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Efficacy of synthetic peptide candidate vaccines against serotype-A foot-and-mouth disease virus in cattle

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Abstract Foot-and-mouth disease (FMD) remains a major threat to livestock worldwide, especially in developing countries. To improve the efficacy of vaccination against FMD, various types of vaccines have been developed, including synthetic peptide vaccines. We designed three synthetic peptide vaccines, 59 to 87 aa in size, based on immunogenic epitopes in the VP1, 3A, and 3D proteins of the A/HuBWH/CHA/2009 strain of the foot-and-mouth disease virus (FMDV), corresponding to amino acid positions 129 to 169 of VP1, 21 to 35 of 3A, and 346 to 370 of 3D. The efficacies of the vaccines were evaluated in cattle and guinea pigs challenged with serotype-A FMDV. All of the vaccines elicited the production of virus-neutralizing antibodies. The PB peptide, which contained sequences corresponding to positions 129 to 169 of V P1 and 346 to 370 of 3D, demonstrated the highest levels of immunogenicity and immunoprotection against FMDV. Two doses of 50 μg of the synthetic PB peptide vaccine provided 100 % protection against FMDV infection in guinea pigs, and a single dose of 100 μg provided 60 % protection in cattle. These findings provide empirical data for facilitating the development of synthetic peptide vaccines against FMD.

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious and devastating disease of livestock that causes significant economic losses worldwide (Perry and Rich 2007). The causative agent, foot-and-mouth disease virus (FMDV), belongs to the genus *Aphthovirus* of the family *Picornaviridae*. The virion exhibits a high potential for genetic and antigenic variation, and the following seven serotypes have been identified: A, O, C, Asia1, SAT1, SAT2, and SAT3 (Grubman and Baxt 2004). The existence of multiple serotypes and subtypes of FMDV that do not induce cross-protective antibodies confounds the efforts of vaccination programs for controlling FMD (Kitching et al. 1989; Knowles and Samuel 2003).

Inactivated virus vaccines have been used to effectively control FMD in many developing countries, and such vaccines have helped eliminate the disease from some areas of the world (Rodriguez and Grubman 2009; Sobrino et al. 2001). These vaccines usually elicit high levels of neutralizing antibodies and offer efficacious protection against homologous serotypes (Doel 2003). However, conventional FMD vaccines have a number of disadvantages, including incomplete inactivation of the virus, difficulty distinguishing vaccinated animals from infected animals, and the need for revaccination using virus strains that are antigenically similar to currently circulating strains (Doel 2003; Rodriguez and Gay 2011; Rodriguez and Grubman 2009). Therefore, subunit vaccines, recombinant virus vaccines, DNA vaccines, and peptide vaccines have been studied extensively to identify safe and effective alternatives to conventional methods of FMD vaccine production (Wigdorovitz et al. 1999; Chan et al. 2001;

Mason et al. 1997; Qian et al. 2004; Chinsangaram et al. 1998; Li et al. 2008).

The structure of the antigenic regions on the surface of the capsid of the FMDV has been characterized (Crowther et al. 1993; Kitson et al. 1990; Collen et al. 1991). A region in the G–H loop of the VP1 capsid protein consisting of amino acid positions 140 to 160 has been identified as the predominant epitope that elicits the production of neutralizing antibodies by B cells (Bittle et al. 1982; Pfaff et al. 1982). By contrast, two regions of the 3A viral protein consisting of amino acid positions 11 to 25 and 21 to 35 in the O1Kb strain of FMDV have been identified as epitopes which elicit T-cell-mediated immunity (Blanco et al. 2001). The immunogenicity of T cell epitopes within the 3D protein of the FMDV has been demonstrated in both cattle (Collen et al. 1998; Yang et al. 2007) and swine (Foster et al. 1998; Garcia-Briones et al. 2004; Gerner et al. 2006).

Because FMDV contains several neutralizing epitopes, it is an attractive target for the production of synthetic peptide vaccines. In contrast to the conventional vaccines prepared from inactivated virus, both the production and application of synthetic peptide vaccines are epidemiologically secure, and the antigenic specificity of peptide vaccines allows discriminating between vaccinated and infected animals. The ease with which peptide vaccines can be redesigned is also of critical importance with regard to controlling the spread of rapidly mutating viruses, such as the FMDV.

Synthetic peptide vaccines corresponding to viral antigenic epitopes can be rapidly synthesized using chemical methods, and such synthetic peptides have been shown to mimic the structure of viral epitopes (Oldstone et al. 1995). Efforts to develop peptides as FMDV vaccines began in the 1980s. In a previous study, a fusion protein corresponding to amino acid positions 133 to 158 of VP1 and 20 to 34 of VP4 in the Asia1 strain of FMDV elicited neutralizing antibodies in guinea pigs but did not provide complete protection against FMDV challenge (Zhang et al. 2002).

A large-scale synthetic peptide vaccination study in 138 cattle evaluated four peptides corresponding to different sequences within the G–H loop of VP1 in serotype C of the FMDV and found that none of the peptides produced protective immunity in greater than 40 % of the vaccinated animals, regardless of the dose or vaccination schedule used (Taboga et al. 1997). A later study reported that a synthetic peptide vaccine against serotype O of the FMDV was effective in swine (Wang et al. 2002). The epitope corresponding to this peptide immunogen, designated as the UBI peptide, spanned the entire G–H loop and extensive flanking sequences (amino acid positions 129–169) of VP1, has a unique consensus sequence to confront the hypervariability of serotype O viruses, and included an artificial Th site. In 20 of the 21 immunized pigs, the UBI peptide vaccine induced protective immunity against a Taiwanese isolate of FMDV O1 using peptide doses as low as 12.5 μg (Wang et al. 2002).

By contrast, a subsequent pilot study of the UBI peptide vaccine in cattle showed that the titers of virus-neutralizing antibodies (VNAs) induced in the vaccinated cattle were relatively low and that UBI peptide vaccination failed to protect cattle against challenge with a serotype-O strain of FMDV at 3 weeks postvaccination (Rodriguez et al. 2003). Another study reported that pigs vaccinated with a dendrimeric peptide vaccine were protected against challenge with FMDV and that their immune response was characterized by high serum titers of VNAs, activated FMDV-specific T cells, and a potent anti-FMDV immunoglobulin-A response (Cubillos et al. 2008).

Although the results of studies investigating the use of FMDV-based synthetic peptide vaccines have been encouraging, no such vaccine has demonstrated a high level of efficacy for inducing protective immunity against serotype-A FMDV strains in cattle. In our current study, we designed three peptide vaccines against the A/HuBWH/CHA/2009 strain of FMDV corresponding to amino acid positions 129–169 of VP1, 21–35 of 3A, or 346–370 of 3D and assessed the level of protection that each provided against challenge with serotype-A FMDV in guinea pigs and cattle. The PB peptide, which contained sequences corresponding to positions 129 to 169 of VP1 and 346 to 370 of 3D, demonstrated the highest levels of immunogenicity and immunoprotection against FMDV.

Materials and methods

Ethics statement

All animal challenge experiments were performed in a secure biosafety level 3 laboratory at the Lanzhou Veterinary Research Institute (LVRI) of the Chinese Academy of Agricultural Sciences (CAAS). All of the animal protocols were approved by the Institutional Animal Use and Care Committee of the CAAS.

Animals

Male and female guinea pigs weighing 250 to 300 g were purchased from the LVRI and were maintained under clean-air conditions with pathogen-free food and water. Twenty-eight healthy cattle that were approximately 1-year old were obtained from a local farm in Gansu, China, with no history of FMD. All of the cattle were confirmed to be seronegative for FMDV using a liquid-phase blocking-sandwich enzyme-linked immunosorbent assay (ELISA) (Hamblin et al. 1986).

Virus and cell lines

The A/HuBWH/CHA/2009 strain of FMDV is an epidemic virulent strain, originating from an outbreak in Wuhan, China, during 2009. It was maintained and available at the LVRI, CAAS. The baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % (*v*/*v*) heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, and 100 IU/mL penicillin at 37 °C in 5 % CO_2. Peptides were designed based on the published amino acid sequences of the VP1, 3A, and 3D proteins of the A/HuBWH/CHA/2009 strain (GenBank accession no. JF792355). A conventional FMDV vaccine prepared by chemical inactivation and mineral-oil emulsification of the A/HuBWH/CHA/2009 strain was used as a positive-control vaccine in both the antibody and virus challenge experiments described below.

Vaccine preparation

Three polyepitope peptides, designated PA, PB, and PC, were designed based on the B and T cell epitopes previously identified in the various structural and nonstructural proteins of the FMDV (Blanco et al. 2001; Garcia-Briones et al. 2004; Kim et al. 2006). The polyepitope peptides were produced using Fmoc-solid-phase synthesis chemistry. The sequence corresponding to amino acid positions 129 to 169 of VP1 in the A/HuBWH/CHA/2009 strain was used as the B cell epitope, with cysteine residues incorporated at positions 134 and 156 for cyclization. The sequences corresponding to amino acid positions 21 to 35 of 3A and/or those corresponding to positions 346 to 370 of 3D were used as T cell epitopes. Adjacent epitope units were joined using the spacer sequence, PPS, to avoid creating junctional epitopes or interfering with processing and presentation in antigen-presenting cells (Livingston et al. 2002). Details of the composition of the peptide vaccines are listed in Table 1. The amino acid sequences of the peptide vaccines were confirmed using mass spectrometry. The purity of the peptide preparations was determined to be greater than 95 % using reverse-phase high-pressure liquid chromatography. All of the peptides were dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.5) and subsequently emulsified in an equal volume of oil adjuvant (ISA 206, Montanide, Seppic, France).

Immunization and virus challenge in guinea pigs

Inoculation of guinea pigs with three different synthetic peptide vaccines

Thirty guinea pigs were randomly divided into five groups of six animals each. Group 1 (negative controls) was immunized by intramuscular injection of the PBS-oil adjuvant mixture. Group 2 (positive controls) was immunized by intramuscular injection of 0.3 mL of the inactivated A/HuBWH/CHA/2009 vaccine. Groups 3, 4, and 5 were immunized by intramuscular injection of 0.3 mL of emulsion containing 75 μg of PA, PB, and PC, respectively. After 3 weeks, the procedure was repeated for booster vaccination. Serum was collected from three guinea pigs randomly selected from each group at 2, 3, 5, and 6 weeks postinoculation and subjected to FMDV detection and VNA tests.

Inoculation of guinea pigs with different doses of peptide PB

Four groups of five guinea pigs each were injected intramuscularly with concentrations of PB ranging from 25 to 250 μg in 0.5 mL of emulsion. The guinea pigs in the positive control group were injected intramuscularly with the inactivated A/HuBWH/CHA/2009 vaccine, and those in the negative control group were injected intramuscularly with the PBS-oil adjuvant mixture. Serum samples were collected at 0, 3, and 6 weeks postimmunization for the FMDV detection and VNA tests.

Table 1 Synthesized amino acid sequences corresponding to epitopes in the 3A, 3D, and VP1 proteins of the A/HuBWH/CHA/2009 strain of FMDV

Peptide name and amino acid positions	Amino acid sequence
PA: 59mer 3A(21–35)~PPS~VP1(129-134(T→C)[a]-156(Q→C)[b]-169)	acetyl-AAIEF FEGMV HDSIK *PPS* VYNGT CKYSA PATRR GDLGS LAARL AACLP ASFNY GAIRA T-amide
PB: 69mer 3D(346–370)~PPS~VP1(129-134(T→C)[a]-156(Q→C)[b]-169)	acetyl-YDLDF EALKP HFKSL GQTIT PADKS *PPS* VYNGT CKYSA PATRR GDLGS LAARL AACLP ASFNY GAIRA T-amide
PC: 87mer 3A(21–35)~PPS~VP1(129-134(T→C)[a]-156(Q→C)[b]-169) ~PPS~3D(346–370)	acetyl-AAIEF FEGMV HDSIK *PPS* VYNGT CKYSA PATRR GDLGS LAARL AACLP ASFNY GAIRA T *PPS* YDLDF EALKP HFKSL GQTIT PADKS-amide

PPS linker sequence shown in italics

[a] N134 of the native VP1 sequence is replaced by C

[b] Q158 of the native VP1 sequence is replaced by C

Virus challenge of vaccinated guinea pigs

Six weeks after the initial inoculation, all of the guinea pigs were challenged with 100 GID_{50} (50 % guinea pigs infectious dose) of homotypic FMDV in 0.2 mL of PBS by subcutaneous injection of 0.1 mL and intradermal injection of 0.1 mL in one rear footpad. All of the guinea pigs were monitored for typical FMD lesions on the footpads for seven consecutive days postchallenge. Animals with primary lesions at the sites of virus injection only were considered protected. Those with secondary lesions on at least one noninoculated foot were considered unprotected (Terpstra et al. 1976).

Immunization and challenge in cattle

Twenty-eight healthy cattle were randomly divided into six groups. Each animal received a single 1-mL intramuscular injection. One group consisted of three animals that unvaccinated as the negative controls. Each of the other five groups consisted of five animals that were inoculated by the inactivated virus vaccine, 100 μg of PA, 100 μg of PC, 100 μg of PB, or 50 μg of PB. Blood samples were collected from each animal on day 21 postimmunization. Antibody response to FMDV serotype A was evaluated using serum neutralization assays (SNAs) in BHK-21 cells.

Challenge infections were performed as previously described in the OIE Manual for the testing of FMD vaccines in cattle. On day 21 postimmunization, all the vaccinated and unvaccinated animals were challenged with an intradermal inoculation of 10^4 50 % ID_{50} of a live, cattle-adapted, homologous strain of FMDV in a volume of 0.1 mL at two sites on the upper surface of the tongue. The cattle were monitored for clinical signs of FMD during the postchallenge period. After 11 days, external and postmortem examinations were performed to indentify FMD-related lesions. Animals with lesions at sites other than the tongue (secondary lesions) at 11 days postchallenge were considered unprotected.

Indirect ELISA detection of specific antibody response in guinea pigs

An indirect ELISA was used to detect FMDV-specific IgG in the serum of vaccinated animals. Ninety-six-well plates were coated overnight at 4 °C with 50 μL of the purified FMDV 146S antigen diluted to a final concentration of 1 μg/mL in 0.05 M bicarbonate buffer (pH 9.6). Following three washes with PBS containing 0.05 % Tween-20 (PBST), the coated plates were blocked for 2 h using PBST containing 3 % (*w*/*v*) bovine serum albumin. After washing, the plates were incubated with serum diluted in PBST (1:100). After incubation for 1 h at 37 °C, the plates were washed again. Horseradish peroxidase-conjugated goat anti-guinea pig IgG (Sigma, St. Louis, MO, USA) was added to each well, and the plates were incubated for 1 h at 37 °C. After a final washing step, the OPD substrate (Sigma, St. Louis, MO, USA) was added, and FMDV reactivity was colorimetrically detected based on optical density (OD) at 492 nm using a spectrophotometer (Jimenez-Clavero et al. 1998). The mean OD of three guinea pigs was calculated to represent the antibody titer of each group.

SNA

The titer of VNA in the serum of vaccinated animals was measured using a SNA in BHK-21 cells, as previously described (Golde et al. 2005). Serum samples were heated at 56 °C for 30 min to inactivate complement. Twofold series dilutions were prepared in RPMI 1640 culture medium. A 50-μL aliquot of each dilution was added to 50 μL of 200 $TCID_{50}$ of serotype-A FMDV, and the mixture was incubated for 1 h at 37 °C in 5 % CO_2. A 50-μL suspension containing 10^5 BHK-21 cells was added to each well, and the plates were incubated for 3 days at 37 °C in 5 % CO_2. The cells were observed for cytopathic effects. The VNA titers were calculated using the Reed-Muench formula as the highest dilution of serum that neutralized 50 % of the virus activity (Reed and Muench 1938) and were expressed as log10 $TCID_{50}$/mL.

Statistical analysis

Data handling and analysis and graphic representation were performed using GraphPad Prism 3.0 software (San Diego, CA). The SEM was used as the error value and one-tailed *t* test was used for comparison. Differences were considered to be statistically significant with $p<0.05$.

Results

Immune response and protective effect of peptide vaccines in guinea pigs

Three synthetic peptide vaccine preparations were tested in guinea pigs. Animals were kept in isolation throughout the experiments. Anti-FMDV antibodies in vaccinated guinea pigs were assayed using the indirect ELISA. The analysis of the antibody titers revealed that none of the groups inoculated with the peptide vaccines developed an anti-FMDV-serotype-A IgG response that was detectable within 2 weeks postimmunization. On day 21 postimmunization, anti-FMDV-serotype-A antibodies were detectable in the guinea pigs immunized with PB but were not detectable in animals immunized with PA or PC. After the booster vaccination, the anti-FMDV-serotype-A antibody levels increased in all of the peptide-vaccinated groups. The anti-FMDV-serotype-A

antibody titers in the PC-vaccinated group increased dramatically, reaching a maximum level at week 5 postimmunization, before gradually declining.

The antibody titers in the PA- and PB-vaccinated groups increased steadily and reached peak levels in week 6. The guinea pigs vaccinated with PA had lower antibody titers than those vaccinated with PB or PC ($p<0.05$). The PB vaccine elicited higher levels of antibody before challenge than that observed for the other peptide vaccines. Throughout the experiments, the guinea pigs inoculated with the inactivated FMDV vaccine had higher levels of antibody, compared to the peptide-vaccinated groups. The guinea pigs inoculated with the PBS-oil adjuvant mixture did not develop an FMDV-specific antibody response (Fig. 1).

To identify the peptides that elicited neutralizing antibodies, the capacity of the various vaccines to induce VNA production was assessed in BHK-21 cells based on the titer of VNAs in the sera collected from the immunized guinea pigs. The data in Table 2 show that each of the three different peptide vaccines elicited a higher neutralizing antibody response against A/HuBWH/CHA/2009 on day 42 postimmunization, with the average VNA titer of 1.96±0.33 and 2.034±0.28 for PA and PB, respectively, which were higher than those of the animals immunized with the inactivated FMDV vaccine (1.581±0.13, $p<0.05$). No detectable FMDV-specific neutralizing antibody was detected at any time point in the guinea pigs immunized with PBS. The titer of VNAs induced by PB was highest, which was followed by that by the PA and PC in descending order.

The guinea pigs were challenged with 100 GID_{50} of homotypic virus, and the protective effects of the peptide vaccines were evaluated. All of the animals in groups 1 and 3 exhibited clinical signs of FMD, including fever, swollen soles, and vesicles at the sites of injection, whereas four of the five animals in each of the other three groups developed primary vesicles. On day 3 postchallenge, four animals in group 1 and two animals in group 3 began to develop secondary vesicles in their noninjected feet. On day 7 postchallenge, all of the guinea pigs vaccinated with the PB vaccine or the inactivated FMDV vaccine demonstrated complete protection against FMDV challenge. All of the animals injected with the PBS-oil adjuvant (negative controls) exhibited severe primary and secondary lesions on their footpads. The PA- and PC-vaccinated groups both demonstrated 66 % (4/6) protection against FMDV challenge. Compared with the PA and PC groups, the PB group demonstrated the highest level of protection. Therefore, the PB vaccine was selected for further evaluation in the subsequent experiments.

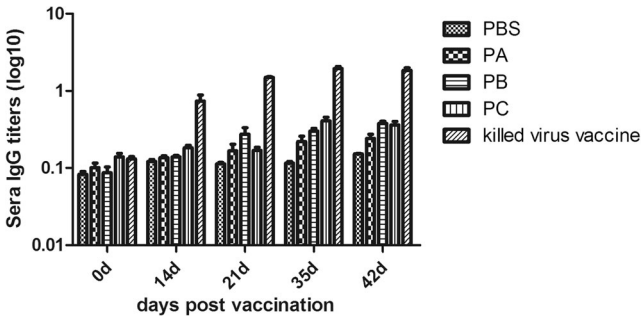

Fig. 1 Time course of antibody production in guinea pigs. Guinea pigs were immunized on days 0 and 21 with synthetic peptides or a PBS-oil adjuvant mixture. Serum samples were collected at weeks 0, 2, 3, 5, and 6. Serum levels of anti-FMDV antibody were measured by an indirect ELISA using inactivated serotype-A FMDV as the antigen. Each data point represents the mean±standard error of measurements for three guinea pigs from each immunization group

Dose dependence of protective immunity in PB-vaccinated guinea pigs

The dose dependence of PB-induced protective immunity was evaluated in doubly immunized guinea pigs using doses of PB ranging from 25 to 250 μg. The titer of VNA in each animal was determined at 0, 3, and 6 weeks postimmunization. As shown in Table 3, the titer of VNA correlated with the dose of PB vaccine administered. In the animals vaccinated with 250 or 100 μg of the PB vaccine, VNAs were clearly detected at 21 days after the first immunization. However, VNAs were not observed in the animals vaccinated with 50 or 25 μg until the second dose of the peptide vaccine was administered. A significant increase in VNA titers was observed following the booster vaccination for all of the dosages used. At 42 days postimmunization, the average VNA titers in the animals vaccinated with 250 (1.8±0.29), 100 (2.4±0.33), or 50 μg (1.73±0.31) of PB in 0.5 mL of emulsion were higher than those in the animals vaccinated with the inactivated FMDV vaccine (1.58±0.14, $p<0.05$). Limited induction of VNAs (1.27±0.45) was observed in the guinea pigs immunized with 25 μg of PB. Guinea pigs immunized with 100 μg of PB had the highest VNA titers ($p<0.05$). No VNA was detected in the guinea pigs immunized with the PBS-oil adjuvant mixture.

All of the animals were experimentally challenged using a fetal FMDV preparation, which caused severe disease in all of the negative control animals. Protective immunity against the FMDV challenge was observed in three of the five animals immunized with 25 μg of PB, whereas all of the animals vaccinated using higher doses of PB remained clinically healthy. These data demonstrated that, in guinea pigs, vaccination using two doses of 50 μg of PB (immunization and booster) provided complete immunoprotection against FMDV.

Immune response and protective effect of cattle to synthetic peptides

Cattle were vaccinated using a single dose of the peptide vaccines. The cattle were challenged on day 21

Table 2 Immunogenicity and immunoprotective effects of peptide vaccines in guinea pigs

Group	Vaccine	Neutralizing antibody titer[a] (average value)		Primary vesicles[b]	Secondary vesicles[b]	Protection rate (%)
		Day 0	Day 42			
1	PBS-oil adjuvant mix	<0.3	<0.3	6/6	6/6	0
2	Inactivated virus/0.3 mL/dose	<0.3	1.581±0.13	5/6	0/6	100
3	PA/75 μg/0.3 mL/dose	<0.3	1.96±0.33	6/6	2/6	66
4	PB/75 μg/0.3 mL/dose	<0.3	2.034±0.28	5/6	0/6	100
5	PC/75 μg/0.3 mL/dose	<0.3	1.73±0.4	5/6	2/6	66

[a] Titer is defined as −log of the minimum dilution capable of maintaining an unaltered cell monolayer

[b] Ratio of number of animals with clinical signs of disease to the total number of animals in each group

postimmunization, and the VNA titer of each was determined in BHK-21 cells using the SNA. The protective effect was assessed by observing the clinical signs of FMD after challenge. The protective responses in cattle against FMDV challenge are summarized in Table 4. Four of the five cattle immunized with inactivated FMDV remained free of clinical signs throughout the postchallenge observation period. By contrast, all three of the nonimmunized cattle had developed clinical signs of FMD on day 2 postchallenge, and these animals exhibited severe disease on day 3 postchallenge and thereafter, including fever, lesions, and vesicle formation. In the cattle immunized with the PA or PC vaccine, 40 % (2/5) of the animals exhibited protective immunity against FMDV challenge, whereas 60 % (3/5) of the cattle immunized with the $PB_{100\ \mu g}$ vaccine were protected. None of the cattle immunized with the $PB_{50\ \mu g}$ vaccine displayed immunoprotection.

All of the cattle immunized with inactivated FMDV showed measurable levels of VNAs by day 21 postimmunization. In the cattle immunized with the synthetic peptide vaccines, there were only three animals (No. 036, No. 039, and No. 046) with titers of VNAs greater than 1.2 on day 21 postimmunization (Table 4). The correlation between the level of VNA and immunoprotection against virus challenge was limited. Immunoprotection against FMDV was observed in some animals with low levels of VNAs (titer <1.2), with animals, No. 041, No. 067, No. 053, and No. 045, exhibiting immunoprotection despite the absence of detectable VNAs. By contrast, animal No. 038, which was immunized with inactivated FMDV, had a relatively high titer of VNAs, but immunoprotection was not conferred.

Discussion

The A/HuBWH/CHA/2009 strain of FMDV used in our current study occurs in many regions of China, and vaccination against it produces suboptimal protection. Synthetic peptides corresponding to protective B and T cell epitopes represent candidates for safer, more effective FMD vaccines. This approach requires information regarding the structure of immunogenic regions of viral proteins and methods of their association into a single construct efficiently. Previous studies have shown that the critical immunodominant conformational epitopes responsible for the induction of FMDV neutralizing antibody are located within the G–H loop of VP1, which spans amino acid positions 131 to 159 in the serotype-O,

Table 3 Dose dependence of the immunogenicity and immunoprotective effects of the PB vaccine in guinea pigs

Group	Vaccine and peptide dose	Neutralizing antibody titer[a] (average value)			Primary vesicles[b]	Secondary vesicles[b]	Protection rate (%)
		Day 0	Day 21	Day 42			
1	PBS	<0.3	<0.3	<0.3	5/5	5/5	0
2	Killed virus vaccine	<0.3	1.389±0.15	1.58±0.14	5/5	0/5	100
3	PB/250 μg/0.5 mL/dose	<0.3	1.73±0.25	1.8±0.29	3/5	0/5	100
4	PB/100 μg/0.5 mL/dose	<0.3	1.5±0.35	2.4±0.33	3/5	0/5	100
5	PB/50 μg/0.5 mL/dose	<0.3	0.6±0.22	1.73±0.31	5/5	0/5	100
6	PB/25 μg/0.5 mL/dose	<0.3	0.381±0.12	1.27±0.45	5/5	2/5	60

[a] Titer is defined as −log of the minimum dilution able to keep unaltered cell monolayer

[b] Ratio of number of animals with clinical signs of disease to the total number of animals in each group

Table 4 Immunogenicity and immunoprotective effects of peptide vaccines in cattle

Vaccine dose (μg)	Animal no.	Titer of virus-neutralizing antibody at day 21 postinoculation (log10)	Lesion scores[a] (day 11)	Protection[b]	Protection rate (%)[c]
Inactivated virus (2 mL)	031	2.7	0	−	4/5
	033	2.3	0	−	
	037	2.1	0	−	
	038	2.7	1	+	
	047	2.1	0	−	
$PA_{100\ \mu g}$	041	<0.9	0	−	2/5
	044	0.9	2	+	
	052	<0.9	3	+	
	054	<0.9	1	+	
	067	<0.9	0	−	
$PC_{100\ \mu g}$	036	1.4	0	−	2/5
	049	<0.9	4	+	
	053	0.9	0	−	
	057	<0.9	5	+	
	070	<0.9	5	+	
$PB_{100\ \mu g}$	035	<0.9	2	+	3/5
	039	1.2	0	−	
	042	<0.9	5	+	
	045	<0.9	0	−	
	046	1.35	0	−	
$PB_{50\ \mu g}$	048	<0.9	2	+	0/5
	051	<0.9	5	+	
	055	<0.9	5	+	
	058	<0.9	4	+	
	061	<0.9	3	+	
Negative control	063	<0.9	4	+	0/3
	068	<0.9	3	+	
	069	<0.9	5	+	

[a] Severity of FMD was scored based on one point for each foot with vesicular lesions and one point for secondary vesicles/erosion of the buccal mucosa (lips, dental pad, or gums), with a score of 5 indicating the highest level of severity

[b] Animals with no secondary lesions (score 0) were considered protected (−), and animals with secondary lesions representing systemic viremia were considered unprotected (+)

[c] The rate of protection (%) was calculated as the number of cattle with no lesions divided by the total number of cattle

serotype-A, and serotype-C strains of FMDV (Kim et al. 2006). Therefore, the G–H loop of VP1 represents an optimal target sequence for the design novel peptide vaccines against FMD. Previous studies have also shown that the incorporation of longer flanking regions of the G–H loop and a cyclic secondary structure induced by the incorporation of cysteine residues positioned 8 Å apart at positions 134 and 158 resulted in high VNA titers in vaccinated animals (Francis et al. 1987; Brown et al. 1999; Zamorano et al. 1995; Valero et al. 1995). Current immunological theory considers the presence of a T-helper epitope in a peptide sequence to be a necessary prerequisite for the efficacious production of VNAs (Francis et al. 1987).

We designed synthetic peptide vaccines which contain sequences corresponding to regions of three different proteins of the FMDV that are highly immunogenic B or T cell epitopes, and they contained extensive flanking sequences and additional cysteine residues to induce a cyclic secondary structure. One B cell epitope corresponded to a sequence in the G–H loop of VP1, and the other two T-helper epitopes corresponded to sequences in the 3A and 3D viral proteins. In contrast to the former peptide design and inactivated virus, the peptide vaccines presented here have several advantage design elements. Peptide vaccines do not involve infectious virus and can easily be designed and chemically synthesized. T cell epitopes derived from the NS protein region were more effective than those that relied on the Th cell epitopes intrinsic to the VP1 region. Longer target site peptides covering the G–H loop domain from amino acids 129–169 were more effective than the 133–158 constructs. Cyclization to constrain a loop conformation like the one found on the VP1 capsid protein was more effective for high neutralization activity.

We evaluated these peptide vaccines in guinea pigs to determine whether they were immunogenic and conferred immunoprotection because previous studies have shown that the guinea pig is a reliable model for evaluating FMD vaccines (Barnett and Statham 2002). The three peptide vaccines induced VNAs and immunoprotective effects in guinea pigs, and the PB vaccine conferred protective immunity comparable to that of an inactivated FMDV vaccine. We also evaluated

the dose dependence of the protective immunity induced by the PB vaccine. Significant differences were observed among the animals immunized with high or low doses of the PB vaccine, which implies that the dose of vaccine administered may be an important determinant for immune response and/or immunoprotection. In guinea pigs, two doses of 50 μg of PB provided complete protection against FMDV challenge. We conducted a third trial using four groups of cattle with five animals in each group that were vaccinated with PA, PC, $PB_{100\ \mu g}$, or $PB_{50\ \mu g}$. After challenge, 80 % protection was observed in the cattle immunized with the inactivated FMDV vaccine. Immunization with PA or PC provided immunprotection in 40 % (2/5) of the cattle challenged with FMDV. Immunization with $PB_{100\ \mu g}$ conferred protective immunity in 60 % (3/5) of the vaccinated cattle, and no immunoprotection was observed in the cattle immunized with $PB_{50\ \mu g}$. Therefore, 50 μg of synthetic peptide was insufficient for developing an efficacious antibody response and immunoprotection against FMDV.

A number of research studies are being carried out to develop synthetic peptide vaccines against viral pathogens including FMDV. Most of them are able to protect laboratory animals such as mice, rabbits, and guinea pigs. However, it is difficult to translate efficacy data from laboratory animals to cattle and swine. The naturally sensitive animals (pigs and cattle) still could not be completely protected from FMDV with the use of synthetic peptides despite the fact that many synthetic peptide vaccines have been described as protecting laboratory animals from the virus. The results in current study are consistent with the general rule. Despite the fact that this vaccine in Guinea pigs have 100 % efficacy, a lower protection of 60 % was achieved in cattle. The different results might be indicative of marked differences in the nature of the protective immune responses to FMDV between laboratory animals and bovine.

There are also differences between the immune responses induced by peptide vaccines and by classical inactivated FMD vaccines. In animals immunized with inactivated vaccines, a good correlation between neutralizing activity in sera and protection has been observed, and such a correlation has not been well established with animals immunized with synthetic antigens. Although both the inactivated virus and PB vaccines conferred similar levels of immunoprotection, the titers of VNAs induced by each differed. This lack of a correlation between immunoprotection and VNA titer is consistent with the findings of previous studies. Antoni et al. (1988) showed that, although cattle immunized with a peptide vaccine had high VNA titers on day 21 postimmunization, they developed generalized lesions after challenge with 10.000 ID_{50} of the homolog virus (Antoni et al. 1988). By contrast, other studies in cattle showed that animals displaying insufficient VNA titers exhibited immunoprotection after virus challenge (Udenfriend et al. 1972). The finding that synthetic peptides induce levels of in vitro neutralizing antibodies that fail to protect cattle from challenge could be due to the fact that peptides induce antibodies of different isotypes (Mulcahy et al. 1990) and lower affinity (Mulcahy et al. 1992) than the conventional whole virus vaccines. Some of the cattle with insufficient antibody titer could be protected after challenge most probably due to an effective T cell reaction that probably provides an essential regulatory role in the induction of protective immunity against FMDV. Altogether, these results suggest that mechanisms other than the direct neutralization of viral particles by anti-FMDV antibodies may play a role in protection (McCullough et al. 1992). These factors may be the variation in immunological parameters between individuals, such as the longevity of the antibody response or the mechanisms involved in the induction of cellular versus humoral immunity (Barnett and Carabin 2002).

Low levels of amino acid variation in nonstructural proteins among different serotypes of FMDV allow the identification of heterotypic T cell epitopes, which are important components of a synthetic vaccine against FMD (Collen 1994). The 3D protein, a viral RNA polymerase, is efficiently recognized by bovine lymphocytes (Collen et al. 1998). A previous study showed that the coexpression of 3D by P1-based DNA vaccines resulted in improved immune responses (Cedillo-Barron et al. 2001). In our current study, all the three peptide constructs contained the major immunogenic region 129–169 of VP1 with cyclization to constrain a loop conformation. The main differences among the three peptide vaccines are the selection of T cell epitopes. We used peptide sequences that corresponded to amino acid positions 346 to 370 of 3D or 21 to 35 of 3A as T cell epitopes. Peptide PA contained the amino acid positions 21 to 35 of 3A, Peptide PB contained the amino acid positions 346 to 370 of 3D, and both the two epitopes were involved into the peptide PC. Our results showed that peptides consisting of the 3D-derived peptide sequence were more highly immunogenic and conferred greater immunoprotection than those observed using the 3A-derived sequence. It can be concluded that peptide 346 to 370 of 3D, containing a powerful T-helper epitope, is responsible for the high immunogenic activity of peptide PB. The lower immunogenic and immunoprotective effects conferred by the 3A- and 3D-derived sequence suggest that adding more epitopes to a peptide construct does not always have a positive effect but rather a negative one on its immunogenic and protection inducing capacity.

Significant efforts have been made toward developing effective peptide vaccines against foot-and-mouth disease (FMD) in cattle (DiMarchi et al. 1986; Morgan and Moore 1990; Taboga et al. 1997; Volpina et al. 1999). But to date, none of them can compete with the conventional inactivated vaccines yet. A synthetic peptide vaccine providing 40 % protection against the FMDV in cattle was reported in 1997 (Taboga et al. 1997). Similar results were obtained by

Rodriguez et al. in 2003. An inactivated vaccine is considered acceptable for use in the field if a minimum of 80 % protection is achieved in cattle (Barteling and Vreeswijk 1991). Eighty percent protection was obtained with the killed virus vaccine used as a positive vaccination control in our experiments (Table 4). With the synthetic peptides tested, the best protection achieved by the PB peptide was 60 %. Although the results of our experiments are encouraging, the synthetic peptide vaccines did not provide a level of immunoprotection in cattle that was comparable to that of the inactivated virus vaccine. But, it remains theoretically possible that full protective responses could be achieved if a suitable design was made. Larger doses of synthetic peptide per immunization might increase the level of immunoprotection provided. Likewise, modifications in the composition of the synthetic polymer and the use of other types of adjuvant might also enhance the immunoprotective effects of the peptide vaccines (Lofthouse et al. 2002). In conclusion, our results provide empirical data for facilitating the development of an efficacious synthetic peptide vaccine against the A/HuBWH/CHA/2009 strain of FMDV for use in cattle. We believe our findings also provide strong support for the use of synthetic peptide vaccines to expedite FMD control and eradication.

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