-Barron et al., 2003; Chinsangaram et al., 1998). Among these strategies, the development of safe and effective recombinant protein FMD vaccines that contain a large foreign protein carrier and FMD immunogenic epitopes constitutes an interesting goal (Zheng et al., 1994).

We have previously developed a recombinant protein FMD vaccine that contains β-galactosidase and a peptide of immunogenic sites consisting of two copies of aa141–160 and one copy of aa21–40 from VP1 (Huang et al., 1999). This fusion protein can elicit high levels of neutralizing antibody and an FMDV-specific proliferative T-cell response in guinea pigs and swine, as well as efficiently protect the immunized animals against viral challenge. However, repeated immunization with this recombinant protein may result in an undesirable immune response due to non-specific immunogenicity generated by the β-galactosidase carrier protein.

To avoid the side effects of excess immunogenicity caused by β-galactosidase, we have designed a new vaccine candidate by replacing β-galactosidase with the swine IgG single heavy chain constant region. The reasons for choosing swine IgG heavy chain constant region as the epitope carrier are: (1) Swine IgG is a self-molecule; (2) immunoglobulins have been suggested as one of the most suitable candidates to replace the microbial protein used as a carrier among self-molecules (Bona et al., 1994). (3) We once chose swine IgG heavy chain as the carrier for FMDV epitopes by replacing the complementary-determining region 3 (CDR3) with FMDV antigenic peptide, but the created chimeric protein can just elicit a weak immune response in guinea pigs and swine and cannot protect efficiently the immunized animals against viral infection (Zhao et al., 2000). In the current study, the fusion protein IgG-FMDV was created by fusing an immunogenic peptide consisting of two copies of aa141–160 and one copy of aa21–40 of serotype O FMDV VP1 to the carboxy terminus of the swine single heavy chain constant region. The IgG-FMDV was expressed in *Escherichia coli* and was then used to immunize guinea pigs and swine. T-cell proliferative responses, neutralizing activities and protections against viral infection elicited by IgG-FMDV were investigated in the immunized animals.

**Results**

**Expression, purification and Western blot assay of IgG-FMDV fusion protein**

The swine IgG single heavy chain constant region was amplified by PCR. An immunogenic peptide consisting of two copies of aa141–160 and one copy of aa21–40 from VP1 protein was fused to the carboxy terminus of the swine IgG single heavy chain constant region to create a chimeric protein (IgG-FMDV) (Fig. 1). The IgG-FMDV fusion protein was expressed in *E. coli* after induction with IPTG (Fig. 2A, lane 2). To confirm the presence of the FMDV insert, the cultures were detected by Western blot analysis using an anti-FMDV antibody. As shown in Fig. 2A, the IgG-FMDV fusion protein could be recognized by anti-FMDV antibody (Fig. 2A, lane 6), whereas no bands were found in the cell extracts without IPTG induction, with the vector without insert, or the extracts without the construct (Fig. 2A, lanes 7–9). Western blot analysis was also performed to confirm the absence of reactivity with preimmune sera of swine used for viral challenge assays. As expected, no bands were found (Fig. 2A, lanes 10–13).

The expressed IgG-FMDV protein, which forms inclusion body, was partially purified and SDS-PAGE showed that the target protein consisted 90% of the total proteins (Fig. 2B, lane 3).

**T-cell proliferative response in guinea pig**

Two groups of guinea pigs each were immunized with 100 μg of purified IgG-FMDV or Gal-FMDV fusion protein...
emulsified with oil adjuvant. The control guinea pigs received PBS or IgG heavy chain constant region without FMDV epitopes. Spleen T-cells from all test groups of guinea pigs were isolated and exposed to five 2-fold serial dilutions of pure FMDV antigen. The proliferative responses were expressed as the stimulation index (SI). In Fig. 3, it can be seen that positive lymphoproliferations (SI > 2) against both IgG-FMDV and Gal-FMDV fusion proteins were detected when T-cells were stimulated with each of the five serial dilutions of pure FMDV antigen. The peak of proliferation was observed at an antigen dilution of 1:10. There is no significant difference between two fusion proteins in eliciting T-cell proliferation response. As the antigen used to stimulate cells was purified FMDV antigen, the T-cell responses were considered FMDV-specific. Spleen T-cells obtained from the control animals did not show any significant level of T-cell proliferation response (SI < 1.5).

**Levels of neutralizing antibody response and viral challenge assay in guinea pigs**

Neutralizing antibody response to FMDV is considered to be the basis of protective immunity (Aggarwal and Barnett, 2002; Doel, 1999). Phagocytosis of virus–antibody complexes, following viral opsonization, may mediate viral clearance in vivo (McCullough et al., 1988). The IgG-FMDV fusion protein contains two copies of the aa141–160 peptide from VP1 protein. This site has been identified as the continuous viral epitope recognized by host B-cells to produce neutralizing antibody (Baxt et al., 1984; Collen et al., 1991; Strohmaier et al., 1982). To examine the levels of neutralizing antibody in the immunized guinea pigs, blood serum was collected before immunization and at weeks 3 and 7 after secondary administration. The neutralizing antibody levels were examined using a suckling mouse protection test. In our previous experience, successful
protection against FMDV infection in swine can usually be achieved if the neutralizing titer in the suckling mouse protection assay is above 2.0. As shown in Table 1, guinea pigs inoculated with 100 μg of IgG-FMDV fusion protein displayed strong neutralizing activities against serotype O FMDV at week 3 (neutralizing titer > 3.75). Although the neutralizing level decreased somewhat at week 7, the neutralizing titer still remained above 3. High neutralizing activities were also observed when guinea pigs were inoculated with Gal-FMDV fusion protein. The negative control guinea pigs injected with either PBS or IgG heavy chain constant region without FMDV epitopes did not develop neutralizing activities.

IgG-FMDV induced T-cell proliferative responses and high levels of neutralizing antibody response. We then test whether IgG-FMDV can protect guinea pigs against FMDV infection. For this purpose, all immunized guinea pigs were challenged by direct inoculation with 100 ID_{50} serotype O FMDV. The animals were then observed daily for clinical signs of FMD. All immunized guinea pigs given IgG-FMDV or Gal-FMDV fusion protein were completely protected and ate and behaved normally during the observation period (Table 1). In contrast, in the negative control group, all the guinea pigs presented characteristic signs of FMD within 3 days post-challenge, those included vesicles on the feet and tongue and high body temperature.

Neutralizing antibody response and viral challenge assays in swine immunized with two inoculations of the fusion proteins

We next sought to determine whether the fusion protein could elicit protective immune responses in swine, which are economically important hosts known to be highly susceptible to FMDV infection. The swine experiment contained two groups of five swine that were each inoculated twice at an interval of 3 weeks with 400 μg of purified IgG-FMDV or Gal-FMDV. The experiment also included five swine that were inoculated with PBS. Neutralizing antibody response and protection of the animals against FMDV challenge were assayed 3 weeks after the secondary inoculation. The results were summarized in Table 2. The neutralizing titers were above 2.5 in both IgG-FMDV- and Gal-FMDV-immunized swine. There was no significant difference in the antibody titer between the two groups. The animals were challenged with serotype O FMDV. All of the immunized animals showed no signs of infection through the course of the experiment. Whereas the animals given PBS developed severe FMD with vesicles and high fever.

Neutralizing antibody response and viral challenge assays in swine immunized with a single inoculation of the fusion proteins

Two inoculations of IgG-FMDV protein in swine were able to induce a high level of neutralizing antibody and protect the animals against viral challenge. We then test the efficiency of a single inoculation of IgG-FMDV protein in swine. Two groups of 10 swine each were immunized with a single inoculation of 800 μg of purified IgG-FMDV or Gal-FMDV protein. The neutralizing antibody levels in sera were assayed at weeks 6 and 10 post-inoculation. As shown in Table 3, the neutralizing antibody level reached approximately 2.7 at week 6 and 2.0 at week 10 in the IgG-FMDV-inoculated group. However, the neutralizing antibody titers in sera from swine inoculated with Gal-FMDV protein were lower than 1.0 at weeks 6 and 10 post-inoculation. The swine were challenged with 100 ID_{50} swine infectious dose FMDV at week 10 post-inoculation. All five swine in the IgG-FMDV-inoculated group showed no FMD clinical symptoms during the 14-day observation period. But one animal receiving Gal-FMDV protein exhibited vesicles on the feet at day 4 post-challenge, and the other four swine also developed severe FMD several days later. This result agrees with our previous study, which showed a single inoculation of Gal-FMDV.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of guinea pigs</th>
<th>Neutralizing titers*</th>
<th>No. protected/ No. challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0</td>
<td>Week 3</td>
<td>Week 7</td>
</tr>
<tr>
<td>IgG-FMDV</td>
<td>6</td>
<td>0</td>
<td>&gt;3.75</td>
</tr>
<tr>
<td>Gal-FMDV</td>
<td>6</td>
<td>0</td>
<td>&gt;3.75</td>
</tr>
<tr>
<td>IgGb</td>
<td>6</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* A mouse protection test was performed to determine the induction of neutralizing antibody responses in tested animals. Titer is the surplus between the log of LD_{50} in experiment group and that in control group. 

b Swine IgG heavy chain constant region without FMDV epitope.
protein could not elicit a protective immune response in guinea pigs.

**Discussion**

It has been shown that fusion protein FMD vaccines have a stronger immunogenicity and longer half-life compared with short peptides corresponding to immunogenic epitopes (Bona et al., 1994; Syred et al., 1987). Immunoglobulin (Ig) has been suggested as an ideal carrier for microbial peptides because of its various advantages such as high efficacy to deliver antigenic peptide, safety, and long half life (Bona et al., 1994). The chimeric protein where the CDR3 region of Ig is replaced with immunogenic epitope was able to induce immune responses efficiently (Zaghouani et al., 1993, 1995). In our previous study, we tried to use swine IgG heavy chain as the carrier for FMDV epitopes. A chimeric protein (L-IgG-FMDV) was constructed by replacing the CDR3 loop of FMDV with the antigenic FMDV peptide (aa141–160)–(aa21–40)–(aa141–160). We believe that the IgG-FMDV has a more adaptable three-dimensional structure than L-IgG-FMDV that exposes the immunogenic sites to the host immune system.

Our previous study showed that a single inoculation of Gal-FMDV only elicits weak immune responses in guinea pigs and swine and cannot protect the immunized animals against FMDV infection, even when up to 5 mg of the fusion protein was used (unpublished data). However, when testing the efficiency of a single inoculation of IgG-FMDV, we found that swine developed protective immune responses. We can offer no definitive explanation for the difference between the immune responses induced by the two fusion proteins. One possibility may be a difference in the uptake of IgG-FMDV and Gal-FMDV by antigen presenting cells. It has been previously shown that Igs are taken up by various types of antigen presenting cells such as dendritic, spleen, and B cells (Bona et al., 1994). In functional assays of the influenza virus hemagglutinin presentation, Ig-peptide was proved to be delivered to T-cells more efficiently than free peptide or influenza virus (Zaghouani et al., 1993). Another possible explanation is that IgG-FMDV is more stable than Gal-FMDV in vivo, resulting in a longer exposure of the immunogen to the immune system. An alternative explanation is that the IgG constant region has inherent immunopotentiating properties when used as a carrier protein fused to the immunogen. Further work is necessary to better understand the nature of the protective immune response to FMDV induced by IgG-FMDV.

Although VP1-based peptide vaccines often induce high level of immunogenicity, they do not always achieve protection against FMDV challenge in livestock. Part of the low efficacy may originate in the hypervariability of the immunogenic sites, expected from the quasi-species genetic structure of FMDV (Grubman and Baxt, 2004). In a large-scale bovine vaccination study using synthetic peptides, Taboga et al. (1997) detected viral escape mutants that were antigenic variants of the challenge virus in vaccinated, unprotected animals. Thus, a possible increase in the efficiency of peptide vaccines may be attained by enlarging the repertoire of independent B cell and T-cell epitopes, together with variant forms of some of them. Another approach to overcome the antigenic variance problem is to incorporate consensus residues into the hypervariable positions of the VP1 sites and this approach has been
shown to provide for broad immunogenicity in swine. Although IgG-FMDV exhibited potency and efficacy in the homologous virus challenge assay, further studies are required to test the efficiency of this vaccine candidate in other species.

Materials and methods

Viruses

The serotype O FMDV used to challenge guinea pigs and swine was strain Hongkong/1999 passaged 2–4 times in sucking mice.

Expression plasmid construction

Plasmid pBSK-IgG carrying the full length of swine IgG heavy chain gene was as described previously (Zhao and Zheng, 1999). PCR was used to amplify the swine single heavy chain constant region from the plasmid pBSK-IgG using a pair of swine IgG gene specific primers: swine IgG-5’ specific primer 5’-ACCGGAATCC (BamHI) TCAGCCCCAAGACGC3’ and swine IgG-3’ specific primer 5’ACCGGAATTCC(EcoRI) TTTACCCTGAGTCTTGC3’. Restriction sites used for subcloning are underlined in each primer.

DNA fragments encoding peptides spanning residues 21–40 and 141–160 of FMDV VP1 protein were chemically synthesized and were ligated into a sequence (aa141–160)–(aa21–40)–(aa141–160). Restriction sites EcoRI and PstI were added to the 5’ and 3’ ends, respectively. This immunogenic sequence and the swine IgG single heavy chain constant region gene were then cloned into the pTricHis vector (Invitrogen, CA, USA) at the BamHI/PstI ends, respectively. This construct thus created encodes a fusion protein (IgG-FMDV) that consists of an immunogenic dominant epitope linked to the carboxy terminus of swine heavy chain constant region without FMDV epitopes (Fig. 1). The DNA sequence was also fused to carboxy terminus of the β-galactosidase gene by cloning into the plasmid pWR590 at EcoRI/BamHI sites as described previously (Huang et al., 1999). This fusion protein was named Gal-FMDV.

Fusion protein expression, purification, and Western blot assays

The IgG-FMDV fusion protein was produced in the E. coli TOP10 strain. Cells were grown at 37 °C to an OD600 of 0.5–0.6, followed by induction with 1 mM of isopropyl-β-D-thiogalactoside (IPTG) for 6 h. Cells were harvested by centrifugation, resuspended and then disrupted by sonication. The lysate was centrifuged and inclusion body pellets were dissolved in 8 M urea. The solubilized samples were then purified by a His-tagged affinity column according to manufacturer’s instructions (Clontech, CA, USA). The Gal-FMDV fusion protein was prepared as described previously (Huang et al., 1999).

The presence of the FMDV insert was confirmed by Western blot analysis. Briefly, a 10% polyacrylamide gel was used for protein separation, and the protein was transferred to a nitrocellulose membrane. The membrane was then incubated with anti-Rabbit FMDV antibody at 4 °C overnight. After washing, the membrane was incubated with anti-guinea pig IgG conjugated with horseradish peroxidase for 1 h at room temperature. The membrane was washed and then incubated with O-phenylenediamine (OPD) containing 0.05% H2O2 (30% W/V) until bands were of the proper intensity.

Vaccine preparation

To prepare oil adjuvant vaccine, the aqueous protein was emulsified with Montanide ISA206 (Seppic, France) to form a water-in-oil-in-water blend. The ratio of aqueous protein to the oil adjuvant was 50:50.

Animals and vaccination

Male and female Dunkan–Hartley guinea pigs weighing 250–300 g and large white swine, 2- to 3-month-old and approximately 40–50 kg, were used in this study. All of the animals were housed at disease secure isolation facilities in an FMDV-free area and were free of previous FMD contact as confirmed by the absence of detectable anti-FMDV antibodies in the serum (Fig. 2A and Tables 1–3). Each guinea pig in a group of six guinea pigs was vaccinated by intramuscular inoculation with 0.2 ml of vaccine containing 50 μg of purified fusion protein. After 3 weeks, the guinea pigs were boosted with the same dose of purified fusion protein. The control groups were inoculated with phosphate-buffered saline (PBS) or the swine heavy chain constant region without FMDV epitopes.

Swine experiment 1: swine immunized with two inoculations of fusion proteins. Fifteen swine were separated into three groups. Two groups of ten swine each was injected intramuscularly in the neck area with 400 μg of purified IgG-FMDV or Gal-FMDV fusion protein. The animals were boosted 3 weeks later with 400 μg of the fusion protein. The negative control group consisted of five unvaccinated swine. Blood samples were obtained for neutralizing antibody level assays at week 3 post-secondary inoculation, and then viral challenge assays were performed.

Swine experiment 2: swine immunized with a single inoculation of fusion proteins. Fourteen swine were separated into three groups. One group of five animals each was inoculated with 800 μg of purified IgG-FMDV protein. The other group of five animals each was inoculated with 800 μg of purified Gal-FMDV protein. The control group contained four animals, which were inoculated with PBS. The neutralizing antibody levels were assayed at weeks 6...
and 10 post-inoculation, respectively, and then viral challenge assays were performed.

**T-cell proliferation assay in guinea pigs**

T-cells were isolated by 1.077 g/ml Percoll solution (Pharmacia) from spleen of guinea pigs 3 weeks after the secondary inoculation and were cultured in triplicate using 96-well flat-bottom plates at a concentration of $1 \times 10^6$ cells/ml. The cells were stimulated with five 2-fold serial dilutions of pure type O FMDV antigen at 37 °C for 4 days. During the last 12 h of culture, each well was pulsed with 0.5 μCi of $[^3]$H]thymidine. The cells were harvested and the uptake of $[^3]$H]thymidine was detected using a liquid scintillation counter (Beckman LS6500). Results were obtained as mean counts per minute (cpm) and expressed as stimulation index (SI; mean cpm of cultures with antigen divided by mean cpm of cultures without antigen). The response was considered significant only when the SI was 2.0 or higher.

**Neutralizing antibody assays**

To determine the induction of neutralizing antibody responses in guinea pigs and swine, a mouse protection test was performed according to the procedures described elsewhere (Huang et al., 1999; Mulcahy et al., 1991). Briefly, suckling mice (6 groups of 10 each) were injected by neck-subcutaneous route with 100 μl of the serum obtained from the fusion protein-immunized animals and control groups. After 24 h, suckling mice were challenged with 100 μl of 10-fold serially diluted virus (i.e., $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$) (serotype O FMDV). The virus dilution required to kill 50% of suckling mice was defined as one half of lethal dose (LD$_{50}$) and estimated by the Reed–Muench method (Reed and Muench, 1938). Neutralizing antibody titer was expressed as the surplus between the log of LD$_{50}$ in the experiment group and that in the control group.

**Viral challenge assays in guinea pigs and swine**

FMDV challenge assays in immunized animals were performed as described previously (Huang et al., 1999). Briefly, 3 weeks after secondary immunization, each guinea pig was inoculated intradermally in each of the rear feet with 0.2 ml of viral solution containing 100 guinea pig infectious dose (100 ID$_{50}$) serotype O FMDV. Challenge tests in swine were carried out by intramuscular injection of each animal with 2 ml of swine infectious dose (100 ID$_{50}$) in the neck region. After challenge assay, all of the animals were examined daily for clinical signs of FMD such as increase in body temperature (above 41 °C) and the appearance of vesicles on the mouth or hooves. Observation was terminated on day 14 post-challenge and the animals were humanely euthanized.

**Acknowledgments**

We are grateful to Dr. Peter Franco for help in preparing this manuscript. This work was supported by China National High Technology Program 863 grant to Z. Z. (2001AA213071).

**References**


