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BARD

FINAL REPORT

PROJECT NO. US-2417-94

**Development of an Empty Viral Capsid Vaccine
against Foot-and-Mouth Disease**

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1999

BARD Scientific Report

Date: May 24, 1999

BARD Project Number: US-2417-94

Project Title: Development of an Empty Viral Capsid
Vaccine against Foot-and-Mouth Disease

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August 7, 1995

Project Start Date: _____

Type of Report: 1st Annual _____ 2nd Annual _____ Final X

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ABSTRACT

Title: Development of an Empty Viral Capsid Vaccine Against Foot-and-Mouth Disease.

Foot-and-mouth disease (FMD), a highly infectious viral disease of cloven-hoofed animals, is economically the most important disease of domestic animals. Although inactivated FMD vaccines have been successfully used as part of comprehensive eradication programs in Western Europe, there are a number of concerns about their safety. In this proposal, we have attempted to develop a new generation of FMD vaccines that addresses these concerns. Specifically we have cloned the region of the viral genome coding for the structural proteins and the proteinase responsible for processing of the structural protein precursor into both a DNA vector and a replication-deficient human adenovirus. We have demonstrated the induction of an FMDV-specific immune response and a neutralizing antibody response with the DNA vectors in mice, but preliminary potency and efficacy studies in swine are variable. However, the adenovirus vector induces a significant and long-lived neutralizing antibody response in mice and most importantly a neutralizing and protective response in swine. These results suggest that the empty capsid approach is a potential alternative to the current vaccination strategy.

OBJECTIVES:

The goal of this proposal is to develop an alternative vaccine against FMD that will be free of FMD infectious material and thus completely safe. This vaccine should be as effective as the current vaccine in protecting animals against homologous virus challenge and also allow distinction between vaccinated and convalescent animals.

This proposal presents our plans to molecularly clone the region of the FMDV genome coding for the structural proteins and the viral proteinase responsible for polyprotein processing into, 1) human adenovirus to produce a recombinant virus vector and 2) a naked DNA vector containing the cytomegalovirus (CMV) promoter for direct DNA delivery to animals. Expression, processing, and assembly of the structural proteins into capsid structures will be examined in mammalian cells infected with the recombinant adenovirus or transfected with the naked DNA vector. The vaccine candidates will be given to mice and guinea pigs and serological responses monitored. Challenge studies will be performed in guinea pigs and in cattle. The animals will be monitored for a neutralizing antibody response after one vaccination, revaccinated if required and subsequently challenged with virus. Future goals include, 1) extending protection to other subtypes or serotypes of FMDV by exchanging sites involved in inducing neutralizing antibody or introducing structural protein genes from more than one serotype into the vaccine, 2) utilizing a nonreplicating adenovirus to reduce concerns about the vector and increase its capacity for capsid genes of other FMDV serotypes, 3) utilizing a bovine adenovirus vector, and 4) examining the cell-mediated immune response.

The only deviation from the original proposal was 1) the use of a replication-deficient adenovirus rather than a live adenovirus and this change in strategy was based on safety concerns, and 2) use of swine rather than guinea pigs and cattle for the efficacy studies. The latter deviation was because the US lab has gained considerable experience with vaccine studies in swine over the past 3-4 years.

Detailed description of research activities and results.

USA

1. Construction of naked DNA containing the foot-and-mouth disease virus (FMDV) serotype A12 empty capsid cDNA construct with wild-type or mutant 3C proteinase coding regions.

a. Expression in a cell-free system. We used an expression vector, pcDNA3, to construct a plasmid, pP12X3C, containing the P1-2A and wild-type (WT) 3C coding regions of FMDV serotype A12 (Fig. 1, line 3). This construct contained a T7 RNA polymerase promoter for analysis of expression *in vitro* and a human cytomegalovirus (CMV) promoter for expression in cell culture and in animals. To examine the requirement for 3C proteinase activity in inducing an immune response, we constructed an additional plasmid coding for a mutant 3C protein. We have previously shown that this 3C mutant enzyme can not process capsid precursor P1-2A. This plasmid, pP12X3C-mut, was constructed by replacing a portion of the 3C coding region with a cDNA containing a mutation at the active site of 3C (cysteine to glycine at amino acid 163[C163G]). Both plasmids were transcribed and translated using a TNT cell-free

system (Promega) and the translation products immunoprecipitated with VP1-specific monoclonal antibodies (mAbs) 6FF5.1.3 and 6EE2.1.2. mAb 6FF5.1.3, which recognizes an epitope within the G-H loop of VP1, immunoprecipitated the processed capsid proteins from pP12X3C translation extracts and unprocessed P1-2A from mutant 3C translation extracts (Fig. 2, lanes 2 and 4; Chinsangaram et al., 1998). These results show that a portion of the processed antigens in WT 3C extracts are assembled, since all three capsid proteins were immunoprecipitated by a VP1-specific mAb. However, mAb 6EE2.1.2, which recognizes an epitope at the carboxy terminus of VP1, only immunoprecipitated the processed capsid proteins from WT 3C translation products and not unprocessed P1-2A from mutant 3C translation extracts (Fig. 2, lanes 5 and 7).

It has been shown by many groups that the presence of an intron flanking the cDNA insert frequently increases the level of gene expression. Therefore, we added a chimeric intron to pP12X3C and produced the plasmid piP12X3C. This plasmid along with pP12X3C and pP12X3C-mut were examined in cell culture and mice.

b. Activity of FMDV empty capsid plasmids in tissue culture and mice.

Expression of the FMDV cassettes driven by the CMV promoter was tested by transfecting BHK cells with lipofectamine or DOTAP, cationic lipids used in liposome mediated transfection. Twenty-four hours post transfection viral antigen expression was assayed by immunoperoxidase staining using mAb's 6FF5.1.3 and 6EE2.1.2. As detected by staining with mAb 6FF5.1.3, cells transfected with the plasmid containing WT 3C and the intron, piP12X3C, expressed 2-3 fold higher levels of viral antigen than

cells transfected with either pP12X3C or pP12X3C-mut (data not shown). Furthermore, similar to expression in a cell-free transcription/translation system, immunoperoxidase staining with mAb 6EE2.1.2 only detected viral antigens in cells transfected with pP12X3C and piP12X3C and not in cells transfected with pP12X3C-mut.

In earlier work, we found that neither intramuscular (IM) nor intradermal (ID) inoculation of mice with the plasmid pP12X3C elicited an efficient immune response in mice. To determine the optimal technique of DNA delivery, groups of mice were inoculated twice with 100 µg pP12X3C or a control plasmid via IM, ID, or with 1 µg DNA by epithelial (gene gun) inoculation. Serum samples from individuals within each group were pooled, heat inactivated at 56°C for 30 min, and the samples examined in plaque reduction neutralization (PRN) and radioimmunoprecipitation (RIP) assays for FMDV-specific antibody responses. As shown in Table 1, analyses of pooled sera from ID- or IM-inoculated mice failed to detect antibodies to FMDV by PRN or RIP assays at 1:4 serum dilution. However, analyses of pooled sera from animals inoculated with pP12X3C by gene gun were positive by both assays after the second inoculation. When individual sera from this group were examined, 4 of 5 mice were found positive by RIP (Fig. 3, lanes 4 to 13) and 3 of these mice developed neutralizing antibodies as detected by PRN (Table 1).

In a concurrent study, gene gun inoculation was chosen as the technique of DNA delivery to determine both the effect of an intron and the role of an active 3C proteinase on induction of an immune response. As shown in Table 1, analyses of pooled serum revealed that only 1 gene gun inoculation of piP12X3C was required to induce an

FMDV-specific antibody response as measured by RIP, while 2 gene gun inoculations were required to induce an RIP response with either pP12X3C or pP12X3C-mut. After 2 gene gun inoculations, 4 of 5 mice inoculated with the piP12X3C developed both an RIP and PRN antibody response similar to pP12X3C (see previous paragraph). Interestingly, serum from 2 of 5 mice inoculated with pP12X3C-mut contained antibodies that could immunoprecipitate FMDV capsid proteins, but neither pooled serum nor individual serum from this group contained antibodies capable of neutralizing the virus (Table 1).

We also attempted to increase the expression level of piP12X3C and enhance the immune response in animals by including the FMDV internal ribosome entry site (IRES) upstream of the P1 coding region. This genetic element is responsible for directing cap-independent translation. The IRES was added to the construct in the absence or presence of the L coding region. The L proteinase shuts off host cell mRNA translation, which is cap-dependent, and could result in enhanced expression of the FMDV cassette regulated by the IRES. All modified constructs expressed FMDV proteins in a cell-free transcription-translation system and in transfected cells, but after gene gun inoculation these genetic elements had either no effect or a negative effect on the antibody response (Table 1).

c. Immune response of swine to FMDV capsid constructs. In a preliminary study one pig was inoculated by gene gun with pP12X3C. After 1 inoculation with 3.5 µg DNA this animal developed a neutralizing antibody response of 1.9 (PRN₅₀). The neutralizing antibody titer was boosted to 2.8 after 3 additional inoculations. In another swine

experiment, piP12X3C and piRESP12X3C were inoculated into swine (3/group) twice by gene gun. The swine did not seroconvert and were not protected from contact challenge. These results suggest that with our present constructs, more than two inoculations are required for production of an immune response and protection.

d. Effect of mouse cytokine genes on the immune response to FMDV capsid constructs. Studies in many systems indicate that some cytokines can function as biological adjuvants. Cytokines such as interleukin 1 and interleukin 2 (IL2) have been shown to enhance the immune response to inactivated FMD vaccines. In addition, granulocyte macrophage colony stimulating factor (GM-CSF) and IL2 genes have been demonstrated to enhance the immune response to inoculation with DNA encoding various immunogens.

To attempt to enhance and direct the immune response towards FMDV DNA vaccination, we subcloned mouse cDNA for IL2 (Genzyme Corp.) and GM-CSF (obtained from DNAX Research Institute of Molecular and Cellular Biology, Inc.) into expression vectors pCI and pSI. In these latter vectors the IL2 and GM-CSF genes were cloned downstream of the CMV promoter, pCI, or the SV40 promoter, pSI. In addition, both of these expression vectors contain an intron. The SV40 promoter and the IL2 or GM-CSF genes were also subcloned into our previously constructed FMDV expression vector, pCI-P12X3C (essentially identical to piP12X3C), to generate the vectors pCI-P12X3C-SI-IL2 or pCI-P12X3C-SI-GMCSF. To demonstrate cytokine expression, the plasmids were transcribed and translated in a cell-free system in the presence of [³⁵S]methionine and the products examined by SDS-polyacrylamide gel

electrophoresis. As shown in Figures 4 and 5, IL2 and GM-CSF were expressed from the vectors that we constructed (lanes 2-4).

To demonstrate that the expressed IL2 and GM-CSF was biologically active, the various plasmids were transfected into BHK-21 cells and after 24 h supernatants and cell lysates were obtained and examined in a thymidine incorporation assay with either HT-2 or NSO cells, cell lines that proliferate in response to mouse IL2 or GM-CSF, respectively. As seen in Figures 6 and 7, supernatants and cell lysates from cells transfected with pCI-P12X3C-SI-IL2 (Fig. 6) and pCI-GMCSF or pCI-P12X3C-SI-GMCSF (Fig. 7) contained biologically active IL2 and GM-CSF, while cells transfected with the control FMDV empty capsid vector, pCI-P12X3C, have no activity.

To examine the effect of the IL2 and GM-CSF genes on the immune response of mice to the FMDV empty capsid construct groups of 3 mice were inoculated IM with 100 µg of various plasmid combinations and boosted IM 5 weeks later with pCI-P12X3C. Mice were bled 3 and 7 weeks post initial inoculation. Serum samples from individuals within each group were pooled and examined in RIP and PRN assays for FMDV-specific antibody responses.

As can be seen in Table 2 only the groups inoculated with either the GM-CSF gene in the same plasmid as the FMDV construct, pCI-P12X3C, (group 2) or as a separate plasmid (group 4) induced an antibody after the first shot. After the booster, group 2 but not group 4 developed a neutralizing antibody response. However, group 4 developed the highest RIP antibody response of all the groups examined.

These results indicate that the GM-CSF gene and to a lesser extent the IL2 gene

enhance the immune response of mice to the FMDV empty capsid cDNA construct. To further examine the apparent adjuvant effect of the GM-CSF gene on the immune response to FMDV and examine its efficacy studies are planned in animals naturally susceptible to FMDV using cytokine genes from these species.

2. Construction of recombinant adenovirus containing FMDV capsid constructs with wild-type or mutant 3C proteinase coding regions.

a. Expression in cell culture. In the first annual report, we discussed the construction of adenovirus transfer vectors containing the FMDV serotype A12 P1-2A and 3C coding regions. By homologous recombination with a plasmid containing defective human adenovirus type 5 DNA a recombinant adenovirus with insertion of the FMDV sequences in the adenovirus early region 3 (E3) would result after transfection of 293 cells. However, our initial attempts to generate recombinant adenoviruses were unsuccessful.

Subsequently we adopted a slightly different strategy. We have cloned the FMDV P1-2A and WT or mutant 3C coding regions into a adenovirus transfer vector, pCA3. This vector contains the human CMV immediate early promoter/enhancer and SV40 polyadenylation signals and would allow insertion of the FMDV sequences into the E1 region of adenovirus under the control of the CMV promoter. The E1 region is required for complete virus replication and thus these recombinant viruses are replication-deficient and can only be generated and grown in 293 cells which contain the adenovirus E1 region. This strategy therefore offers safety advantages as compared to our previous strategy.

Even though human replication-deficient recombinant adenoviruses can not complete a full virus replication cycle in cell culture except for 293 cells, numerous workers have shown that they can infect cells and induce foreign gene expression and can also elicit immune responses and successfully protect animals from virulent pathogen challenge. Using this strategy we have successfully generated recombinant adenoviruses containing FMDV P1-2A and WT (Ad5-P12X3CWT) or mutant 3C (Ad5P12X3CMUT).

High-titered purified stocks of these viruses have been produced and FMDV gene expression was examined with both viruses in 293 cells. Immunoprecipitation of infected cell cytoplasmic extracts with anti-VP2 serum, which recognizes epitopes on VP0 the precursor of VP4 and VP2, P1-2A, VP0, VP3, and VP1 are synthesized (Fig. 8, lane 3). This result demonstrates not only production of P1-2A and processing by the 3CWT protease to yield VP0, VP3, and VP1, but the likely formation of higher order structures as shown by coprecipitation of VP0, VP3, and VP1 with VP2 antiserum. In Ad5-P12X3CMUT infected cells, P1-2A is synthesized, but is not processed (Fig. 8, lane 4), since processing requires 3CWT protease.

To further examine for the assembly of FMDV capsid structures, monospecific polyclonal or monoclonal antibodies (mAb) prepared against FMDV structural proteins were used. Monospecific VP1 and VP2 antiserum precipitates VP0, VP3 and VP1 (Fig. 9, lanes 1-2). Monoclonal antibody 6EE2, which recognizes an epitope on VP1, also precipitates all three structural proteins (Fig. 9, lane 3). Most significantly mAb 2PD11, which is a conformation-dependent antibody that recognizes an epitope found on the surface of intact virus particles that includes regions of VP3 and VP1 precipitates VP0

as well as VP3 and VP1 (Fig. 9, lane 4). In summary, these results indicate that at least some of the capsid proteins expressed in Ad5-P12X3CWT infected cells are present in a particle form which is structurally and antigenically similar to intact capsids. Similar results were obtained in HeLa cells, a cell line that is nonpermissive for replication-defective adenoviruses. In contrast, in 293 cells infected with Ad5-P12X3CMUT, monospecific antibodies against VP1 and VP2 only precipitated P1-2A (Fig. 9, lanes 5-6). Interestingly mAb 6EE2 immunoprecipitates very little P1-2A, possibly the result of the specificity of this antibody for protein in the native conformation (Fig. 9, lane 7). Most significantly is the almost complete absence of precipitation of P1-2A with mAb 2PD11, a further indication of the specificity of this antibody for a structural motif only present on virus capsids.

b. Immune response of mice to Ad5-P12X3C inoculation. BALB/c mice were inoculated IM (5/group) with 5×10^7 pfu/mouse of Ad5-P12X3XWT or Ad5-P12X3CMUT. As a control, mice (2/group) were inoculated with phosphate buffered saline (PBS). The mice were bled at 3 and 5 weeks postinoculation, boosted at 6 weeks with an equivalent amount of virus, and bled at weeks 8, 11 and 21. Sera from individual mice at 3 and 5 weeks postinoculation were assayed by RIP and pooled serums from each group were assayed by PRN.

Five week serum from all individual mice inoculated with either virus contained antibodies against the structural proteins VP0, VP3, and VP1 (Fig. 10, lanes 3-12). The representative control mouse serum did not immunoprecipitate any viral proteins (Fig. 10, lane 2). The three week sera showed the same results, although signals with these

sera were weaker. By PRN, only the group inoculated with Ad5-P12X3CWT developed a low but detectable neutralizing antibody response by 5 weeks postinoculation (Fig. 11). Two weeks after the boost there was a dramatic increase in the level of neutralizing antibody in the group inoculated with Ad5-P12X3CWT (Fig. 11). The increase in titer after the boost occurred in all mice in this group (Table 3). Furthermore, the titer of this group increased by week 11 and by week 21 (15 weeks postboost) this group still had a significant neutralizing antibody titer suggesting that the adenovirus vaccine may impart long lasting immunity against FMDV to inoculated animals. In contrast, the control group and the Ad5-P12X3CMUT group never developed any neutralizing antibody response (Fig. 11).

Thus, utilizing either naked DNA or a recombinant adenovirus as a delivery vehicle, only FMDV empty capsid constructs with an active 3C proteinase are able to induce a neutralizing antibody response in mice. These results suggest that processing of the capsid protein precursor is essential for inducing this type of antibody response.

c. Potency and efficacy of Ad5-P12X3C in swine. Two groups of swine, 3 animals per group, were inoculated IM with 1×10^8 pfu/swine Ad5-P12X3CWT or PBS and boosted at 4 weeks with an equivalent amount of Ad5-P12X3CWT virus or PBS. Serum samples were obtained weekly and assayed for an FMDV-specific immune response by RIP and PRN. Swine inoculated with Ad5-P12X3CWT developed a significant FMDV-specific neutralizing antibody response by 2 weeks postinoculation (Fig. 12A). The titers decreased over the next 2 weeks, increased 2-8 fold after the boost, and stabilized prior to challenge. The control group did not develop an FMDV-specific neutralizing

antibody response (Fig. 12B). The Ad5-P12X3CWT inoculated animals developed an antibody response against the FMDV structural proteins as assayed by RIP (Fig. 13, lanes 2-4), while the control group did not immunoprecipitate any viral proteins prior to challenge (Fig. 13, lanes 5-7).

At 8 weeks post initial inoculation, control animal #3 was inoculated intradermally in the snout and by a combination of intradermal and skin scarification on one foot with a total of 5×10^4 infectious units of virulent cattle passaged FMDV serotype A12. All the animals were monitored for clinical signs of disease including fever, ie., 40°C or more for 2 consecutive days, and lesions. Animal #3 developed a fever by 2 days postchallenge (dpc) and lesions on all 4 feet by 3 dpc. The remaining two uninoculated control animals developed a fever and vesicular lesions on all 4 feet by 4 dpc (Table 4).

In contrast, the vaccinated animals were significantly protected from clinical disease. One of the vaccinated animals, WT#3, did not develop any clinical signs of FMD, while the other two animals in this group developed disease signs that were significantly reduced as compared to the animals in the control group (Table 4), ie., no fever but lesions on only 1 or 2 digits.

Serological analysis demonstrated that after challenge the directly inoculated swine as well as the two uninoculated control animals seroconverted, ie., all had a significant FMDV-specific neutralizing antibody response (Table 4) and antibodies against the structural and in some cases nonstructural proteins 3D and 2C (Fig. 13, lanes 11-13, [1 wpc], 17-19[2 wpc]). The PRN titer of the vaccinated animal that was completely protected from disease only increased 5-fold after challenge (Table 4).

These results demonstrate that a replication-defective Ad5-FMDV recombinant virus containing a WT FMDV 3C proteinase is a viable FMD vaccine candidate.

d. Examination of vaccine dose on potency and efficacy. To attempt to improve the FMDV-specific immune response of swine to Ad5-P12X3CWT inoculation, we examined the effect of the amount and number of doses of this vaccine. Groups of swine were inoculated IM with either a low dose, 1×10^8 pfu/animal, or a high dose, 5×10^8 pfu/animal, and either boosted at 4 weeks postinoculation with a like dose or not boosted (Table 5). The animals inoculated with a high dose of virus developed a significant PRN response by 1 wpi, while animals inoculated with the low dose only developed a PRN response by 2 wpi (data not shown) as found in the previous experiment (Fig. 12A). By 4 wpi animals in all the virus inoculated groups had similar PRN titers (data not shown). The PRN titers of the animals boosted with either dose increased 2-4 fold. After 8 weeks the PRN titers were higher in the animals given 2 inoculations as compared to a single inoculation and all virus inoculated animals developed a FMDV-specific antibody response as assayed by RIP (Table 5).

At 7 weeks postinoculation animal #158 was directly inoculated as in the previous experiment with animal virulent virus. This animal developed typical clinical signs of FMD, seroconverted, and demonstrated antibodies against the viral nonstructural proteins (Table 5). The two noninoculated control animals, #156 and #157, also developed FMD, although #156 had a mild disease. All three animals inoculated with two low doses of Ad5-P12X3CWT were completely protected from disease. However, the PRN titers did increase from 8-10 fold indicative of limited replication. In the group

inoculated with two high doses of virus, one animal was completely protected, while the other two animals developed some disease. The groups inoculated with only 1 low or high dose of virus also demonstrated some protection, but not as significant as the animals inoculated with two doses.

In a similar experiment groups of swine were inoculated IM with two low doses of virus, a low dose and a high dose, a low dose and boosted with plasmid DNA, ie., pCI-P12X3C, or a low intranasal dose and boosted with a low IM dose (Table 6). After challenge the control animals all developed severe clinical signs of disease including lesions on all 4 feet and the snout. In the group inoculated with a low dose and boosted with a high dose two of three animals were completely protected and the third animal in this group had very mild disease, ie., no fever and only 1 lesion. Only two animals were inoculated with two low doses of virus (one animal in this group died early in the experiment from unrelated causes), one was completely protected, while the other animal developed severe disease. In the group inoculated with a low dose and boosted with the FMDV empty capsid cDNA construct, one animal was completely protected, a second animal had only mild disease, ie., one lesion and no temperature, while the third animal had severe disease including fever and multiple lesions. In the group inoculated IN with a low dose and boosted IM with a low dose, one animal was completely protected, a second had mild disease, and the third developed severe disease including fever and lesions on all four feet and the snout.

We have also examined the immune response of the swine to adenovirus. Our preliminary results by RIP and PRN indicate that the swine had been pre-exposed

presumably to porcine adenovirus prior to the start of our experiments. However, a neutralizing antibody response was not induced until after the second inoculation.

Figure 1

Figure 2.

Figure 3.

Fig. 4. Expression of mIL2 in a cell-free system

Figure 5. Expression of mGM-CSF in a cell-free system

Figure 6. Biological Assay of mIL-2 Expressed in HT2 Cells

Figure 7. Biological Activity of mGM-CSF Expressed in NSO Cells

Figure 8.

Figure 9.

Figure 10.

Figure 11.

Figure 12.

Figure 13.

TABLE 1. Antibody response to DNA vaccination

Group #	Route/DNA ^a	PRN ₅₀ group (individuals) ^b	RIP group (individuals) ^c
1	ID/pcDNA3-βgal-2x	-	-
2	IM/pcDNA3-βgal-2x	-	-
3	GG/pcDNA3-βgal-1x	-	-
4	GG/pcDNA3-βgal-2x	-	-
5	GG/pP12X3C-mut-1x	-	-
6	GG/pP12X3C-mut-2x	- (-, -, -, -)	+ (+, -, -, +)
7	ID/pP12X3C-2x	-	-
8	IM/pP12X3C-2x	-	-
9	GG/pP12X3C-1x	-	-
10	GG/pP12X3C-2x	1.9 (2.5, 2.2, -, -, 1.9)	+ (+, +, +, -, +)
11	GG/piP12X3C-1x	- ^d (-, -, -, -)	+ (-, +, -, +, -)
12	GG/piP12X3C-2x	2.5 (2.6, -, 2.2, 2.6, 2.5)	+ (+, -, +, +, +)
13	GG/pIRES P12X3C-1x	-	-
14	GG/pIRES P12X3C-2x	- (-, -, -, -)	+ (+, -, -, +, -)
15	GG/piIRES P12X3C-1x	- (-, -, -, -) ^f	+ (+, +, -, -, -)
16	GG/piIRES P12X3C-2x	1.9 (2.2, 2.2, -, >2.8, 2.3)	+ (+, +, -, +, +)
17	GG/pIRESLP12X3C-1x	-	-
18	GG/pIRESLP12X3C-2x	-	-
19	GG/piIRESLP12X3C-1x	-	-
20	GG/piIRESLP12X3C-2x	-	-

^a Groups of 5 mice were inoculated once (1x) or twice (2x) intradermally (ID), intramuscularly (IM), or epithelially (GG) with the indicated plasmid.

^b Log of serum dilution giving a 50% reduction in PFU. Serum samples tested at 1:4 starting dilution for pooled samples (1:20 for each individual) and at 1:20 starting dilution for individual samples.

^c Tested at 1:20 dilution for pooled samples (1:100 for each individual) and at 1:20 dilution for individual samples.

^d Starting dilution at 1:200

^e Starting dilution at 1:25

^f Starting dilution at 1:62

Table 2. Immune response of mice inoculated with various plasmids.

Group No.	First Shot		Second Shot	First Bleed	Second Bleed
	Inoculum 1 (µg)	Inoculum 2 (µg)	Inoculum (100 µg)	PRN _{50/70} ; RIP	PRN _{50/70} ; RIP
1	pCI-P12X3C-SI-mIL2 (100)	-	pCI-P12X3C	-/-;-	-/-;+
2	pCI-P12X3C-SI-mGMCSF (100)	-	pCI-P12X3C	-/-;+	1:80/1:20;++
3	pCI-P12X3C (100)	pCI-mIL2 (100)	pCI-P12X3C	-/-;-	1:40/1:40;+
4	PCI-P12X3C (100)	pCI-mGMCSF (100)	pCI-P12X3C	-/-;+	-/-;+++
5	PCI-P12X3C (100)	-	pCI-P12X3C	-/-;-	-/-;+

TABLE 3
PRN₇₀ titers of serum from individual mice
inoculated with Ad5-P12X3CWT.

Five weeks post inoculation ¹	Eight weeks post inoculation ¹
<1:20	1:160
1:20	1:160
1:40	1:320
1:40	1:1280
1:80	1:2560

¹ Identity of individual mice is uncertain, so PRN₇₀ values are listed in arbitrary order of increase in the PRN₇₀ titer.

TABLE 4
Summary of immune and clinical responses to vaccination and challenge.

Swine	Vaccine	Pre-Challenge			Post-Challenge				
		PRN ¹ 0 wpi ⁵	PRN ¹ 8 wpi ⁵	RIP ² 8 wpi ⁵ s/ns	PRN ¹ 1 wpc ⁶	PRN ¹ 2 wpc ⁶	RIP ² 2 wpc ⁶ s/ns	Fever ³	Lesion ⁴ Score
WT1	P12X3CWT	<4	128	+/-	128	10240	+/-	-	2
WT2	P12X3CWT	<4	256	+/-	128	10240	+/-	-	1
WT3	P12X3CWT	<4	256	+/-	128	1280	+/-	-	0
C1	none	8	8	-/-	256	25600	+/-	+	16
C2	none	4	<4	-/-	128	25600	+/+	+	16
C3	none	<4	4	-/-	8192	12800	+/+	+	16+snout

¹ The number listed is the highest fold dilution resulting in a plaque reduction of 70%.

² A positive result indicates that the sera from this animal is able to immunoprecipitate protein as determined by PAGE analysis. s = structural proteins VP0, VP1, VP3. ns = nonstructural proteins 3D and/or 2C.

³ Fever is scored as 2 or more consecutive days of temperature over 40°C

⁴ The Lesion Score is the number of digits on which the swine exhibited lesions. Swine have 4 digits per foot.

⁵ wpi = weeks post-inoculation

⁶ wpc= weeks post-challenge

TABLE 5

Effect of number and amount of doses on immune induction and protection.

Swine No.	Vaccine	Pre-Challenge			Post-Challenge				Lesion ⁴ Score
		PRN ¹ 0 wpi ⁵	PRN ¹ 8 wpi ⁵	RIP ² 8 wpi ⁵ s/ns	PRN ¹ 1 wpc ⁶	PRN ¹ 2 wpc ⁶	RIP ² 2 wpc ⁶ s/ns	Fever ³	
145 ⁷	P12X3CWT	8	400	+/-	320	3200	+/-	-	0
146 ⁷	P12X3CWT	8	320	+/-	160	3200	+/-	-	0
147 ⁷	P12X3CWT	4	320	+/-	640	3200	+/-	-	0
148 ⁸	P12X3CWT	<4	40	+/-	640	16000	+/-	-	0
149 ⁸	P12X3CWT	4	320	+/-	64000	128000	+/-	-	1
154 ⁸	P12X3CWT	<4	160	+/-	12800	128000	+/+	+	6
150 ⁹	P12X3CWT	32	320	+/-	800	128000	+/+	+	2
151 ⁹	P12X3CWT	8	320	+/-	640	64000	+/-	+	2
152 ⁹	P12X3CWT	<4	400	+/-	640	12800	+/-	-	0
153 ¹⁰	P12X3CWT	16	160	+/-	6400	16000	+/+	+	1
155 ¹⁰	P12X3CWT	<4	160	+/-	320	6400	+/-	-	0
144 ¹⁰	P12X3CWT	8	80	+/-	1600	64000	+/+	+	5
156	none	8	64	-/-	32000	25600	+/+	+	16+snout
157	none	4	8	-/-	1600	16000	+/-	-	1
158	none	<4	<8	-/-	32000	12800	+/+	+	13

¹The number listed is the highest fold dilution resulting in a plaque reduction of 70%²A positive result indicates that the sera from this animal is able to immunoprecipitate protein as determined by PAGE analysis. S = structural proteins VP0, VP3, VP1. ns = nonstructural proteins 3D and/or 2C.³Fever is scored as 2 or more consecutive days of temperature over 40°C after challenge.⁴The lesion score is the number of digits on which the swine exhibited lesions. Swine have 4 digits per foot.⁵wpi = weeks postinoculation⁶wpc = weeks postchallenge⁷Each animal in this group was inoculated with 1x10⁸ pfu and boosted at 4 wpi with a like dose.⁸Each animal in this group was inoculated once with 1x10⁸ pfu.⁹Each animal in this group was inoculated with 5x10⁸ pfu and boosted at 4 wpi with a like dose.¹⁰Each animal in this group was inoculated once with 5x10⁸ pfu.

TABLE 6

Effect of amount, route, and type of boost on immune induction and protection.

Swine No.	Vaccine	Pre-Challenge			Post-Challenge				
		PRN ¹ 0 wpi ⁵	PRN ¹ 7 wpi ⁵	RIP ² 7 wpi ⁵ s/ns	PRN ¹ 1 wpc ⁶	PRN ¹ 2 wpc ⁶	RIP ² 2 wpc ⁶ s/ns	Fever ³	Lesion ⁴ Score
25	none	<8	64	-/-	800	8000	+/-	+	15+snout
26	none	<8	8	-/-	>25600	16000	+/-	+	13+snout
27	none	<8	32	-/-	16000	32000	+/-	+	16+snout
28 ⁷	P12X3CWT	<8	>1280	+/-	400	12800	+/-	-	0
29 ⁷	P12X3CWT	<8	80	+/-	4000	64000	+/-	+	10
30 ⁸	P12X3CWT	16	1280	+/-	800	3200	+/-	-	0
32 ⁸	P12X3CWT	<8	640	+/-	400	3200	+/-	-	0
33 ⁸	P12X3CWT	8	320	+/-	400	64000	+/-	-	1
34 ⁹	P12X3CWT	<8	320	+/-	200	32000	+/-	-	1
35 ⁹	P12X3CWT	16	320	+/-	200	>25600	+/-	-	0
36 ⁹	P12X3CWT	<8	320	+/-	400	64000	+/-	+	5
37 ¹⁰	P12X3CWT	8	160	+/-	3200	12800	+/-	-	1
38 ¹⁰	P12X3CWT	<8	320	+/-	400	>25600	+/-	-	0
39 ¹⁰	P12X3CWT	8	160	+/-	>25600	32000	+/-	+	16+snout

¹The number listed is the highest fold dilution resulting in a plaque reduction of 70%

²A positive result indicates that the sera from this animal is able to immunoprecipitate protein as determined by PAGE analysis. S = structural proteins VP0, VP3, VP1. ns = nonstructural proteins 3D and/or 2C.

³Fever is scored as 2 or more consecutive days of temperature over 40°C after challenge.

⁴The lesion score is the number of digits on which the swine exhibited lesions. Swine have 4 digits per foot.

⁵wpi = weeks postinoculation

⁶wpc = weeks postchallenge

⁷Each animal in this group was inoculated IM with 1x10⁸ pfu and boosted IM at 4 wpi with a like dose.

⁸Each animal in this group was inoculated IM with 1x10⁸ pfu and boosted IM at 4 wpi with 5x10⁸ pfu.

⁹Each animal in this group was inoculated with 1x10⁸ pfu and boosted IM at 4 wpi with 400µg pCI-P12X3C DNA.

¹⁰Each animal in this group was inoculated IN with 1x10⁸ pfu and boosted IM at 4 wpi with a like dose.

Israel

Expression, processing, and assembly of FMDV clones in BHK-21 cells.

a. Materials and Methods

1. Molecular biology procedures

All procedures were according to Molecular Cloning, Sambrook et al, 1989 2nd edition. All DNA analysis were performed using Genetic Computer Group (GCG) software.

2. Transfection of plasmids

Transfection was done on 50-70% confluent cells. Three µg of DNA in 100 µl of OptiMEM and 18 µl of LipofectAMINE plus (GibcoBRL) in 100µl of OptiMEM (GibcoBRL) were combined, mix gently and incubated at room temperature for 20 minutes. The mixture was added to 800 µl of OptiMEM and then to cells that were washed twiced. Five hours later, OptiMEM was replaced with 4 ml of Eagle medium with 5% calf serum, 10% (3% triphospho broth) and 1% antibiotics and antifungicides. At designated times the cells were taken for analysis.

3. ELISA

Each well of the microtiter plate were coated with 20mg/ml of 16 mer VP1 peptide in 0.1M Tris HCl pH8.8 for 16 hr. at room temperature. The content of each of the wells were discarded and to each of the wells 250 µl of 50% fetal calf serum (FCS) in PBS for saturation for 1 hr. at room temperature and washed 5 times with PBS + 0.05 % Tween 20. To each of the wells 200 µl of serum at different dilutions in PBS+0.5% FCS were added for 1 hr. at room temperature. Washed 5 times with PBS + 0.05 % Tween

20 and reacted with 200 μ l of anti-rabbit peroxidase 1:40000 dilution in PBS+0.1% FCS for 1 hr. at room temperature and washed 5 times with PBS + 0.05 % Tween 20. The reaction was done by adding 200 μ l of peroxides substrate. Results were read 30 min. later at OD₄₁₀.

4. Fixation and slide preparation

At designated times the cells were washed with PBS + 0.05% TWEEN-20. Then methanol at -20°C was added for 5 min. Next acetone at -20°C was added for 5 min. The cells were washed with PBS + 0.05% TWEEN-20 and blocking was performed with 5% skim milk in PBS + 0.05% TWEEN-20. The first antibody (1:100) in PBS + 0.05% TWEEN-20 was added for 60 min. incubation at room temperature and washed 5 times with PBS + 0.05% TWEEN-20. Second antibodies (1:100) in PBS + 0.05% TWEEN-20 were added for 1 hr. at room temperature and washed 5 times with PBS + 0.05% TWEEN-20 and the slides were prepared with Mounting media (BioRad). For detecting Asia1 VP1 (1:100) Guinea Pig anti-FMDV Asia 1 as first antibodies and as second antibodies (1:100) **CyTM5-conjugated** affini pure Goat anti-guinea pig IgG F(ab')₂ was used. For O1 (G) rabbit anti-16 mer peptide O1 (G) and (1:100) **FITC-conjugated** affini pure Sheep anti-rabbit IgG as the first and second antibodies.

5. DNA vaccination

To introduce the DNA we used the Agene gun@ (BioRad Hercule, CA)

6. Preparing plasmid DNA for biobalistic bombardments

To 25 mg gold (1.65 μ), 100 μ l of 0.05 M spermidine was added and vortexed for a few seconds, then sonicated for 3-5 seconds using an ultrasonic cleaner to break up

gold clumps. To the gold and spermidine mixture, 100 μ l of 1 μ g/ μ l plasmid was added. The DNA, spermidine and gold were mixed by vortexing for ~5 sec. While vortexing the mixture at moderate rate on a variable speed vortexer, 100 μ l of 1 M CaCl₂ was added dropwise and the mixture was left to precipitate at room temperature for 10 min. The microcarrier was spin for 15 sec. and the supernatant was removed. The pellet was washed three times with 1 ml of freshly opened 100% ethanol. After the last wash the pellet was resuspend in the ethanol solution and transferred to a 15 ml disposable polypropylene centrifuge tube with a screw cap. The microfuge tube was rinsed once with 200 μ l of 0.05 mg/ml of PVC in ethanol that was added to the centrifuge tube. Ethanol/PVP solution was added to final volume of 1.5-2 ml. The gold DNA suspension was used to coat tubes with the tubing prep station (BioRad). DNA was introduced by the gene gun (BioRad).

b. Results

1. Cloning FMDV O1 (G) sequences and constructing FMDV (G) expression plasmids

For cloning, we applied the long-and-accurate RT-PCR (LA-RT-PCR) technique to amplify viral fragments from FMDV O1 Geshur (G) for cloning. For The RT step we used the AMV RT and for the PCR step we used ex-taq (Takara, Osaka Japan). We amplified a 3.4 kbp segment which started from the first AUG at the 5' end and contained the L, VP4, VP2, VP3, VP1 and the 2A protease. The 3' end of the fragment is beyond the 2A cleavage site ensuring proper cleavage by the 2A protease. The fragment was cloned into the pTarget Δ Tfi I to create pKVI307 (Figure 1). The vector

pTargetT Δ Tfi I a derivative of pTargetT (Promega, Madison WI) contains the CMV promoter/enhancer; a chimeric intron consisting of the (3-globin 5' donor site and immunoglobulin gene heavy chain variable region branch and 3'-acceptor) and the late SV40 poly A adenylation sequences without the selective neo marker that was removed. The 3C fragment was amplified and cloned in frame with the 3.4 Kbp pTargetT Δ Tfi I insert to create pKVI318. The entire clone was sequenced and from the sequence data we could show that there is no stop codon in the expressing frame and that the clone contains all L, 2A and 3C cleavage sequences ensuring proper processing of viral proteins upon introducing the clone into cells. Since the L protease inhibits the translation of the cap dependent RNA, we set up experiments to exchange the L sequences with two IRES's, one from our Geshur isolate and the other from EMC virus (Fig. 1). LA-PCR was performed on pKVI318 by using a forward primer corresponding to sequences after the L 3' end that contains a Xba I site. The reverse primer is compatible with the vector T7 sequencing that contains a Bam HI site. Following PCR and ligation pKVI318 Δ L plasmid lacking the L sequences was created. To the BamH I and Xba I sites we introduced the EMCV IRES after amplification using primers corresponding to the 5' and 3' of the IRES containing the BamH I and Xba I respectively, to generate pKVI324 (Fig. 1) Another expression vector we prepared is a plasmid that contains the 3CD sequences to create pKVI326. This plasmid is important since the efficiency of viral proteins processing by the 3CD polypeptide is superior than with 3C by itself. To do so, we PCR'd the 3CD 2.25 kb fragment and replaced the 3C gene in pKVI324 with the newly synthesized fragment to create pKVI326.

We also prepared an expression vector containing O1 (G) and Asia 1 VP1 together with O1 (G) 3C all in the same frame and containing the O1 proteolytic sequence between each of the genes. The three genes were cloned into pVCL1010 expression vector (Vical, CA) to create pKVI335 (Fig. 1).

At every step in the cloning process we sequenced each of clones to ensure the authenticity of the sequences. As a result the entire coding sequences of O1 (G) was elucidated. There is 88% nucleotide homology between O1 (K) and O1 (G) where there is 95% amino acids (AA) homology between the two isolates. The two viruses share the same viral protease's AA cleavage sequences (Fig. 2) (Paper in preparation).

2. Using 16 mer VP1 amino acids peptide to raise anti-O1 (G) VP1 antibodies

One of the problems we faced was inadequate anti-VP1 antibodies for testing the presence of VP1 in infected and transformed cells. To overcome the problem a 16 mer peptide VRGDLQVLARKAARPL corresponding to aa 144-159 of the O1 (G) VP1 was prepared and bound to KLH. The bound peptide was used to inject rabbits in complete Freund's adjuvant three times at 4 week intervals. Serum was taken before each injection and tested by ELISA against FMDV O1 (G) and 16 mer peptide. After the second and the third injection the antibody titer reached at least 1:24,000 against the entire virus and 1:15,000 against the 16 mer peptide (Paper in preparation).

3. Expression of FMDV constructs

BHK cells were transformed with pKVI318 carrying the L, P1, 2A, 3C in pTargetT Δ Tfi I, pKVI325 carrying the EMC IRES, P1, 2A, 3C, pKVI326 carrying the EMC IRES, P1, 2A, 3CD, and pKVI335 carrying the O1 (G) VP1, Asia 1 VP1 and O1 (G) 3C as one open

reading frame in pVCL1010 vector (Fig. 1). As control for transformation efficiency we transformed cells with pEGFP (Clontech) containing the EGFP gene. Two days later at least 50% of the cells were transformed. FMDV protein expression was tested by fixing and reacting cells with anti-VP1 antibodies that were prepared against the O1 (G) VP1 16 mer peptide. To stain the cells, FITC conjugated sheep anti-rabbit antibodies were reacted with the treated cells. As for the cells transformed with pKVI335 carrying Asia 1 VP1 we used guinea pig anti-Asia1 antibodies and CyTM5-conjugated affinity pure goat anti-guinea pig IgG F(ab)₂. All preparations were checked using fluorescent microscope using A-492nm E-520nm filter for FITC and A650 E670nm filter for Cy5. As can be seen all transformed cells were specifically stained (Figs. 3 and 4). Cells that were transformed with pKVI326 and pKVI335 were stained with FITC (Figs. 4A and 5A respectively) suggesting that both plasmids drove the expression of O1 (G) VP1. Cells transformed with pKVI335 carrying both O1 and Asia1 VP1 genes were stained also with Cy5 suggesting that in addition to O1 (G) VP1 Asia 1 VP1 was expressed as well (Fig. 4B). In Figure 5C we show the expression of both proteins in pKVI335 transformed BHK cells as they were doubled stained after reacting with anti-O1 (G) VP1 and anti-Asia1. Control cells transformed with the pVCL1010 vector did not stain (Fig. 4E). As positive control we show the staining of O1 (G) and Asia 1 infected pig kidney cells (Figs. 3B and 4D respectively).

All the data presented here suggested that all plasmids that were examined could drive the expression of viral proteins. To show viral protein processing, transfected cells extracts were used in Western analysis using the anti-O1 VP1 antibodies

described earlier (Fig. 5). It can be seen that a band similar in size to VP1 in FMDV infected cells (Fig. 5, lane 8) is present in extracts from pKVI335, 326, 324 and 318 (Fig. 5, lanes 2, 4-6 respectively). In the control BHK cells transfected with pVCL1010 the same protein band could not be detected (Fig. 5, lane 7). The results of both experiments strongly suggested that the viral proteins translated by all tested plasmids are correctly processed (Fig. 5).

4. DNA Vaccination

Pathogen free female BALB/c mice (6-8 weeks old) were used for vaccination in 6 mice/group (Table 1). The plasmids used for the experiment are, pKVI326 containing EMCV IRES, P1, 2A and 3CD sequences and as a control pTarget Δ Tfi I was used. DNA was introduced by Agene gun@ (BioRad, USA) in the abdomen of the mice after shaving. To each mouse we introduced 1 μ g of DNA that was coated on 1.65 micron gold particles. Twenty four hours and 48 hours later each of the mice was injected with 200 units cytokines in PBS+20mg/ml BSA as listed in Table 1. Two weeks later a booster injection was given and 24 hours later the same cytokines were injected, 14 days later, serum was taken and tested for the presence of anti-FMDV antibodies.

We tested for the presence of anti-FMDV antibodies by ELISA after coating with 16 mer O1 (G) VP1 peptide. We combined 100 μ l from each of the mice from each group for use in the analysis. As can be seen (Table 1) in two groups there is induction of anti-O1 (G) VP1, one group are mice that were administered IL-2. The second group is the one that no cytokines were administered. In all other groups there was no induction of anti-VP1 antibodies. Additional experiments are in progress to further enhance the

immune response to our vaccine candidates.

5. Legends to Figures

Fig.1. Schematic representation of FMDV expression plasmids. A. pTarget Δ Tfi. B. pPVI324. C. pKVI326. D. pKVI335.

Fig. 2. The entire nucleotide sequence of O1 (G) FMDV open reading frame.

Fig. 3. Expression of FMDV VP1 pKVI326 transfected BHK cells as detected by immunofluorescent technique. A. Detection of O1 (G) VP1 in pKVI326 transfected BHK cells. B. FMDV O1 (G) infected pig kidney cells. C. Uninfected BHK cells.

Fig. 4. Expression of O1 (G) and Asia 1 VP1 in pKVI335 transfected BHK cells. A. Detection of O1 (G) VP1 in pKVI335 transfected BHK cells. B detection of Asia 1 VP1 in pKVI335 transfected BHK cells. C. Double detection of O1 (G) and Asia 1 VP1 in pKVI335 BHK transfected cells. D. Detection of Asia 1 VP1 in Asia 1 infected pig kidney cells. E. Control BHK cells transfected with pVCL1010.

Fig. 5. Expression and processing of FMDV proteins in transfected cells as detected by Western analysis. Lane 1. BHK cell transfected with the vector pVCL1010. Lane 2, BHK cell transfected with the pKVI335. Lane 4. BHK cell transfected with the pKVI326. Lane 5. BHK cell transfected with the pKVI324. Lane 6. BHK cell transfected with the pKVI318. Lane 7. BHK cell. Lane 8. FMDV O1 (G) infected BHK cells.

Figure 1.

A.

B.

C.

D.

Fig. 1

Figure 2. Nucleotide sequence of O1(G) FMDV open reading frame

1	A TTG GGC CGA CGT CGC ATG CTC CCG GCC GCC ATG GCC GCG GGA TTC	46
47	ACT CTA GAC ATG AAT ACA ACT GAC TGT TTT ATC GCT CTG TTA ACC GCT	94
1	Met Asn Thr Thr Asp Cys Phe Ile Ala Leu Leu Thr Ala	13
95	TTC AGA GAG ATT AAG TCA CTG TTT CTT TCA CAA ACA CAA GGT AAA ATG	142
14	Phe Arg Glu Ile Lys Ser Leu Phe Leu Ser Gln Thr Gln Gly Lys Met	29
143	GAG TTC ACA CTT CAC AAC GGT GAA AAG AAG ACT TTT TAT TCT AGA CCC	190
30	Glu Phe Thr Leu His Asn Gly Glu Lys Lys Thr Phe Tyr Ser Arg Pro	45
191	AAC AAC CAC GAC AAC TGC TGG CTG AAC GCC ATC CTC CAG TTG TTC AGG	238
46	Asn Asn His Asp Asn Cys Trp Leu Asn Ala Ile Leu Gln Leu Phe Arg	61
239	TAC GTT GAT GAG CCT TTC TTT GAC TGG GTC TAT GAT TCA CCG GAA AAC	286
62	Tyr Val Asp Glu Pro Phe Phe Asp Trp Val Tyr Asp Ser Pro Glu Asn	77
287	CTT ACC CTT GAT GCC ATC AAA CAA TTG GAA GAC ATT ACT GGT CTT GAA	334
78	Leu Thr Leu Asp Ala Ile Lys Gln Leu Glu Asp Ile Thr Gly Leu Glu	93
335	CTG CAC GAG GGT GGG CCA CCC GCC CTC GTC ATT TGG AAC ATC AAA CAC	382
94	Leu His Glu Gly Gly Pro Pro Ala Leu Val Ile Trp Asn Ile Lys His	109
383	TTG CTC CAC ACC GGG ATC GGC ACT GCC TCA CGC CCT AGT GAG GTG TGC	430
110	Leu Leu His Thr Gly Ile Gly Thr Ala Ser Arg Pro Ser Glu Val Cys	125
431	ATG GTC GAC GGA ACG GAC ATG TGT TTG GCT GAC TTT CAC GCT GGC ATT	478
126	Met Val Asp Gly Thr Asp Met Cys Leu Ala Asp Phe His Ala Gly Ile	141
479	TTC CTG AAG GGA CAG GAA CAC GCC GTG TTC GCC TGC GTC ACC TCC AAC	526
142	Phe Leu Lys Gly Gln Glu His Ala Val Phe Ala Cys Val Thr Ser Asn	157
527	GGG TGG TAC GCG ATC GAT GAC GAG GAC TTC TAC CCT TGG ACG CCG GAC	574
158	Gly Trp Tyr Ala Ile Asp Asp Glu Asp Phe Tyr Pro Trp Thr Pro Asp	173
575	CCG TCC GAC GTC CTG GTA TTT GTC CCG TAC GAT CAA GAA CCG CTC AAC	622
174	Pro Ser Asp Val Leu Val Phe Val Pro Tyr Asp Gln Glu Pro Leu Asn	189
623	GGA GAA TGG AAA GCA AAA GTT CAG AAG CGA CTT AAG GGG GCC GGG CAA	670
190	Gly Glu Trp Lys Ala Lys Val Gln Lys Arg Leu Lys Gly Ala Gly Gln	205
671	TCC AGT CCG GCA ACC GGG TCA CAG AAC CAA TCA GGC AAC ACC GGG AGC	718
206	Ser Ser Pro Ala Thr Gly Ser Gln Asn Gln Ser Gly Asn Thr Gly Ser	221
719	ATC ATC AAT AAT TAC TAC ATG CAG CAG TAC CAA AAC TCC ATG GAC ACA	766
222	Ile Ile Asn Asn Tyr Tyr Met Gln Gln Tyr Gln Asn Ser Met Asp Thr	237
767	CAA CTT GGT GAC AAC GCT ATA AGC GGA GGC TCC AAT GAA GGG TCC ACG	814
238	Gln Leu Gly Asp Asn Ala Ile Ser Gly Gly Ser Asn Glu Gly Ser Thr	253
815	GAC ACA ACT TCC ACC CAC ACA AAT AAC ACA CAG AAC AAC GAC TGG TTC	862
254	Asp Thr Thr Ser Thr His Thr Asn Asn Thr Gln Asn Asn Asp Trp Phe	269
863	TCA AAG CTG GCC AGT TCC GCT TTT AGC GGA CTT TTC GGC GCT CTT CTC	910
270	Ser Lys Leu Ala Ser Ser Ala Phe Ser Gly Leu Phe Gly Ala Leu Leu	285
911	GCC GAC AAG AAA ACC GAG GAG ACC ACC CTC CTC GAG GAC CGC ATC CTC	958
286	Ala Asp Lys Lys Thr Glu Glu Thr Thr Leu Leu Glu Asp Arg Ile Leu	301
	VP2	
959	ACC ACC CGC AAC GGA CAC ACC ACC TCG ACA ACC CAT TCG AGT GTT GGA	1006
302	Thr Thr Arg Asn Gly His Thr Thr Ser Thr Thr His Ser Ser Val Gly	317
1007	GTC ACG TAC GGG TAC GCA ACA GCT GAG GAC TTT GTG AGT GGG CCT AAC	1054
318	Val Thr Tyr Gly Tyr Ala Thr Ala Glu Asp Phe Val Ser Gly Pro Asn	333
1055	ACA TCT GGC CTT GAA ACC AGA GTT GCT CAG GCA GAG CGG TTC TTC AAA	1102
334	Thr Ser Gly Leu Glu Thr Arg Val Ala Gln Ala Glu Arg Phe Phe Lys	349

Figure 2.

1103	ACC CAC CTG TTT GAC TGG GTC CCC AGT GAC CCG TTC GGA CGG TGC CAT	1150
350	Thr His Leu Phe Asp Trp Val Pro Ser Asp Pro Phe Gly Arg Cys His	365
1151	CTG CTG GAA CTC CCA ACT GAA CAC AAA GGT GTC TAC GGC AGC CTG ACT	1198
366	Leu Leu Glu Leu Pro Thr Glu His Lys Gly Val Tyr Gly Ser Leu Thr	381
1199	GAC TCG TAT GCA TAT ATG AGA AAT GGT TGG GAC GTT GAA GTC ACT GCA	1246
382	Asp Ser Tyr Ala Tyr Met Arg Asn Gly Trp Asp Val Glu Val Thr Ala	397
1247	GTG GGA AAT CAG TTT AAT GGA GGA TGC CTG CTG GTG GCC ATG GTG CCA	1294
398	Val Gly Asn Gln Phe Asn Gly Gly Cys Leu Leu Val Ala Met Val Pro	413
1295	GAG TTG TCC TTC AGC AAG AGA GAG TTA TAC CAA TTC ACG TTC TTT CCC	1342
414	Glu Leu Ser Phe Ser Lys Arg Glu Leu Tyr Gln Phe Thr Phe Phe Pro	429
1343	CAT CAG TTC ATC AAA CCT TCG AAC GAA ATG ACG GCA CAC ATC ACT GTG	1390
430	His Gln Phe Ile Lys Pro Ser Asn Glu Met Thr Ala His Ile Thr Val	445
1391	CCC TTT GTT GGG GTC AAC CGC TAC GAC CAG TAC AAG ACA CAC AAG CCT	1438
446	Pro Phe Val Gly Val Asn Arg Tyr Asp Gln Tyr Lys Thr His Lys Pro	461
1439	TGG ACC CTT GTG GTT ATG GTT GTA GCC CCT TTG ACC GTC AAC AAC GAA	1486
462	Trp Thr Leu Val Val Met Val Val Ala Pro Leu Thr Val Asn Asn Glu	477
1487	GGT GCC CCA CAA ATC AAG GTT TAC GCC AAC ATC GCC CCC ACC AGC GTG	1534
478	Gly Ala Pro Gln Ile Lys Val Tyr Ala Asn Ile Ala Pro Thr Ser Val	493
1535	CAC GTC GCA GGT GAG TTC CCT TCC AAA GAG GGG ATC TTT CCT GTG GCT	1582
494	His Val Ala Gly Glu Phe Pro Ser Lys Glu Gly Ile Phe Pro Val Ala	509
1583	TGT AGC GAT GGT TAC GGC GGT TTG GTG ACC ACG GAC CCG AAG ACG GCT	1630
510	Cys Ser Asp Gly Tyr Gly Gly Leu Val Thr Thr Asp Pro Lys Thr Ala	525
1631	GAC CCC GCC TAC GGG AAG GTG TTC AAC CCC CCT CGC AAC ATG TTG CCA	1678
526	Asp Pro Ala Tyr Gly Lys Val Phe Asn Pro Pro Arg Asn Met Leu Pro	541
1679	GGG CGG TTC ACC AAC TTC CTT GAT GTG GCT GAG GCG TGC CCA ACG TTT	1726
542	Gly Arg Phe Thr Asn Phe Leu Asp Val Ala Glu Ala Cys Pro Thr Phe	557
1727	ATG CGC TTC GAA GGT GGC GTA CCA TAC GTG ACC ACG AAG ACG GAT TCG	1774
558	Met Arg Phe Glu Gly Gly Val Pro Tyr Val Thr Thr Lys Thr Asp Ser	573
1775	GAC AGG GTG CTC GCT CAA TTT GAC TTG TCT TTG GCA GCA AAA CAC ATG	1822
574	Asp Arg Val Leu Ala Gln Phe Asp Leu Ser Leu Ala Ala Lys His Met	589
1823	TCA AAT ACC TTT CTG GCA GGT CTT GCC CAG TAC TAC GCG CAG TAC AGC	1870
590	Ser Asn Thr Phe Leu Ala Gly Leu Ala Gln Tyr Tyr Ala Gln Tyr Ser	605
1871	GGC ACT ATC AAC CTG CAT TTC ATG TTC ACA GGG CCC ACT GAC GCG AAA	1918
606	Gly Thr Ile Asn Leu His Phe Met Phe Thr Gly Pro Thr Asp Ala Lys	621
1919	GCG CGT TAC ATG ATT GCA TAT GCC CCT CCT GGC ATG GAG CCG CCC AAG	1966
622	Ala Arg Tyr Met Ile Ala Tyr Ala Pro Pro Gly Met Glu Pro Pro Lys	637
1967	ACA CCC GAG GCG GCT GCC CAC TGC ATT CAC GCT GAG TGG GAC ACA GGG	2014
638	Thr Pro Glu Ala Ala Ala His Cys Ile His Ala Glu Trp Asp Thr Gly	653
2015	TTG AAC TCA AAA TTC ACC TTC TCA ATC CCT TAC CTC TCA GCG GCT GAC	2062
654	Leu Asn Ser Lys Phe Thr Phe Ser Ile Pro Tyr Leu Ser Ala Ala Asp	669
2063	TAC GCG TAC ACC GCG TCC GAC ACT GCT GAG ACC ACA AAT GTA CAG GGA	2110
670	Tyr Ala Tyr Thr Ala Ser Asp Thr Ala Glu Thr Thr Asn Val Gln Gly	685
2111	TGG GTT TGC CTG TTT CAA ATA ACA CAC GGG AAA GCT GAC GGT GAC GCA	2158
686	Trp Val Cys Leu Phe Gln Ile Thr His Gly Lys Ala Asp Gly Asp Ala	701
2159	CTG GTC GTT CTG GCT AGC GCC GGT AAG GAC TTT GAG CTG CGT TTA CCA	2206
702	Leu Val Val Leu Ala Ser Ala Gly Lys Asp Phe Glu Leu Arg Leu Pro	717
2207	GTG GAT ACC CGC ACA GAG ACC ACC TCT GCG GGC GAG TCG GCT GAC CCC	2254
718	Val Asp Thr Arg Thr Glu Thr Thr Ser Ala Gly Glu Ser Ala Asp Pro	733

Figure 2.

2255	GTG ACC GCC ACC GTT GAG AAC TAC GGT GGT GAG ACA CAG GTC CGG AGG	2302
734	Val Thr Ala Thr Val Glu Asn Tyr Gly Gly Glu Thr Gln Val Arg Arg	749
2303	CGC CAA CAC ACG GACGTC TCT TTC ATA TTG GAC AGA TTT GTG AAA GTA	2350
750	Arg Gln His Thr Asp Val Ser Phe Ile Leu Asp Arg Phe Val Lys Val	765
2351	ACA CCA AAA GAT CAA ATT AAC GTG TTG GAC CTG ATG CAA ACC CCT GCT	2398
766	Thr Pro Lys Asp Gln Ile Asn Val Leu Asp Leu Met Gln Thr Pro Ala	781
2399	CAC ACT CTG GTG GGA GCA CTC CTT CGC GCT GCC ACC TAC TAC TTT GCA	2446
782	His Thr Leu Val Gly Ala Leu Leu Arg Ala Ala Thr Tyr Tyr Phe Ala	797
2447	CAC TTC CAA CTC GCA CTC AAG CAC GAG GGG AAC CTC TCT TGG GTC CCG	2494
798	Asp Leu Glu Val Ala Val Lys His Glu Gly Asn Leu Ser Trp Val Pro	813
2495	AAC GGG GCG CCC GAG AAA GCG TTG GAC AAC ACC ACC AAC CCA ACA GCT	2542
814	Asn Gly Ala Pro Glu Lys Ala Leu Asp Asn Thr Thr Asn Pro Thr Ala	829
2543	TAC CAC AAG GCC ACA CTC ACC CGA CTT GCA CTG CCT TAC ACG GCA CCA	2590
830	Tyr His Lys Ala Thr Leu Thr Arg Leu Ala Leu Pro Tyr Thr Ala Pro	845
2591	CAC CGC GTG TTG GCT ACT GTG TAC AAC GGG AAC TGT AGG TAT GGT GAG	2638
846	His Arg Val Leu Ala Thr Val Tyr Asn Gly Asn Cys Arg Tyr Gly Glu	861
2639	GGA CAC GTG GCC AAT GTG AGA GGT GAC CTG CAA GTG CTG GCC CGA AAA	2686
862	Gly His Val Ala Asn Val Arg Gly Asp Leu Gln Val Leu Ala Arg Lys	877
2687	GCG GCG AGA CCG CTG CCC ACC TCC TTC AAC TAC GGT GCC ATC AAA GCT	2734
878	Ala Ala Arg Pro Leu Pro Thr Ser Phe Asn Tyr Gly Ala Ile Lys Ala	893
2735	ACT CGG GTG ACC GAG CTG CTT TAC CGC ATG AAG AGG GCT GAG ACA TAC	2782
894	Thr Arg Val Thr Glu Leu Leu Tyr Arg Met Lys Arg Ala Glu Thr Tyr	909
2783	TGT CCC CGG CCT CTA TTG GCC ATT CAC CCA AGT GAA GCT AGA CAC AAG	2830
910	Cys Pro Arg Pro Leu Leu Ala Ile His Pro Ser Glu Ala Arg His Lys	925
2831	CAC AAA ATA GTG GCA CCT GTG AAA CAG CTT CTG AAC TTT GAC CTG CTC	2878
926	His Lys Ile Val Ala Pro Val Lys Gln Leu Leu Asn Phe Asp Leu Leu	941
2879	AAG TTG GCA GGG GAC ATG GAG TCC AAC CCT GGG CCC TTC TCC TTC TCC	2926
942	Lys Leu Ala Gly Asp Met Glu Ser Asn Pro Gly Pro Phe Ser Phe Ser	957
2927	GAC GTT AGG TCA AAT TTC ACC AAG TTG GTG GAC ATC ATC AAC CAG ATG	2974
958	Asp Val Arg Ser Asn Phe Thr Lys Leu Val Asp Ile Ile Asn Gln Met	973
2975	CAG GAG GAC ATG TCA ACA AAA CAC GGA CCT GAC TTT AAC CGG TTG GTG	3022
974	Gln Glu Asp Met Ser Thr Lys His Gly Pro Asp Phe Asn Arg Leu Val	989
3023	ACC GCA TTT GAG GAA TTG GCC ACT GGA GTG AAG GCT ATC AGG ACC GGT	3070
990	Thr Ala Phe Glu Glu Leu Ala Thr Gly Val Lys Ala Ile Arg Thr Gly	1005
3071	CTG GAC GAG GCC AAG CCC TGG TAC AAA CTC ATT AAG CTC TTA AGC GCC	3118
1006	Leu Asp Glu Ala Lys Pro Trp Tyr Lys Leu Ile Lys Leu Leu Ser Ala	1021
3119	CTG TCG TGC ATG GCC GCT GTA GCA GCA CGG TCA AAG GAC CCA GTC CTT	3166
1022	Leu Ser Cys Met Ala Ala Val Ala Ala Arg Ser Lys Asp Pro Val Leu	1037
3167	GTG GCT ATC ATG CTG GCT GAC ACC GGT CTC GAG ATT CTG GAC AGC ACA	3214
1038	Val Ala Ile Met Leu Ala Asp Thr Gly Leu Glu Ile Leu Asp Ser Thr	1053
3215	TTT GTC GTG AAG AAG ATC TCG GAC TCA CTT TCC AGT CTC TTC CAC GTG	3262
1054	Phe Val Val Lys Lys Ile Ser Asp Ser Leu Ser Ser Leu Phe His Val	1069
3263	CCG GCC CCC GTC TTC AGT TTC GGA GCC CCG ATA CTG CTG GCT GGG TTG	3310
1070	Pro Ala Pro Val Phe Ser Phe Gly Ala Pro Ile Leu Leu Ala Gly Leu	1085
3311	GTC AAA GTC GCC TCG AGT TTC TTC CGG TCA ACA CCC GAA GAC CTT GAG	3358
1086	Val Lys Val Ala Ser Ser Phe Phe Arg Ser Thr Pro Glu Asp Leu Glu	1101
3359	AGA GCA GAG AAA CAG CTC AAA GCA CGT GAC ATC AAC GAC ATC TTC GCC	3406
1102	Arg Ala Glu Lys Gln Leu Lys Ala Arg Asp Ile Asn Asp Ile Phe Ala	1117

Figure 2.

3407	ATT CTC AAG AAT GGC GAG TGG CTG GTC AAA CTG ATT CTT GCC ATC CGC	3454
1118	Ile Leu Lys Asn Gly Glu Trp Leu Val Lys Leu Ile Leu Ala Ile Arg	1133
3455	GAC TGG ATC AAG GCA TGG ATC GCC TCA GAA GAG AAG TTT GTC ACT ATG	3502
1134	Asp Trp Ile Lys Ala Trp Ile Ala Ser Glu Glu Lys Phe Val Thr Met	1149
3503	ACA GAT TTG GCG CCG GGC ATC CTT GAG AAA CAG CGG GAC TTG CAC GAC	3550
1150	Thr Asp Leu Ala Pro Gly Ile Leu Glu Lys Gln Arg Asp Leu His Asp	1165
3551	CCC AGC AAA TAC AAG GAC GCC AAG GAA TGG CTC GAC AAC GCG CGC CAA	3598
1166	Pro Ser Lys Tyr Lys Asp Ala Lys Glu Trp Leu Asp Asn Ala Arg Gln	1181
3599	GCG TGT TTG AAG AGC GGG AAC GTC CAC ATT GCC AAC CTG TGC AAA GTC	3646
1182	Ala Cys Leu Lys Ser Gly Asn Val His Ile Ala Asn Leu Cys Lys Val	1197
3647	GTC ACC CCA GCA CCC AGC AGG TCG AGA CCC GAA CCT GTG GTC GTT TGC	3694
1198	Val Thr Pro Ala Pro Ser Arg Ser Arg Pro Glu Pro Val Val Val Cys	1213
3695	CTC CGT GGC AAA TCC GGC CAG GGA AAG AGC TTC CTT GCG AAC GTG CTC	3742
1214	Leu Arg Gly Lys Ser Gly Gln Gly Lys Ser Phe Leu Ala Asn Val Leu	1229
3743	GCA CAA GCA ATT TCC ACA CAC TTC ACT GGC AGA ACC GAT TCA GTT TGG	3790
1230	Ala Gln Ala Ile Ser Thr His Phe Thr Gly Arg Thr Asp Ser Val Trp	1245
3791	TAC TGT CCA CCT GAC CCT GAC CAC TTC GAC GGT TAC AAC CAG CAG ACC	3838
1246	Tyr Cys Pro Pro Asp Pro Asp His Phe Asp Gly Tyr Asn Gln Gln Thr	1261
3839	GTT GTA CTG ATG GAT GAT TTG GGC CAC AAC CCT GAC GGC AAG GAC TTT	3886
1262	Val Val Leu Met Asp Asp Leu Gly His Asn Pro Asp Gly Lys Asp Phe	1277
3887	AAA TAC TTT GCC CAC ATG GTC TCA ACT ACG GGG TTC ATC CCG CCC ATG	3934
1278	Lys Tyr Phe Ala His Met Val Ser Thr Thr Gly Phe Ile Pro Pro Met	1293
3935	GCT TCA CTC GAC GAC AAG GGA AAA CCT TTT AAC AGC AAA GTC ATC ATC	3982
1294	Ala Ser Leu Asp Asp Lys Gly Lys Pro Phe Asn Ser Lys Val Ile Ile	1309
3983	GCC ACC ACC AAC CTG TAC TCG GGT TTC ACC CCG AGG ACC ATG GTG TGC	4030
1310	Ala Thr Thr Asn Leu Tyr Ser Gly Phe Thr Pro Arg Thr Met Val Cys	1325
4031	CCT GAC GCG CTG AAC CGC AGG TTT CAC TTT GAC ATT GAC GTG AGT GCC	4078
1326	Pro Asp Ala Leu Asn Arg Arg Phe His Phe Asp Ile Asp Val Ser Ala	1341
4079	AAG GAT GGG TAC AAA ATT AAT AAC AAA TTG GAC ATA ACC AAA GCT CTT	4126
1342	Lys Asp Gly Tyr Lys Ile Asn Asn Lys Leu Asp Ile Thr Lys Ala Leu	1357
4127	GAG GAC ACC CAC ACC AAC CCA GTG GCA ATG TTT CAA TAT GAC TGT GCC	4174
1358	Glu Asp Thr His Thr Asn Pro Val Ala Met Phe Gln Tyr Asp Cys Ala	1373
4175	CTT CTC AAC GGC ATG GCC GTT GAG ATG AAG AGA ATG CAA CAG GAT GTG	4222
1374	Leu Leu Asn Gly Met Ala Val Glu Met Lys Arg Met Gln Gln Asp Val	1389
4223	TTC AAG CCC CAA CCA CCT CTT CAA AAC GTG TAC CAA CTT GTT CAA GAG	4270
1390	Phe Lys Pro Gln Pro Pro Leu Gln Asn Val Tyr Gln Leu Val Gln Glu	1405
4271	GTG ATT GAT CGG GTT GAG CTC CAC GAG AAA GTG TCG AGC CAC CCA ATC	4318
1406	Val Ile Asp Arg Val Glu Leu His Glu Lys Val Ser Ser His Pro Ile	1421
4319	TTT AAA CTG ATC TCA ATT CCT TCC CAA AAA TCC GTG CTG TAC TTT CTT	4366
1422	Phe Lys Leu Ile Ser Ile Pro Ser Gln Lys Ser Val Leu Tyr Phe Leu	1437
4367	ATT GAG AAG GGC CAA CAC GAG GCT GCA ATT GAA TTC TTT GAG GGA ATG	4414
1438	Ile Glu Lys Gly Gln His Glu Ala Ala Ile Glu Phe Phe Glu Gly Met	1453
4415	GTG CAT GAC TCC ATC AAG GAG GAG CTC CGG CCT CTT ATC CAA CAG ACC	4462
1454	Val His Asp Ser Ile Lys Glu Glu Leu Arg Pro Leu Ile Gln Gln Thr	1469
4463	TCA TTT GTG AAA CGC GCC TTT AAG CGC CTG AAG GAA AAC TTT GAG ATT	4510
1470	Ser Phe Val Lys Arg Ala Phe Lys Arg Leu Lys Glu Asn Phe Glu Ile	1485
4511	GTT GCC CTG TGT TTG ACT CTT TTG GCA AAC ATA GTG ATC ATG ATC CGC	4558
1486	Val Ala Leu Cys Leu Thr Leu Leu Ala Asn Ile Val Ile Met Ile Arg	1501

Figure 2.

4559	GAG ACT CGC AGG AGA CAG CAG ATG GTG GAT GAT GCA ATG AAT GAG TAC	4606
1502	Glu Thr Arg Arg Arg Gln Gln Met Val Asp Asp Ala Met Asn Glu Tyr	1517
4607	ATC GAG AAA GCA AAC ATC ACC ACA GAT GAC AAG ACT CTT GAC GAG TGT	4654
1518	Ile Glu Lys Ala Asn Ile Thr Thr Asp Asp Lys Thr Leu Asp Glu Cys	1533
4655	GAA AAG AAC CCT TTG GAG ACT AGC GGT GCC AGT ACG GTT GGC TTC AGA	4702
1534	Glu Lys Asn Pro Leu Glu Thr Ser Gly Ala Ser Thr Val Gly Phe Arg	1549
4703	GAG AGA ACT CCT CCG GGG CAC AAG GTG AGT GAT GAC GTG AAC TCC GAG	4750
1550	Glu Arg Thr Pro Pro Gly His Lys Val Ser Asp Asp Val Asn Ser Glu	1565
4751	CCC ACC AAA CCC GTG GAA GAA CAA CCA CAA GCA GAA GGA CCC TAC GCC	4798
1566	Pro Thr Lys Pro Val Glu Glu Gln Pro Gln Ala Glu Gly Pro Tyr Ala	1581
4799	GGA CCA CTC GAG CGT CAG AAG CCT CTG AAA GTG AAA GCC AAG CTG CCA	4846
1582	Gly Pro Leu Glu Arg Gln Lys Pro Leu Lys Val Lys Ala Lys Leu Pro	1597
4847	CAG CAG GAG GGA CCC TAC GCT GGC CCG ATG GAG AGA CAG AAA CCA CTG	4894
1598	Gln Gln Glu Gly Pro Tyr Ala Gly Pro Met Glu Arg Gln Lys Pro Leu	1613
4895	AAA GTG AAA GCA AAA GCC CCG GTC GTA AAG GAA GGA CCT TAC GAG GGA	4942
1614	Lys Val Lys Ala Lys Ala Pro Val Val Lys Glu Gly Pro Tyr Glu Gly	1629
4943	CCG GTG AAG AAA CCT GTC GCT TTG AAA GTG AAA GCC AAG AAC TTG ATT	4990
1630	Pro Val Lys Lys Pro Val Ala Leu Lys Val Lys Ala Lys Asn Leu Ile	1645
4991	GTC ACT GAG AGT GGT GCC CCC CCG ACC GAC TTG CAA AAG ATG GTC ATG	5038
1646	Val Thr Glu Ser Gly Ala Pro Pro Thr Asp Leu Gln Lys Met Val Met	1661
5039	GGC AAC ACC AAG CCC GTC GAA CTC ATC CTC GAC GGG AAG ACA GTA GCC	5086
1662	Gly Asn Thr Lys Pro Val Glu Leu Ile Leu Asp Gly Lys Thr Val Ala	1677
5087	ATC TGT TGT GCT ACT GGA GTG TTT GGC ACT GCC TAC CTC GTG CCT CGT	5134
1678	Ile Cys Cys Ala Thr Gly Val Phe Gly Thr Ala Tyr Leu Val Pro Arg	1693
5135	CAC CTT TTC GCA GAA AAG TAC GAC AAG ATC ATG CTG GAC GGC AGA GCC	5182
1694	His Leu Phe Ala Glu Lys Tyr Asp Lys Ile Met Leu Asp Gly Arg Ala	1709
5183	ATG ACA GAC AGT GAC TAC AGA GTG TTT GAG TTT GAG ATT AAA GTA AAA	5230
1710	Met Thr Asp Ser Asp Tyr Arg Val Phe Glu Phe Glu Ile Lys Val Lys	1725
5231	GGG CAG GAC ATG CTC TCA GAC GCC GCA CTC ATG GTG CTC CAC CGC GGG	5278
1726	Gly Gln Asp Met Leu Ser Asp Ala Ala Leu Met Val Leu His Arg Gly	1741
5279	AAC CGC GTG AGA GAC ATC ACG AAA CAC TTT CGT GAC ACA GCA AAA ATG	5326
1742	Asn Arg Val Arg Asp Ile Thr Lys His Phe Arg Asp Thr Ala Lys Met	1757
5327	AAG AAA GGC ACC CCC GTC GTT GGT GTG ATC AAC AAT GCC GAC GTT GGG	5374
1758	Lys Lys Gly Thr Pro Val Val Gly Val Ile Asn Asn Ala Asp Val Gly	1773
5375	AGA TTG ATT TTC TCT GGT GAG GCC CTC ACC TAC AAG GAC ATT GTA GTG	5422
1774	Arg Leu Ile Phe Ser Gly Glu Ala Leu Thr Tyr Lys Asp Ile Val Val	1789
5423	TGC ATG GAT GGC GAC ACC ATG CCT GGC CTC TTT GCC TAC AGA GCA GCC	5470
1790	Cys Met Asp Gly Asp Thr Met Pro Gly Leu Phe Ala Tyr Arg Ala Ala	1805
5471	ACC AAG GCT GGT TAC TGC GGA GGA GCC GTT CTT GCT AAG GAC GGA GCT	5518
1806	Thr Lys Ala Gly Tyr Cys Gly Gly Ala Val Leu Ala Lys Asp Gly Ala	1821
5519	GAC ACA TTC ATC GTT GGC ACT CAC TCT GCG GGT GGC AAT GGA GTT GGA	5566
1822	Asp Thr Phe Ile Val Gly Thr His Ser Ala Gly Gly Asn Gly Val Gly	1837
5567	TAT TGC TCA TGC GTT TCC AGG TCC ATG CTT TTG AAA ATG AAG GCA CAC	5614
1838	Tyr Cys Ser Cys Val Ser Arg Ser Met Leu Leu Lys Met Lys Ala His	1853
5615	ATT GAC CCC GAA CCA CAC CAC GAG GGG TTG ATT GTG GAC ACC AGA GAT	5662
1854	Ile Asp Pro Glu Pro His His Glu Gly Leu Ile Val Asp Thr Arg Asp	1869
5663	GTG GAA GAG CGC GTA CAC GTT ATG CGC AAA ACC AAG CTT GCA CCC ACC	5710
1870	Val Glu Glu Arg Val His Val Met Arg Lys Thr Lys Leu Ala Pro Thr	1885

Figure 2.

5711	GTG	GCA	CAC	GGC	GTG	TTT	AAT	CCT	GAA	TTT	GGC	CCC	GCC	GCC	TTG	TCC	5758
1886	Val	Ala	His	Gly	Val	Phe	Asn	Pro	Glu	Phe	Gly	Pro	Ala	Ala	Leu	Ser	1901
5759	AAC	AAG	GAC	CCG	CGG	TTG	AAC	GAA	GGA	GTT	GTC	CTT	GAC	GAG	GTC	ATC	5806
1902	Asn	Lys	Asp	Pro	Arg	Leu	Asn	Glu	Gly	Val	Val	Leu	Asp	Glu	Val	Ile	1917
5807	TTC	TCC	AAA	CAC	AAG	GAA	GAC	ACC	AAG	ATG	TCC	GGG	GAG	GAC	AAA	GCG	5854
1918	Phe	Ser	Lys	His	Lys	Glu	Asp	Thr	Lys	Met	Ser	Gly	Glu	Asp	Lys	Ala	1933
5855	CTG	TTT	CGC	CGC	TGT	GCT	GCT	GAC	TAC	GCG	TCA	CGC	CTG	CAT	AGT	GTG	5902
1934	Leu	Phe	Arg	Arg	Cys	Ala	Ala	Asp	Tyr	Ala	Ser	Arg	Leu	His	Ser	Val	1949
5903	TTG	GGT	ACG	GCA	AAT	GCC	CCA	TTA	AGC	ATC	TAC	GAG	GCA	ATC	AAG	GGC	5950
1950	Leu	Gly	Thr	Ala	Asn	Ala	Pro	Leu	Ser	Ile	Tyr	Glu	Ala	Ile	Lys	Gly	1965
5951	GTC	GAC	GGA	CTT	GAC	GCC	ATG	GAA	CCA	GAC	ACC	GCG	CCT	GGC	CTT	CCC	5998
1966	Val	Asp	Gly	Leu	Asp	Ala	Met	Glu	Pro	Asp	Thr	Ala	Pro	Gly	Leu	Pro	1981
5999	TGG	GCT	CTC	CAG	GGG	AAA	CGC	CGC	GGT	GCG	CTC	ATC	GAC	TTC	GAG	AAC	6046
1982	Trp	Ala	Leu	Gln	Gly	Lys	Arg	Arg	Gly	Ala	Leu	Ile	Asp	Phe	Glu	Asn	1997
6047	GGC	ACG	GTC	GGA	CCC	GAG	GTT	GAA	GCT	GCC	CTG	AAG	CTC	ATG	GAG	AAA	6094
1998	Gly	Thr	Val	Gly	Pro	Glu	Val	Glu	Ala	Ala	Leu	Lys	Leu	Met	Glu	Lys	2013
6095	AGA	GAA	TAC	AAA	TTT	GCA	TGC	CAG	ACT	TTC	CTG	AAG	GAC	GAG	ATC	CGC	6142
2014	Arg	Glu	Tyr	Lys	Phe	Ala	Cys	Gln	Thr	Phe	Leu	Lys	Asp	Glu	Ile	Arg	2029
6143	CCG	ATG	GAG	AAA	GTA	CGT	GCC	GGC	AAG	ACT	CGC	ATT	GTC	GAT	GTC	CTG	6190
2030	Pro	Met	Glu	Lys	Val	Arg	Ala	Gly	Lys	Thr	Arg	Ile	Val	Asp	Val	Lcu	2045
6191	CCA	GTT	GAA	CAC	ATT	CTT	TAC	ACC	AGG	ATG	ATG	ATT	GGC	AGA	TTT	TGT	6238
2046	Pro	Val	Glu	His	Ile	Leu	Tyr	Thr	Arg	Met	Met	Ile	Gly	Arg	Phe	Cys	2061
6239	GCT	CAA	ATG	CAC	ACG	AAC	AAC	GGC	CCG	CAG	ATT	GGT	ACA	GCG	GTC	GGT	6286
2062	Ala	Gln	Met	His	Thr	Asn	Asn	Gly	Pro	Gln	Ile	Gly	Thr	Ala	Val	Gly	2077
6287	TGT	AAC	CCT	GAT	GTT	GAT	TGG	CAG	AGA	TTT	GGA	ACA	CAC	TTC	GCC	CAA	6334
2078	Cys	Asn	Pro	Asp	Val	Asp	Trp	Gln	Arg	Phe	Gly	Thr	His	Phe	Ala	Gln	2093
6335	TAT	AGA	AAC	GTA	TGG	GAT	GTG	GAC	TAT	TCG	GCC	TTT	GAC	GCT	AAC	CAT	6382
2094	Tyr	Arg	Asn	Val	Trp	Asp	Val	Asp	Tyr	Ser	Ala	Phe	Asp	Ala	Asn	His	2109
6383	TGC	AGT	GAC	GCC	ATG	AAC	ATC	ATG	TTC	GAG	GAG	GTG	TTC	CGC	ACG	GAG	6430
2110	Cys	Ser	Asp	Ala	Met	Asn	Ile	Met	Phe	Glu	Glu	Val	Phe	Arg	Thr	Glu	2125
6431	TTC	GGT	TTC	CAC	CCA	AAC	GCT	GAG	TGG	ATC	CTG	AAG	ACT	CTT	GTG	AAC	6478
2126	Phe	Gly	Phe	His	Pro	Asn	Ala	Glu	Trp	Ile	Leu	Lys	Thr	Leu	Val	Asn	2141
6479	ACG	GAG	CAC	GCG	TAC	GAG	AAC	AAA	CGC	ATC	ACT	GTT	GAG	GGC	GGG	ATG	6526
2142	Thr	Glu	His	Ala	Tyr	Glu	Asn	Lys	Arg	Ile	Thr	Val	Glu	Gly	Gly	Met	2157
6527	CCG	TCT	GGT	TGT	TCC	GCA	ACA	AGC	ATC	ATC	AAC	ACA	ATT	TTG	AAC	AAC	6574
2158	Pro	Ser	Gly	Cys	Ser	Ala	Thr	Ser	Ile	Ile	Asn	Thr	Ile	Leu	Asn	Asn	2173
6575	ATT	TAC	GTG	CTT	TAC	GCT	CTG	CGT	AGA	CAC	TAT	GAG	GGA	GTT	GAG	CTG	6622
2174	Ile	Tyr	Val	Leu	Tyr	Ala	Leu	Arg	Arg	His	Tyr	Glu	Gly	Val	Glu	Leu	2189
6623	GAC	ACG	TAC	ACC	ATG	ATC	TCC	TAC	GAG	GAT	GAC	ATC	GTG	GTT	GCA	AGT	6670
2190	Asp	Thr	Tyr	Thr	Met	Ile	Ser	Tyr	Glu	Asp	Asp	Ile	Val	Val	Ala	Ser	2205
6671	GAT	AAC	GAT	CTG	GAC	TTT	GAG	GCT	CTT	AAG	CCC	CAC	TTC	AAA	TCT	CTT	6718
2206	Asp	Asn	Asp	Leu	Asp	Phe	Glu	Ala	Leu	Lys	Pro	His	Phe	Lys	Ser	Leu	2221
6719	GGC	CAA	ACC	ATC	ACT	CCA	GCT	GAC	AAA	AGC	GAC	AAA	GGT	TTT	GTT	CTT	6766
2222	Gly	Gln	Thr	Ile	Thr	Pro	Ala	Asp	Lys	Ser	Asp	Lys	Gly	Phe	Val	Leu	2237
6767	GGT	CAC	TCC	ATT	ACC	GAT	GTC	ACT	TTC	CTC	AAA	AGA	CAC	TTC	CAC	ATG	6814
2238	Gly	His	Ser	Ile	Thr	Asp	Val	Thr	Phe	Leu	Lys	Arg	His	Phe	His	Met	2253
6815	GAC	TAT	GGA	ACT	GGG	TTT	TAC	AAA	CCT	GTG	ATG	GCC	TCG	AAG	ACC	CTC	6862
2254	Asp	Tyr	Gly	Thr	Gly	Phe	Tyr	Lys	Pro	Val	Met	Ala	Ser	Lys	Thr	Leu	2269

Figure 2.

6863	GAG GCC ATC CTC TCC TTT GCA CGC CGT GGG ACC ATA CAG GAG AAG TTG	6910
2270	Glu Ala Ile Leu Ser Phe Ala Arg Arg Gly Thr Ile Gln Glu Lys Leu	2285
6911	ATC TCC GTG GCA GGA CTC GCC GTC CAC TCA GGA CCT GAC GAG TAC CGG	6958
2286	Ile Ser Val Ala Gly Leu Ala Val His Ser Gly Pro Asp Glu Tyr Arg	2301
6959	CGT CTC TTT GAG CCT TTT CAA GGC CTC TTT GAG ATT CCA AGC TAC AGA	7006
2302	Arg Leu Phe Glu Pro Phe Gln Gly Leu Phe Glu Ile Pro Ser Tyr Arg	2317
7007	TCA CTT TAC CTG CGT TGG GTG AAC GCC GTG TGC GGT GAC GCG AAT T	7052
2318	Ser Leu Tyr Leu Arg Trp Val Asn Ala Val Cys Gly Asp Ala Asn	2332

Fig. 3

Fig. 4

Figure 5.

Table 1.

Anti-16mer O1 (G) VP1 antibodies induction in DNA vaccinated mice. Each group consists of serums from 6 mice equally mixed.

Group	DNA	Interleukin	OD ₄₁₀ at 1:81 dilution	OD(sample)- OD(7)	<u>OD(sample)</u> OD(6)
1	pKVI326	IL-2	0.092	0.042	2.2
2	pKVI326	IL-2,4,5	0.052	0.002	0.1
3	pKVI326	IL-4,5,6	0.059	0.009	0.47
4	pKVI326	IL-12	0.065	0.015	0.79
5	pKVI326	None	0.109	0.059	3.1
6	pTargerT ΔTI I	None	0.069	0.019	1
7	None	None	0.05	0	

Evaluation of the research achievement.

The U.S. lab constructed DNA vectors containing the P1 and 3C regions of an FMDV laboratory strain, serotype A12, and demonstrated that these plasmids induced an FMDV-specific immune response in mice when inoculated by gene gun but not by the IM route. However, an active 3C coding region was essential for induction of a neutralizing antibody response. The Israeli lab made similar constructs, but used a field strain, O1 Geshur, currently present in Israel. It was necessary for the Israeli lab to clone and sequence the genome of this field isolate, since only limited information was previously available. An initial study in mice demonstrated induction of an antibody response with an O1(G) cDNA containing plasmid in the presence or absence of IL-2 protein.

To enhance the immune response after IM inoculation the U.S. lab constructed plasmids containing the murine IL2 and GM-CSF genes and successfully boosted the antibody response to the FMDV serotype A12 containing plasmids, but not the neutralizing antibody response. Preliminary results in swine suggest that numerous inoculations are required to induce an antibody response with our serotype A12 containing plasmids.

The generation of recombinant adenoviruses allowed us to examine a completely different mechanism of antigen delivery. Delivery of antigens via a recombinant virus is significantly more efficient than transfection with DNA. Furthermore, adenoviruses are respiratory pathogens and thus infection of animals with a recombinant adenovirus resembles the natural route of FMDV infection perhaps resulting in the induction of important immune mechanisms. Our results with the replication-deficient Ad5-P12X3XWT (serotype A12) support the above comments. In potency and efficacy studies in swine the

Ad5-P12X3CWT induced a significant neutralizing antibody response and protected or significantly reduced the disease severity of more than 80% of the animals vaccinated.

Description of the cooperation.

Dr. Yehuda Stram the coinvestigator from the Kimron Institute visited Plum Island in the fall of 1995 and discussed the project with the U.S. investigators, Drs. Grubman and Mason. Discussions via fax continued throughout 1995 and 1996 between Drs. Stram and Grubman. In August, 1996 Dr. Grubman visited the Kimron Institute and discussed the status of the research with Dr. Stram, Ms. Luna Benveniste (a graduate student funded by this BARD grant), and the Israeli coinvestigator Dr. Hagai Yadin. Dr. Stram and Ms. Benveniste visited Plum Island in September, 1996 and continued discussions of the project with the U.S. investigators Drs. Grubman and Mason as well as Dr. Jarasvech Chinsangaram the Research Associate recruited by the U.S. with the BARD funds. In addition, Drs. Stram and Grubman have been in contact by e-mail, fax, and telephone over the three year period of the BARD.

Conclusions

We have demonstrated that a vaccine approach using an FMDV cDNA construct containing the capsid and wild-type 3C proteinase coding regions as an immunogen is a viable strategy. Our results indicate that the use of a replication-deficient adenovirus vector is a more efficient mechanism of delivery of the FMDV genes to susceptible animals than DNA vaccination. In efficacy studies involving 26 swine, 46% of the Ad5-P12X3CWT-

inoculated animals were completely protected and the severity of the disease was significantly reduced in 35% of the animals, ie, a lesion score of 2 or less. Thus far, the best vaccine regimen is either two low doses of virus, ie, 62.5% complete protection (5 of 8 animals), or a low followed by a high dose, ie, 67% complete protection (2 of 3 animals).

Certainly improvements in vaccine formulation are required to elicit higher antibody titers and otherwise boost the host immune response. Based on our work in mice, we would expect that IL-2 and GM-CSF would perform this function. We have recently obtained the genes for porcine IL-2 and GM-CSF and plan to produce replication-deficient Ad5-P12X3CWT viruses containing these genes. Similar experiments are planned with bovine cytokines.

There are numerous benefits to agriculture in both the U.S. and Israel based on the development of our FMD capsid vaccine approach. These advantages include 1) no infectious FMDV required or produced, 2) no tissue culture passage of FMDV required and thus no selection of antigenic variants, 3) no large scale biocontainment facilities needed for production, 4) the possibility of producing this type of vaccine on the U.S. mainland, 5) the ability to easily distinguish vaccinated from infected or convalescent animals using current diagnostic technology, since the coding region for the viral 3D RNA polymerase, ie, also termed virus-infection associated antigen, VIAA, is not present in our cDNA constructs, and 6) in the event of an FMDV outbreak it may not be necessary to slaughter vaccinated animals as USDA policy now requires with the current inactivated vaccine.

List of Publications

1. Chinsangaram, J., Beard, C., Mason, P.W., Zellner, M.K., Ward, G., and Grubman, M.J. Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *J. Virol.* 72:4454-4457, 1998.
2. Beard, C., Ward, G., Rieder, E., Chinsangaram, J., Grubman, M. J., and Mason, P.W. Development of DNA vaccines for foot-and-mouth disease, evaluation of vaccines encoding replicating and nonreplicating nucleic acids in swine. *J. Biotechnology*. In press. 1999.
3. Mayr, G.A., Chinsangaram, J., and Grubman, M.J. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. Submitted *Virology*.