IMMUNOGENICITY OF A RECOMBINANT FUSION PROTEIN OF TANDEM REPEAT EPITOPES OF FOOT-AND-MOUTH DISEASE VIRUS TYPE ASIA 1 FOR GUINEA PIGS

Q. ZHANG[1*], Y.Q. YANG[2], Z.Y. ZHANG[1], L.LI[2], W.Y. YAN[1], W.J. JIANG[2], A.G. XIN[2], C.X. LEI[1], Z.X. ZHENG[1]

[1]State Key Laboratory of Genetic Engineering, School of Life Science, Fudan University, Shanghai 200433, P.R. China; [2]Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory, Kunming, Yunnan, P.R. China

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Summary. – In this study, the sequences of capsid protein VP1 regions of YNAs1.1 and YNAs1.2 isolates of foot-and-mouth disease virus (FMDV) were analyzed and a peptide containing amino acids (aa) 133–158 of VP1 and aa 20~34 of VP4 of FMDV type Asia 1 was assumed to contain B and T cell epitopes, because it is hypervariable and includes a cell attachment site RGD located in the G-H loop. The DNA fragments encoding aa 133–158 of VP1 and aa 20~34 of VP4 of FMDV type Asia 1 were chemically synthesized and ligated into a tandem repeat of aa 133–158–20~34–133–158. In order to enhance its immunogenicity, the tandem repeat was inserted downstream of the β-galactosidase gene in the expression vector pWR590. This insertion yielded a recombinant expression vector pAS1 encoding the fusion protein. The latter reacted with sera from FMDV type Asia 1-infected animals *in vitro* and elicited high levels of neutralizing antibodies in guinea pigs. The T cell proliferation in immunized animals increased following stimulation with the fusion protein. It is reported for the first time that a recombinant fusion protein vaccine was produced using B and T cell epitopes of FMDV type Asia 1 and that this fusion protein was immunogenic. The fusion protein reported here can serve as a candidate of fusion epitopes for design of a vaccine against FMDV type Asia 1.

Key words: FMD; FMDV type Asia 1; VP1; epitope; recombinant protein; immunogenicity; guinea pig

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hooved animals, mostly cattle and pigs (Bachrach, 1968; Brown, 1990; Tulasiram *et al.*, 1997). The disease has a potential to cause explosive epidemics and heavy economic losses to the agricultural industry worldwide. FMD is caused by FMDV (the species *Foot-and-mouth disease virus*, the genus *Aphthovirus*, the family *Picornaviridae*). The FMDV virion consists of a single-stranded positive-sense RNA genome of about 8.5 kbp enclosed within an icosahedral capsid comprising 60 copies each of four structural polypeptides VP1-4 (Acharya *et al.*, 1989; Burroughs *et al.*, 1971; King and Newman, 1980). FMDV shows a high genetic and antigenic variability, which is reflected in seven serotypes (O, A, C, Asia 1, SAT1, SAT2, and SAT3) and many subtypes (Domingo *et al.*, 1990). The FMD control is mainly implemented by use of chemically inactivated whole virus vaccines (Barteling and Vreeswijk, 1991). Viral infection and immunization with conventional vaccines usually elicit high levels of circulating neutralizing antibodies, which correlate with protection against homologous and antigenically related viruses (van Bekkum, 1969). However, chemically inactivated vaccines pose a risk of virus release during vaccine production (Barteling and

*E-mail: qz980@yahoo.com; fax: +8621-65104949.

Abbreviations: aa = amino acid; APC = antigen-presenting cell; ConA = concanavalin A; CPE = cytopathic effect; FMD = foot-and-mouth disease; FMDV = FMD virus; IPTG = isopropyl-thiogalactoside; MHC = major histocompatibility complex; nt = nucleotide; PBS = phosphate-buffered saline; PD_{50} = 50% protection dose; RT-PCR = reverse transcription–polymerase chain reaction; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SI = stimulation index

Vreeswijk, 1991). This limitation has led to the search for alternative safe subunit immunogens.

To produce a safer FMD vaccine, we used genetic engineering techniques to develop a recombinant fusion protein vaccine. Protection against FMDV infection is associated with induction of neutralizing antibodies. Therefore, attempts have been made to identify peptides capable of eliciting protective humoral responses. There is considerable evidence showing that VP1 is an immunodominant antigen (Tulasiram and Suryanarayana, 1998; Tulasiram *et al.*, 1997). However, the immunogenicity of VP1 isolated from either the virus or a protein expressed in *Escherichia coli* by DNA recombinant techniques is very low compared with the intact virus particle (Kleid *et al.*, 1981). An alternative approach to increasing the immunogenicity of FMDV is the use of biosynthetic peptides containing B and T cell epitopes. A region located in the G-H loop at aa 140–160 of VP1 of FMDV has been identified as the main continuous viral epitope recognized by host B lymphocytes ("B cell epitope") producing neutralizing antibodies. Synthetic peptides containing the continuous B cell epitope located in this antigenic region have been shown to induce virus-neutralizing antibodies. But the immunity induced in natural hosts by these synthetic antigens was poor due to the lack of adequate epitopes recognized by T cells ("T cell epitopes"), which have major implications for rational design of a vaccine containing T and B cell epitopes. It is known that the VP4 region of aa 20–34 that is highly conserved among different FMDV serotypes is a dominant T cell determinant in cattle (van Lierop *et al.*, 1995). But there is little knowledge about FMDV type Asia 1 immunogenic B cell epitopes (Butchaiah and Morgan, 1997).

In this study, in order to elucidate the structure of B cell epitopes, we analyzed the sequences of VP1 regions of YNAs1.1 and YNAs1.2 isolates of FMDV, compared them with those of Israeli and Indian isolates and concluded that a peptide consisting of aa 133–158 of VP1 of FMDV type Asia 1 might be an important B cell epitope. On the basis of the above sequences of B and T cell epitopes, we constructed a prokaryotic recombinant expression vector pAS1, which encoded a fusion protein of β-galactosidase and the tandem repeat of B and T cell epitopes located at aa 133–158–20~34–133–158 of FMDV type Asia 1. The immunogenicity of the expressed fusion protein was assayed.

Materials and Methods

Viruses. A wild type isolate YNAs1.1 originated from unvaccinated cattle in the Menglian County, Yunnan Province, P.R. China. The Menglian County is situated close to the border between P.R. China and Burma. The Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory typed the YNAs1.1 isolate as a strain of Asia 1 serotype of FMDV. YNAs1.2 is an FMDV serotype Asia 1 attenuated strain for cattle.

Reverse transcription–polymerase chain reaction (RT-PCR). YNAs1.1 and YNAs1.2 strains were grown in BHK-21 cells. Virus was extracted by repeated freezing and thawing of infected cells. Cell debris was removed by low-speed centrifugation.The virus in the supernatant was sedimented by ultracentrifugation. Total RNAs of YNAs1.1 and YNAs1.2 strains were extracted from above virus preparations with TRIzol Reagent (Gibco BRL). The region corresponding to nucleotides (nts) 36–633 of VP1 gene was amplified by RT-PCR using specific primers (a sense primer P13, 5'-GCGAATCAGCAGATCCAGTCAC-3' and an antisense primer P7, 5'-GAAGGGCCCAGGGTTGGACTC-3') according to the protocol of the One-Step™ RT-PCR System (Gibco BRL) with a slight modification. Namely, the cycles consisted of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min, and extension at 72°C for 1 min. The RT-PCR was carried out in a Thermal Cycler (Perkin Elmer Cetus, USA). Finally, the reaction mixture was incubated at 72°C for 10 mins for obtaining full length products. The PCR products were analyzed by electrophoresis in 1% agarose gels. The amplified PCR products were purified according to the manufacturer's protocol. The purified PCR products were cloned and sequenced according to the protocol for the pGEM-T Vector System (Promega) and the T7 Sequencing Kit (Pharmacia), respectively.

Construction of a recombinant expression vector pAS1. The DNA fragments coding for aa 133–158 of VP1 and aa 20~34 of VP4 (Stram *et al.*, 1994) of FMDV type Asia 1 were chemically synthesized and used for construction of a tandem repeat gene with *Eco*RI (5'-end) and *Bam*HI (3'-end) sites (5'-*Eco*RI-133~158–20~34–133~158-*Bam*HI-3') (Fig. 1). This tandem repeat gene was inserted into the expression vector pWR590 downstream of the b-galactosidase gene using the terminal *Eco*RI and *Bam*HI sites (Guo *et al.*, 1985). The insertion yielded the recombinant expression vector pAS1.

Expression of the recombinant fusion protein. E. coli strain C600 transformed with the vector pAS1 was grown overnight at 37°C. Then the culture was diluted 1:10 with a fresh LB medium containing ampicillin (100 μg/ml) and incubated for 2.5 hrs at 37°C. The protein expression was induced by isopropylthiogalactoside (IPTG) in final concentration of 50 μg/ml and the culture was incubated for further 4 hrs.

Western blot analysis. Protein samples were electrophoresed in a discontinuous 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) and blotted onto a nitrocellulose membrane. After blocking with 5% skim milk in the phosphate-buffered saline (PBS) with 0.02% Tween-20, the blot was incubated with a guinea pig antiserum against FMDV type Asia 1 ("FMDV type Asia 1 antiserum") at 37°C for 1.5 hr, washed 3 times with PBS and incubated with an 1:1000 dilution of a guinea pig antiserum against an IgG-horseradish peroxidase conjugate at 37°C for 1.5 hr. For staining, diaminobenzidine (Sigma) and 0.003% H_2O_2 (v/v) were used as chromogen and substrate, respectively, and 0.04% (w/v) $NiCl_2$was used to enhance the color. The reaction was stopped with distilled water.

Animal vaccination with the fusion protein. The fusion protein was prepared from the inclusion bodies of *E. coli*. It was then dissolved in urea, purified as described earlier by Strebel *et al.*

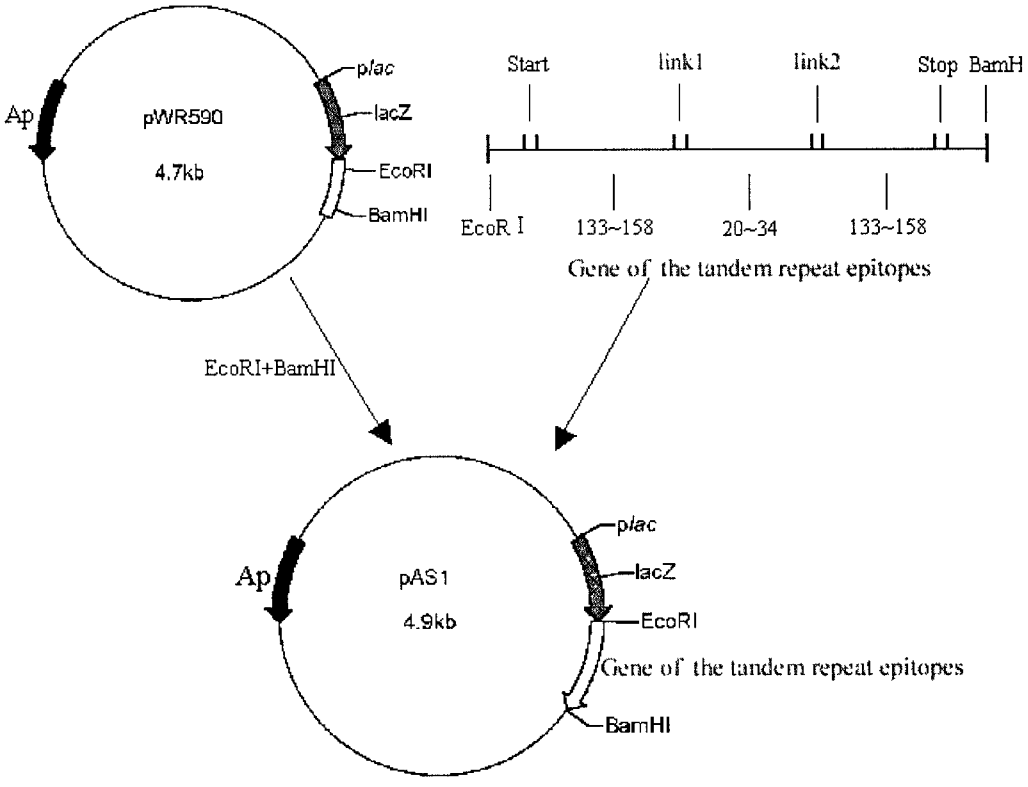

Fig. 1
Construction of recombinant vector pAS1

The synthetic gene of the tandem repeat peptide consisting of aa 133~158–20~34–133~158 of FMDV type Asia 1 was inserted into the *Eco*RI and *Bam*HI sites of the vector pWR590 down the ß-galactosidase gene to yield the recombinant vector pAS1. plac = promoter of lacZ gene; lacZ = ß-galactosidase gene; 133~158 = aa 133~158 of VP1 of FMDV type Asia 1; 20~34 = aa 20~34 of VP4 of FMDV type Asia 1.

(1986) and emulsified with the adjuvant Montanide ISA 206 (1:1) (Seppic, France). FMDV Asia 1 antibody-free guinea pigs were initially injected intramuscularly with 400 µg of the fusion protein and boosted 2 weeks later with an equal amount of the fusion protein. Three weeks after the booster, the blood was taken and was allowed to clot at 37°C for 30 mins. The clots were allowed to contract overnight at 4°C and the serum was aspirated, clarified by centrifugation and stored at -20°C.

Assay of neutralizing antibodies. Neutralizing antibody activity of the serum from immunized guinea pigs was determined in a 96-well flat-bottomed microplates using BHK-21 cells. The wells were observed for cytopathic effect (CPE) and the neutralization titer of a serum was expressed as the final dilution of a serum that neutralized 50% of the virus activity.

Assay of T cell proliferation. The T cells isolated from the spleens of immunized guinea pigs were resuspended in the RPMI 1640 medium containing 10% fetal bovine serum, 5 x 10^{-5} mol/l ß-mercaptoethanol, 100 µg/ml penicillin and 100 µg/ml streptomycin to 4 x 10^6 cells/ml. An 150 µl aliquot of the T cell suspension per well was added to the 96-well round-bottomed plate. A total of 50 µl of the diluted pure FMDV Asia1 antigen or medium was added to each well in triplicate. The cells were incubated at 37°C for 72 hrs and then 1 µCi of [^{3}H]-thymidine per well was added. The cells were incubated at 37°C for 12 hrs, harvested and the incorporation of [^{3}H]-thymidine was measured in a liquid scintillation counter. In addition, 12.5 µg/ml concanavalin A (ConA, Sigma) was used as a polyclonal stimulator and a positive control. The results were expressed as stimulation index (SI).

Results

Analysis of coding VP1 nucleotide sequences of YNAs1.1 and YNAs1.2 strains of FMDV type Asia 1

The VP1 gene, particularly the major B cell epitope region is hypervariable. To detect the B cell epitopes the regions of VP1 of the field strain YNAs1.1 and those of the vaccine strain YNAs1.2 were sequenced and analyzed. The RT-PCR resulted in the amplification of a 676 bp product (Fig. 2). No non-specific products could be seen in the gel indicating that the amplification was specific.

The 676 bp PCR products which included 54 nt from the 2A/2B protein were then cloned and the sequences of nt 36–

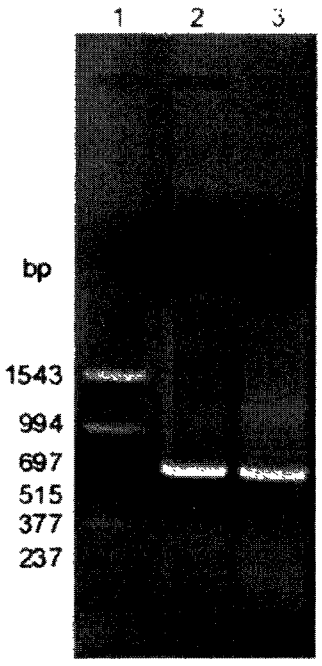

Fig. 2
Agarose gel electrophoresis of PCR products of YNAs1.1 and YNAs1.2 strains of FMDV type Asia 1

PCR products were electrophoresed in agarose gels. DNA size markers (lane 1). YNAs1.1 and YNAs1.2 isolates (lanes 2 and 3, respectively).

633 of VP1 of the YNAs1.1 and YNAs1.2 strains were determined. They were identical (Fig. 3). The sequence of the YNAs1.1 strain was submitted to GeneBank where it is stored under Acc. No. AF241566. The nt sequences and the deduced aa sequences corresponding to nt 36–633 of VP1 gene of the YNAs1.1and YNAs1.2 strains were compared with those of the Israeli and Indian strains (type Asia 1) by the Wisconsin PackageTM Program. These nt sequences exhibited a homology of 82.11% and 88.07%, respectively, while the deduced aa sequences had an 87.94% and 93.47% homology, respectively, with the VP1 region of the Israeli and Indian strains, respectively. The main alterations in aa seqences among them were located in distinct clusters between aa 35 and 48, 80 and 101, and 134 and 155, respectively (Fig. 4).

Western blot analysis of the expressed fusion protein

According to the above nt sequences of VP1 and the reported nt sequences of VP4 of FMDV type Asia 1 the prokaryotic expression vector pAS1 was constructed (Fig. 1). The expressed proteins of pWR590 (negative control) and pAS1 were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Results of the Western blot analysis of the proteins is shown on Fig. 5. The fusion protein reacted with a serum from a FMDV type Asia 1-infected animal; it recognized a band of the M_r expected for the fusion protein, but it didn't recognize the protein expressed by the control plasmid pWR590.

Assay of neutralizing antibodies

To determine whether the fusion protein was able to elicit a specific antibody response, guinea pigs were immunized with a partially purified preparation of the fusion protein. The serum obtained from immunized guinea pigs on the 20th day after the booster neutralized the FMDV type Asia 1. The protective capacity of the immune serum was determined by observing cytopathic effect (CPE). The results summarized in Table 1 showed that the fusion protein containing β-galactosidase and the tandem repeat of aa 133–158–20~34–133–158 elicited a high neutralizing antibody response.

Assay of T cell proliferation

To determine the cellular response, splenic T cells were obtained from guinea pigs vaccinated with the fusion protein from the recombinant vector pAS1 or from non-vaccinated guinea pigs. The splenic T cells were cultured with a diluted FMDV type Asia 1 antigen or with a fresh medium alone. The results shown in Fig. 6 indicated that only T cells obtained from the guinea pigs immunized with the fusion protein proliferated.

Discussion

FMDV type Asia 1 is one of the prevailing FMDV types. Amino acid substitutions in VP1 (Meyer *et al.*, 1994; Strohmaier *et al.*, 1982), particularly those in the hypervariable epitope region, result in a major immunogenic and antigenic diversity (Domingo *et al.*, 1980; Mateu *et al.*, 1988; Tulasiram and Suryanarayana, 1998; Tulasiram *et al.*, 1997). Analysis of the hypervariable region is essential for knowledge of the antigenic structure of a particular serotype and a rational vaccine design. The results of the analysis of VP1 region of the field strain YNAs1.1 and the attenuated strain YNAs1.2 indicate that FMDV type Asia 1 has a distinct antigenic structure. The PCR product from nt 37–633 of VP1 corresponded to aa 13–211. The cell attachment site RGD is situated at aa 143–145 in the serotype Asia 1 unlike in other serotypes where it is present at aa 145–147. The main alterations in the aa sequence indicate that they are located in distinct clusters of aa 35–48, 80–101 and 134–155 of VP1; aa 80–101 of VP1 of YNAs1.1 and YNAs1.2 strains are highly homologous to those of the Indian strain type Asia 1 and other FMDV types, which are relatively highly conserved (Reddy *et al.*, 1999). Many alterations in this region of the Israeli isolate are due to a frameshift mutation caused by a nt 250 deletion and a nt 284 insertion (Stram *et al.*, 1994). The significance of many alterations in the Israeli isolate in the segment of concern needs further research.

Major B cell epitopes of other FMDV types display several features: they are located in the G-H loop which includes the cell attachment site RGD; they are hypervariable (Domingo *et al.*, 1980; Mateu *et al.*, 1988; Tulasiram and Suryanarayana, 1998; Tulasiram *et al.*, 1997).

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YNAs1.1 36   CACCACAGTTGAGAACTACGGAGGAGAGACTCAGACAGCCCGCCGGCTC
YNAs1.2 36   -----------------------------------------------
India   36   ----------------------------------T-G-----A-----A
Israel  36   A-----G--------G--T-------------------A-A-----T

YNAs1.1 85   CACACTGACGTTGCCTTTGTTCTCGACAGGTTTGTGAAACTCACACAG
YNAs1.2 85   -----------------------------------------------
India   85   -------------T-----------------------------C***
Israel  85   ----------C-----CA----T--------------------TGCT

YNAs1.1 133  CTCAAGAACACCCAAACTCTTGATCTTATGCAAATCCCTTCACATACG
YNAs1 2 133  -----------------------------------------------
India   130  -C-----------G-T---------C-----G-----C-----C---
Israel  133  -C--------T------C--C-----C-----G-----C-----C--A

YNAs1.1 181  CTTGTTGGGGCGCTACTTCGGTCTGCGACGTACTACTTCTCAGACCTG
YNAs1.2 181  -----------------------------------------------
India   178  --G-----A---T----C-----C----------------G------
Israel  181  --G-----A--A--------T-------------------------

YNAs1.1 229  GAGGTTGCGATTGTCCACACAGGCTCGGTCACATGGGTGCCTAACGGC
YNAs1.2 229  -----------------------------------------------
India   226  ---------C----T----------A--------------C--T---
Israel  229  -----C---C----------GCCCGGTCACCTGGGTGCCCAACGGCG

YNAs1.1 277  GCACCCAAGGACGCCTTGGACAACCACACCAACCCAACCGCCTACCAA
YNAs1.2 277  -----------------------------------------------
India   274  --G---------------------------------G--T--------G
Israel  277  GGCCCAAC---T--TC-AA-------------------T-----T--G

YNAs1.1 325  AAGCAACCCATTACCCGCCTGGCACTCCCTTACACCGCTCCCCACCGT
YNAs1.2 325  -----------------------------------------------
India   322  ----------C----------G-----C-------------------
Israel  325  ----------C----------------C--------C-----T---

YNAs1.1 373  GTGCTTGCAACAGTGTACAACGGGAAGACAACCTACGGGGAAGAGTCC
YNAs1.2 373  -----------------------------------------------
India   370  -----G-------------------------G------AC-C-AC--
Israel  373  -----G----------------------GGC-G---------AC-A--

YNAs1.1 421  ACAAGACGCGGTGACTTTGCAGCCCTCGCGCAAAGGTTGAGCCGCCGG
YNAs1.2 421  -----------------------------------------------
India   418  --GC-G--T------C-----T-TT--T--A--GC--G-A---AA-A--
Israel  421  T----G-----C---A-G--G--------A----------GCT---

YNAs1.1 469  TTGCCCACCTCCTTCAATTACGGTGCTGTGAAGGCTGACACCATCACC
YNAs1.2 469  -----------------------------------------------
India   466  C----------------C-----------------------------G
Israel  469  C----------------C-----C--C--------C----------T

YNAs1.1 517  GAGCTGTTGATCCGCATGAAGCGCGCGGAAACATACTGCCCCAGGCCT
YNAs1.2 517  -----------------------------------------------
India   514  ----------------------T-----G-------------------
Israel  517  -----T--------------------C--G-----T-----T-----C

YNAs1.1 565  TTGCTGGCTCTTGATACCACCCAAGACCGCCGTAAACAGGAGATCATT
YNAs1.2 565  -----------------------------------------------
India   562  -----A--------C---------C----------G-----------
Israel  565  --A--A--C------C-----T--G--------C---------------

YNAs1.1 613  GCACCCGAGAAACAGGCTCTA  633
YNAs1.2 613  --------------------  633
India   610  -----T-----G--A-T-T-G  630
Israel  613  -----T-----G----T-T-G  633
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Fig. 3
Alignment of nucleotide sequences of VP1genes of different FMDV type Asia 1 strains and isolates

YNAs1.1 (Acc. No. AF241566) and YNAs1.2 (not submitted to GenBank) strains, and Indian (Acc. No. Y09949) and Israeli (Acc. No. U01207) isolates. (-) stands for the same nucleotide, while (*) stands for deletion of a nucleotide.

As aa 134–155 of VP1 of YNAs1.1, YNAs1.2, Indian and Israeli isolates differ from one another and include the cell attachment site RGD located in the G-H loop, we deduced that this region of VP1 might be an important B cell epitope.

The VP4 region of aa 20~34, which is the only T cell epitope, cannot elicit a neutralizing antibody (van Lierop *et al.*, 1995). Nevertheless, our results demonstrate that the recombinant fusion protein of the tandem repeat epitopes 133–158–20~34–133–158 elicited a high level of neutralizing antibodies in guinea pigs. In addition, we constructed also a pAS1 vector with deleted aa 20~34, pAS1-D and found out that a fusion protein expressed by pAS1-D elicited in guinea pigs also high levels of neutralizing antibodies, even though these levels were a little lower than those elicited by the fusion protein expressed by pAS1 (data not shown). This confirms

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YNAs1.1   13  TTVENYGGETQTARRLHTDVAFVLDRFVKLTQLKNTQTLDLMQIPSHTLV
YNAs1.2   13  ------------------------------------------------
India     13  ----------S-------------------*P----------------
Israel    13  -----K----------------I--------AP--I--------------

YNAs1.1   63  GALLRSATYYFSDLEVAIVHTGSVTWVPNGAPKDALDNHTNPTAYQKQPI
YNAs1.2   63  ------------------------------------------------
India     62  ----------------L-------------------------------
Israel    63  ----------------L---AR-PGC-TAR-T----N-Q-----------

YNAs1.1  113  TRLALPYTAPHRVLATVYNGKTTYGEESTRRGDFAALAQRLSRRLPTSFN
YNAs1.2  113  ------------------------------------------------
India    112  ------------------------TQP-----L-V------N-------
Israel   113  --------------------RP---TTS----M--------A-------

YNAs1.1  163  YGAVKADTITELLIRMKRAETYCPRPLLALDTTQDRRKQEIIAPEKQAL  211
YNAs1.2  163  ------------------------------------------------
India    162  ----------------------------------H----------V-  210
Israel   163  ------------------------------------------------V-  211
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Fig. 4
Alignment of amino acid sequences deduced from nucleotide sequences shown in Fig. 3

The alignment shows aa changes. Numbers indicated positions of aa in VP1 of FMDV type Asia 1. (-) stands for the same aa, while (*) stands for deletion of an aa. Regions of main alterations in aa sequences are underlined.

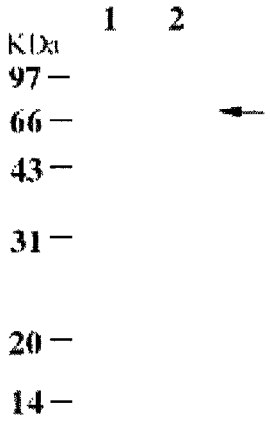

Fig. 5
Western blot analysis of fusion protein

Proteins were separated by SDS-PAGE and subjected to Western blot analysis using an FMDV type Asia 1 antiserum. Fusion protein (lane 1), protein expressed by vector pWR590 (negative control, lane 2). The arrow indicates the fusion protein. Protein size markers are shown on the left.

Table 1. Neutralizing antibody titers in immunized guinea pigs by microneutralization test[a]

Proteins	Sera dilutions	Ratios of protected animals to total animals	Sum of the ratios	Neutralizing antibody titers (PD_{50})[b]
The protein of pWR590	1:4 to 1:1024	0/10	0	> 1:4
	1:4	10/10		
	1:8	10/10		
	1:16	10/10		
	1:32	10/10		
The fusion protein	1:64	10/10	6.9	1:331
	1:128	8/10		
	1:256	6/10		
	1:512	3/10		
	1:1024	2/10		

[a]Two groups of animals were included in the experiment. One consisted of animals immunized with the protein from vector pWR590 (negative control), the other consisted of animals immunized with the fusion protein from the recombinant vector pAS1. Each group contained 10 guinea pigs.
[b]The neutralization titer of a serum was expressed as PD_{50}, the dilution of a serum that neutralized 50% of the virus activity. The titer was calculated by the Spearman-Karber method (Finney, 1978).

that the peptide consisting of aa 133–158 of VP1 of FMDV type Asia1 is indeed an important B cell epitope.

The virulence of FMDV is closely related to the gene variation. The genetic changes selected during the adaptation of a clonal population of FMDV to the guinea pig have been analyzed by Nunez *et al.* (2001). The analysis of the virus directly recovered from the lesions developed in animals revealed the selection of variants with two aa substitutions in non-structural proteins, I by T at position 248 in 2C and Q by R at position 44 in 3A. In further passages, an additional

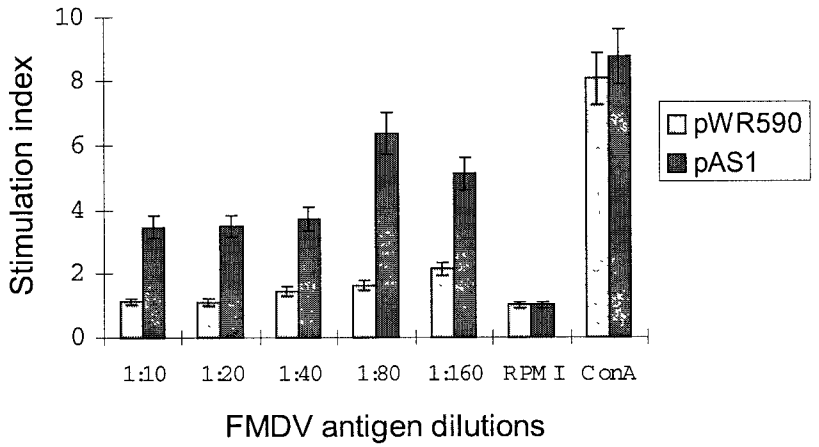

Fig. 6
T cell proliferation stimulated by viral protein in immunized guinea pigs

Guinea pigs were grouped as described in Table 1, the footnote[a]. T cells were stimulated with a purified viral protein. The proliferation effect was determined by comparing the [^{3}H]-thymidine taken up by the cells incubated with and without the viral protein. Stimulation index is the ratio of the mean cpm of the stimulated to that of the unstimulated culture. Bars represent standard deviation.

mutation, L to P at position 147 was selected in an important antigenic site located in the G-H loop in VP1. The aa substitution of Q by R at position 44 in 3A, either alone or in combination with the replacement of I by T at position 248 in 2C, was sufficient to give FMDV the ability to produce lesions. These results imply that one or very few replacements in non-structural viral proteins, which should be within reach of the mutant spectra of replicating viral quasi species, may result in adaptation of a virus to a new animal host. Nucleotide sequences of the YNAs1.1 and YNAs1.2 strains were identical. This implies that the difference between them is located at other region(s) of the VP1 gene. The mechanism of attenuation of virulence of the YNAs1.2 strain can be elucidated by analysis of the genomes of the YNAs1.1 and YNAs1.2 strains.

It has been reported that the murine antibody response to FMDV is T cell-dependent (Collen *et al.*, 1989). Thus, FMDV T cell determinants should enhance the immunogenicity of a synthetic peptide vaccine against FMDV infection. The recognition of T cell epitopes by lymphocytes of different species and individuals is restricted by the polymorphism of the major histocompatibility complex (MHC) molecules, which are responsible for the presentation of foreign antigens by antigen-presenting cells (APCs) (Germain, 1999). Therefore, the identification of T cell epitopes capable of inducing an effective response, while being widely recognized by MHC alleles frequent in natural populations of host species, is a problem for the development of new vaccines, particularly those based on synthetic peptides (Rowlands, 1994). The VP4 region of aa 20~34 was shown to be recognized as a dominant helper T cell (Th) determinant in cattle, which satisfies all criteria for a T cell epitope to be functionally relevant in a peptide vaccine: (i) it is highly MHC-promiscuous, (ii) its sequence is highly conserved in different FMDV types, and (iii) most importantly, it induces a virus-specific T cell response (van Lierop *et al.*, 1995).

Our results are consistent with the above conclusion and the following assumption. Only those T cells proliferate which originate from the guinea pigs immunized with the recombinant fusion protein (SI = 6.39). The extracellular fusion protein enters APCs through endocytosis and is cleaved to peptide fragments within endosomes, where the fragments associate with MHC class II proteins. Then this complex is transported to the cell surface and is presented to the receptor on the CD4-positive helper cell. The result of this series of events is the activation of helper T cells to produce various lymphokines, e.g. IL-2 as well as the IL-2 receptor. IL-2, also known as the T cell growth factor, stimulates in helper T cells multiple functions but some become "memory" cells, which are capable of being rapidly activated upon exposure to antigen at a later time. The activated CD4-positive T cells also produce another lymphokine called gamma interferon (γ-INF) , which increases the expression of MHC class II proteins in APCs. This enhances the ability of APCs to present the antigen to T cells and upregulates the immune response. Killed viral vaccines do not activate cytotoxic (CD8-positive) T cells, because the virus does not replicate in these cells and therefore viral epitopes are not presented in association with MHC class I proteins (Levinson and Jawetz, 2000). This implies that the T cell epitope from VP4 (aa 20~34) of the

fusion protein mainly activates CD4-positive T cells because it is a T_h epitope.

The colinear expression of T-helper and B cell epitopes in peptide vaccines can result in the enhancement of antibody responses (Borrás-Cuesta *et al.*, 1987; Collen *et al.*, 1991). The design of functional combinations of T cell and B cell epitopes is rather empirical (Cox *et al.*, 1988; Shaw *et al.*, 1993) and should maintain the immunostimulatory ability of the B and T cell components. In this study, the results showed that (i) the recombinant fusion protein reacted with sera from FMDV type Asia 1-infected animals *in vitro* and elicited a high level of neutralizing antibodies in guinea pigs, and (ii) the T cell proliferation in immunized animals increased following stimulation with the FMDV type Asia 1 antigen. This elucidated our observation that the fusion protein (aa 133–158–20~34–133–158) exhibited immunogenicity. To our knowledge, this is the first report on a recombinant fusion protein vaccine containing a tandem repeat of B and T cell epitopes of FMDV type Asia 1 that exhibits immunogenicity. The tandem repeat of concern can serve as a candidate for a vaccine containing fused epitopes against FMDV type Asia 1.

Epitopes by themselves are able to elicit an immune response (Bittle *et al.*, 1982). The FMDV epitope consisting of aa 141–160 was found to be immunogenic when delivered in liposomes in polymerized form (Francis *et al.*, 1987). However, peptides alone are poorly immunogenic and carriers should be always added to improve their immunogenicity because of their ability to enhance the T-helper cells response (Broekhuijsen *et al.*, 1986). In our study, in order to enhance the immunogenicity of the epitopes, β-galactosidase was chosen as the carrier of the epitopes. The tandem repeat consisting of aa 133–158–20~34–133–158 was inserted downstream of the C-terminus of the β-galactosidase in the vector pWR590. β-galactosidase has a long life span in *E. coli* and the fusion with β-galactosidase can prolong half-life of a short-living molecule. Therefore, the β-galactosidase carrier may overcome the problem of rapid degradation of foreign epitopes and lead to a continuous exposure to the host, thus enhancing the immunogenicity of foreign epitopes for the host. A fusion protein vaccine has several additional advantages: (i) it is a recombinant DNA vaccine devoid of deleterious effects due to incomplete inactivation of virus or the escape of the virus from vaccine manufacturing facilities; (ii) it is easy to construct; (iii) it is stable; (iv) it is easy to produce in large quantities by fermentation, and (v) it is of low cost. In our experiments, the vaccine protein yield reached up to 1.4 g/l (data not shown).

Variation of serotypes in FMDV is due to aa substitutions in VP1, which is a major immunogen. Type-specific neutralizing antibodies have no cross protection against different virus serotypes (Tulasiram *et al.*, 1997). In our study, we selected the specific B epitope of YNAS1.1 and YNAS1.2 strains and a FMDV with relatively conserved T cell epitope to produce a vaccine against FMDV type Asia 1. The B and T cell response in guinea pigs induced by the recombinant fusion protein of the tandem repeat epitopes indicated that this vaccine design was correct. In addition, a chemically inactivated YNAs1.2 whole virion vaccine provided a complete protection against FMDV type Asia 1 challenge in cattle (data not shown). This implies that the recombinant fusion protein vaccine of the tandem repeat epitopes containing aa 133–158–20~34–133–158 of VP1 may be specific and effective against FMDV circulating in cattle. This needs to be confirmed by virus-challenge experiments.

To summarize, here it is reported for the first time that (i) a recombinant fusion protein vaccine was produced using B and T cell epitopes of FMDV type Asia 1, (ii) the vaccine exhibited immunogenicity, (iii) the peptide containing aa 133–158 of VP1 of FMDV type Asia 1 is an important B cell epitope, and (iv) the tandem repeat containing aa 133–158–20~34–133–158 of VP1 can serve as a candidate of fused epitopes for vaccine design against FMDV type Asia 1.

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