A Bicistronic DNA Vaccine Containing Gene of FMDV and Bovine IFN-α Can Prime Humoral and Cellular Immune Responses of Guinea Pigs

Huichen GUO, Shiqi SUN, Jiangtao MA, Zaixin LIU*, Xiangtao LIU, Qingge XIE

State Key Laboratory of Veterinary Etiological Biology and Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Xujiaping 1, Lanzhou, Gansu, 730046, The People’s Republic of CHINA

Received: 04.10.2006

Abstract: The study was conducted to evaluate the immunogenicity and protective efficacy of a DNA vaccine against foot-and-mouth disease virus (FMDV). Genes encoding the P1, 2A, 3C of FMDV O/China99 and bovine IFN-α were cloned into pcDNA3.1 (+) expression vector under CMV promoter and FMDV IRES control, respectively. The recombinant plasmids were administered to guinea pigs by intramuscular injection with mono- or bicistronic expression plasmids and aurintricarboxylic acid (ATA). After 2 sequential vaccinations with plasmid DNA, all of guinea pigs were challenged with 100 ID50 FMDV O/China99. Anti-FMDV antibodies were detected by ELISA and neutralization assay, and the phenotyping of T-cell subpopulation in the peripheral blood were performed by flow cytometry. Vaccination of guinea pigs with mono- or bicistronic expression plasmids induced humoral and cellular immune responses to FMDV antigens. In the challenge test, a part of the animals were protected against the challenge of a virulent virus. This study demonstrates that the delivery of FMDV antigens via bicistronic vectors is feasible. Further experimentation with bicistronic delivery systems is required for the optimization and refinement of DNA vaccines to effectively prime protective immune responses against foot-and-mouth disease (FMD).

Key Words: Foot-and-mouth disease virus, DNA vaccine, aurintricarboxylic acid, IFN-α, Bicistron expression vector

Introduction

FMDV is a picornavirus that affects artiodactyls, especially cattle and swine. The virion consists of a single-strand, positive-sense RNA genome packaged in an icosahedrally symmetric capsid composed of 60 copies each of 4 structural proteins, VP1 to VP4. FMD is one of the most contagious animal diseases. Control of the disease in endemic areas is carried out with inactivated whole virus vaccines that induce a serotype-restricted, short-lived protection, making frequent revaccination a prerequisite in control campaigns (1). Furthermore, the presence of residual live viruses in these inactivated vaccines and the escape of viruses from vaccine production plants have been implicated in FMD outbreaks (2).

Direct injection of DNA into animals is a novel and promising method for delivering specific antigens for immunization. Endogenous expression of antigen from DNA introduced into host cells leads to the production of structurally and conformationally relevant molecules of the antigen that are able to activate specific immunity. Plasmid DNA vaccination can protect against many viral, fungal and parasitic diseases in different animal models (3-5). However, a general problem with plasmid DNA is that the vaccines alone are often poorly immunogenic. In recent years, there has been great interest in modulating the immune responses by inoculating with vectors encoding cytokines or other costimulatory molecules and antigens.

Type I interferon (IFN-α) not only play a key role in defense against pathogens but also have potent immunomodulatory activities (6,7). Chinsangaram et al. (8) also demonstrated that IFN-α of porcine or bovine origin and porcine IFN-β could exert an inhibitory effect...
on FMDV in cells from homologous and heterologous species. Therefore, we select bovine IFN-α as a native adjuvant and hope it can also exert its immune function in cattle and pig.

On the other hand, nuclease activity present within tissues contributes to the rapid clearance of injected DNA and therefore may reduce the transfection activity of directly injected transgenes. Aurintricarboxylic acid (ATA) is a general inhibitor of nucleases (9). Glasspool-Malone et al. (10) found that intratracheal administration of a nuclease inhibitor (ATA) with naked DNA (0.5 µg ATA/g body weight) enhanced direct transfection efficacy in macaque lung by over 86-fold and in mouse lung by over 54-fold. According to these investigations, we used the ATA in DNA direct injection of guinea pigs in order to enhance direct transfection efficacy of DNA vaccine in vivo and immune response against FMDV antigen.

Bicistronic vectors as a kind of multivalent DNA vaccine expression systems may enable more efficient delivery of DNA vaccine and promote immune responses. Bicistronic plasmids utilize an internal ribosome entry site (IRES) that is placed between 2 coding regions and can allow ribosomes to attach to mRNA, and then the downstream coding sequence of IRES can be translated, while the upstream sequence is translated by cap-dependent mechanisms (11,12). Presently, viral bicistronic and polycistronic vectors are widely used in the research of cancer gene therapy. Nonviral bicistronic vector is also used as a DNA vaccine for several viruses (13,14). But so far, there has been no bicistronic or polycistronic DNA vaccine in FMD gene vaccination. So, according to that bicistronic delivery of DNA vaccines may have the potential to enhance the ability of first-generation DNA vaccines to prime an immune response prior to a FMDV infection. We attempted to analyze the immune response induced in guinea pigs by a recombinant bicistronic DNA vaccine pcDNA/PIF that expresses the precursor polyprotein (P1), non-structural protein 3C which ensures the processing of the polyprotein precursor to produce 3 FMDV capsid proteins (VP0, VP3, VP1), and bovine IFN-α. The effect of bicistronic immunization of guinea pigs on protection after FMDV O/China99 challenge was also assessed.

Materials and Methods

Construction of recombinant plasmid

The bicistronic DNA vaccine pcDNA/PIF was constructed by a 3-way ligation of IFN, IRES and P12X3C. In brief, the bovine IFN-α gene was obtained from pcDNA3.1/IFN (15) by PCR technology and the EcoRI site and XhoI site were introduced into 5’ end and 3’ end of IFN fragment respectively. FMDV IRES gene with BamHI and EcoRI was obtained from pGEM/IRES (unpublished data) by PCR technology. The gene P12X3C was obtained from pcDNA3.1/P12X3C (16) by PCR technology and SpeI and BamHI restriction sites were introduced at each end. Meanwhile, the plasmid pcDNA3.1 (+) (Invitrogen) was digested by SpeI and XhoI endonucleases. Then the digested products were ligated and the resultant plasmid pcDNA/PIF was further characterized by restriction endonuclease analysis, PCR amplification, and sequencing to authenticate the DNA sequences.

Immunization

All guinea pigs were immunized as described previously (16).

Group A: FMD conventional vaccine; Group B: pcDNA/P12X3C; Group C: pcDNA/P12X3C+pcDNA/IFN; Group D: pcDNA/PIF; Group E: pcDNA3.1(+); Group F: DPBS.

Measurement of anti-FMDV antibodies level

Blood samples were analyzed by indirect ELISA as previously described (16).

Detection of specific neutralizing antibodies

Micro-neutralization assays were performed as previously described (16).

Flow cytometry analyses

For the phenotype of T-cell subpopulation in the peripheral blood by flow cytometry, single-labeling methods were employed for defining different subpopulations. To this end, the following mouse monoclonal antibodies (mAb) reactive with guinea pig leukocyte cluster of differentiation (CD) antigens were used: mouse anti guinea pig CD4 (Serotec) and mouse anti guinea pig CD8 (Serotec). The blood cells (100 µl) were incubated with 10 µl of fluorescein isothiocyanate (FITC)-conjugated mouse anti guinea pig IgG per 10⁶ leukocytes for 30 min at room temperature in the dark.
Controls without any reagents were included for each animal. In each test, 10,000 cells were counted using a FACSscan flow cytometer (Becton Dickinson) for data acquisition, and data were analyzed using the CellQuest software (Becton Dickinson).

**Challenge of animals**

All guinea pigs were challenged by footpad injection of 0.2 ml 100 ID50 FMDV O/China99 (offered by State Key Laboratory of Veterinary Etiological Biology and Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, China) at the third week after secondary vaccination. All guinea pigs were examined for characteristic FMD lesions on the footpad for 7 days after challenge. The lesion appeared at the site of virus injection, without spreading to other feet, was referred to as indicator of partial infection and on both back sole as indicator of whole infection (17).

**Results**

**Specific antibody response to FMDV**

As shown in Figure 1, specific antibody of animals immunized with conventional vaccine and recombinant plasmids emerged after the first vaccination, and subsequently these antibodies decreased in some sort. But except for guinea pigs of group A, the antibody level of guinea pigs in group B, C, D, which were increased after the second vaccination, was almost no more than that induced at the second week after the first vaccination and held a steady level on the whole. The antibody level of control group (group E and group F) did not change after inoculation.

**Induction of neutralizing antibodies after DNA immunization**

Neutralizing antibody determinations were performed in serum sera sampled before (day 0) and after vaccination (days 14, 28, 42, and 56). As shown in Figure 2, all guinea pigs of the experimental group developed neutralizing antibody after the first vaccination. Except for guinea pigs in group A (immunized with FMDV conventional vaccine), neutralizing antibody titers of other groups decreased at the first week after the second vaccination. Subsequently, the neutralization titers of all of guinea pigs increased (group B, group C, and group D) or maintained (group A).

![Figure 1](image-url)  
**Figure 1.** Alternation in FMDV antibody levels of immunized guinea pigs. Animals were immunized with different reagents at week 0 and 4. Sera were collected at the indicated times, and antibody levels were measured by ELISA at 1:16 dilution and indicted by OD value. The result was obtained from average of 3 sera in each group.

![Figure 2](image-url)  
**Figure 2.** Neutralizing antibody was measured in 6 groups of animals. Serum samples were taken on day 0, day 14, day 28, day 42, and day 56 before and after vaccination. Titers are expressed as log10 TCID50/ml measured on BHK-21 cells and calculated by the Spearman-Karber method. The result was obtained from average of 3 sera in each group. The results of group E and group F are intersection of abscissa at Y-coordinate.
The phenotyping change of T-cell subpopulation of guinea pigs vaccinated with pcDNA/PIF

Flow cytometric analysis (Figure 3) showed that, in all tested groups, the ratio of both CD4+ and CD8+ peripheral blood T-cell subpopulation increased significantly in comparison with negative controls (group E and group F) after prime immunization, but this ratio decreased at the forth week. And the ratio of CD4+ and CD8+ peripheral blood T-cell subpopulation increased further at the second week after boost immunization, which is similar to the change of ratio after first immunization. Subsequently this ratio decreased at the forth week.

Protection result of guinea pigs

In challenge test, all guinea pigs were subcutaneously and intradermally challenged with 0.2 ml 100 ID50 of live virus on left rear foot at the fourth week after the second vaccination. The positive (group A) and negative (group E and group F) controls followed expectations, yielding protection or disease, respectively (Table). The negative-control animals and blank-control animals that were not protected developed vesicles in both rear feet at day 2 after challenge.

Discussion

Vaccination continues to be the most successful procedure for preventing infectious diseases in animals and humans. The development of new-generation vaccine systems to prevent FMD is needed to overcome the disadvantages of the currently used inactivated whole virus vaccines. The cotransfer of different genes into a cell can be achieved by mixing different plasmids, but gene expression from such mixtures of plasmids is not balanced. The reason is that the uptake of DNA is a matter of statistical variation; the lifetime of different mRNAs varies depending on the nature of the transcript. Even if the same promoters/enhancers are used on different plasmids, protein expression of different genes, and thereby antigen presentation, will vary significantly. This uncoupled expression could cause problems in DNA vaccination in which controlled coexpression of different antigens is an essential prerequisite for the construction of a polyvalent vaccine (18).

The discovery of cap-independent translational initiation elements (IRES, internal ribosomal entry sites) allowed the construction of bi- or polycistronic expression plasmids where several proteins are separately translated from one mRNA (19). Efficient IRES elements have been identified in picornaviruses and other viruses (20-22).
Because foot-and-mouth disease virus IRES provides high-titer bicistronic vectors with high-level 2-gene expression (23), we selected the IRES element of FMDV to initiate the translation of proteins (IFN) downstream in this study. In this communication, we describe the construction of a plasmid carrying FMDV sequences. The advantage of such a construct is its ability to express and process all structural proteins to form an empty viral capsid that will be a superior immunogenic entity as it can initiate the immune system better than separate viral proteins like VP1 itself. It was observed that viral structural proteins have the tendency to induce the humoral response, whereas nonstructural proteins seem to be more effective in inducing the cellular immune system (17). Thus, this particular construct was built in mind to express a mixture of both structural and nonstructural viral proteins.

As the result showed, the FMDV antibodies were induced after the first immunization but only the FMDV antibodies of guinea pigs vaccinated with FMD conventional vaccine increased significantly after the second immunization. Although the FMD antibodies of guinea pigs in other groups (except for group E and group F) also increased, it is not significant in comparison with that of guinea pigs in group A. This result is similar to previous studies (15,16). The neutralization antibody titers showed the same diversification after the prime immunization as the specific antibody titers; however, neutralizing antibody titers increased after boost immunization. It can be postulated that the protein expressed after boost immunization is neutralized by antibodies induced after prime immunization; subsequently enough protein is expressed by plasmid DNA and this induces more distinct neutralizing antibody response than prime immunization. It is reasoned that the DNA plasmid needs the process of adsorption, entrance, translation, and expression when inoculated to animal, so it can be assumed that DNA vaccine stimulates the immune system to produce antibodies for a longer time than conventional vaccine obtained from organism antigen.

<table>
<thead>
<tr>
<th>Serial number of guinea pigs</th>
<th>Group A*</th>
<th>Group B</th>
<th>Group C*</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protection</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
<td>None</td>
<td>Total</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Total</td>
<td>None</td>
<td>None</td>
<td>Total</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>NT</td>
<td>partial</td>
<td>NT</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Severity of Symptoms

<table>
<thead>
<tr>
<th>Severity of Symptoms</th>
<th>Group A*</th>
<th>Group B</th>
<th>Group C*</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>6</td>
<td>NT</td>
<td>Mild</td>
<td>NT</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
</tr>
</tbody>
</table>

Rate of protection (%) = Number of guinea pigs no lesions on both rear feet/Total number of guinea pigs.

Table. Protection of guinea pigs from viral challenge.
But on the other hand, it is thought that the dosage of DNA vaccine is not enough and need to be improved. Additionally, a method to deliver adequate amounts of the desired gene resulting in adequate expression of the encoded protein is needed.

In general, expression of CD4 and of CD8 also defines 2 major functional subpopulations of T lymphocytes. CD4⁺ T cells generally function as T helper (TH) cells and are class-II restricted; CD8⁺ T cells generally function as T cytotoxic (TC) cells and are class-I restricted. Thus the ratio of TH to TC cells in a sample can be approximated by assaying the number of CD4⁺ and CD8⁺ T cells. This ratio is comparatively fixed in normal peripheral blood, but diseases or other disorders may significantly alter it. We used flow cytometric analysis to evaluate the T-cell immune response induced by plasmid DNA. Flow cytometric analysis showed that the ratio of CD4⁺ and CD8⁺ lymphocytes increased firstly after prime immunization or boost immunization and then decreased. This situation is consistent with changes in the level of specific antibody and neutralizing antibody responses. It illuminated that DNA vaccine is inclined to mainly induce CD4⁺ lymphocytes differentiate firstly and then CD8⁺ lymphocytes. This result further confirmed mechanism of T-cell immune response induced by DNA vaccine once again (24).

Combined with the results of FMDV specific and neutralizing antibody responses, it seems that IFN-α did exert its adjuvant function unsatisfactorily, especially, in immune response induced by bicistronic plasmid pcDNA/PIF. According to the literature (25), we presume that the IRES is not optimal for driving translation and expression of IFN-α and the quantity of IFN-α expressed is too little to exert its adjuvant function. So it will be attempted to construct a bicistronic plasmid in which gene of IFN-α can be translated upstream of IRES and compare the immune enhancement with the bicistronic plasmid reported in this study. Another explanation could be low-level transfection of plasmid DNA in vivo, which leads to low-level expression of antigen carried by recombinant plasmid. So much more work might improve the efficiency of this approach including optimize plasmid, dosage of DNA vaccine, immune programme, and so on.

Consistent with our expectation, guinea pigs inoculated with plasmid pcDNA/PIF were partially protected from virulent virus challenge. It is supposed that the size of plasmid pcDNA/PIF is larger than that of pcDNA/P12X3C and pcDNA/IFN, which reduced the transfection efficiency of plasmid in vivo. However, the guinea pigs inoculated with pcDNA/P12X3C were protected partially in comparison with the guinea pigs coimmunized with pcDNA/P12X3C and pcDNA/IFN. It is reasoned that bovine IFN-α gene in pcDNA/IFN can be expressed more efficiently than that of pcDNA/PIF and then exerts its adjuvant function in coimmunization group with pcDNA/P12X3C and pcDNA/IFN more markedly than that of group immunized with pcDNA/PIF, which can also be revealed from results of humoral and cellular immune response.

Although guinea pigs immunized with plasmid pcDNA/PIF were not protected totally in comparison with guinea pigs coimmunized with pcDNA/P12X3C and pcDNA/IFN, the level of specific antibody and neutralization antibody as well as extent of T-cell immune response showed no significant difference from that of guinea pigs coimmunized with pcDNA/P12X3C and pcDNA/IFN. So taken into account the usage of DNA vaccine, bicistronic constructs are more convenient than coimmunization of plasmid DNA.

Taken together, the results of this paper indicate that bicistronic vectors can induce humoral immune response as efficiently as monocistronic constructs or coimmunization of monocistronic constructs. It suggested that bicistronic constructs thus offer a novel approach for the design of FMD polyvalent vaccines that efficiently induce humoral and cellular immune responses and exploit new ways for FMD genetic immunization.

**Acknowledgements**

This work was supported by a grant from “973” Major State Basic Research Development Program of China (NO.2005CB523201). We thank Madam Zhang xiaoli and Tian hong for helping T-lymphocytes separation, Mr. Ling xi for animal experiments.
References


