Immunity Evaluation of an Experimental Designed Nanoliposomal Vaccine Containing FMDV Immunodominant Peptides

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals. The particular virus that causes FMD disease is called FMD virus that is a member of the Aphthovirus genus in the Picornaviridae family. FMD virus has a single strain positive RNA genome with a length of 8500 nt with one ORF which are trapped in an icosahedral capsid protein. This virus genome doesn't have proofreading property which tends to highly mutagenesis. It has seven serotypes, such as, O, A, ASIA, SAT1, SAT2 and C and a lot of subtypes. Iran is an endemic region of foot-and-mouth disease. Vaccination of susceptible animals with inactivated whole-virus vaccine is the only way to control the epidemic in many developing countries. Today, conventionally attenuated and killed virus vaccines are being used worldwide. In Iran, animals have been vaccinated every 105 days with an inactivated FMD vaccine. Although commercially available FMD vaccines are effective, they provide short-term immunity requiring regular boosters. A new FMD vaccine is needed to improve immunization, safety
and long-term immune responses. A synthetic peptide vaccine is one of the safe and important vaccines. Peptide vaccine has low immunogenicity and applying strong adjuvant is necessary. Nano liposomes as new adjuvants could be used to improve the immune response. In the current study, Nano liposomal carrier using Dimiristoyl phosphatidyl choline (DMPC), Dimiristoylphosphoglycerol (DMPG) and Cholesterol (Chol) as an adjuvant containing two immunodominant synthetic FMDV peptides were selected. The liposomal formulations were characterized due to various physicochemical properties. The size, zeta potential and encapsulation efficiency were optimized and the nanoliposome obtained was suitable as a vaccine. The efficacy of vaccines in guinea pigs as an animal model has been evaluated. Indirect ELISA was used to detect FMDV-specific IgG. The study indicates that although antibody titer was observed, the amount was lower compared to the groups that received inactivated virus-containing liposomes. In addition, the results show that liposome is an appropriate adjuvant compared to other adjuvants such as Alum and Freund and can act as a depot and induce immune response.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious and devastating disease of cloven-hoofed animals that can cause significant economic losses. Foot-and-mouth disease is characterized by various clinical signs such as fever, lameness, the formation of vesicles on the feet, mouth, and tongue, decreased milk production, loss of animal power and fertility. Vaccination of susceptible animals with inactivated whole-virus vaccine is the only way to control the epidemic in many developing countries(1). The commercially tetravalent vaccine available in Iran produced by the Razi vaccine and serum research Institute (Karaj, Iran)(2), containing serotypes A, Asia1 and two strains of serotype O is typically produced from BHK21 cell culture supernatants from foot-and-mouth disease virus (FMDV)-infected cells, chemically inactivated, then purified and eventually formulated with an adjuvant. The use of the inactivated virus as a vaccine has a number of disadvantages, such as incomplete inactivation of the virus, the problem of separation between vaccinated animals and infected animals, and the need for booster vaccination every four months. These limitations have led to studies of a safe alternative vaccine (1,3). The use of new technologies to develop alternative vaccines such as subunit vaccines, recombinant virus vaccines, DNA vaccines, empty capsid vaccines and peptide vaccines has been studied to identify safe and effective alternatives to conventional methods of production of FMD vaccines(4–6). Non-living vaccine antigens, such as peptide vaccines due to their synthetic nature are often poorly immunogenic and require selected strong and safe adjuvants to increase their immunogenicity(7). FMDV contains several neutralizing epitopes and is therefore a good candidate for peptide vaccine(8). In 2014 Zhongwang Zhang and et al. applied FMD virus peptides with Montanide adjuvant and provided 60% protection in cattle(8). In recent years, liposomes and its Nano-structure based as Nano-liposomes have been widely used as an adjuvant for vaccines (9). In 2013 Feng-Shan Gao and Et al applied nanoliposom as an adjuvant containing T cell epitope peptide of FMD virus and achieved 25 to 60% protection in the mice (10). Nano-Liposomes act as depots for the slow release of antigen and cell-presenting antigen adjuvants. Lipid composition, charge, size, size distribution, entrapment and the status of the antigens within the liposome can be chosen to achieve strong and effective immune response(11).

In the present research nanoliposomal carrier as an adjuvant containing two different peptide used. Peptide 1 (VP1 141-161, ATNVRGDLQVLAQKAARTLP) is embedded in a B-cell epitope in positions 141-161 of the FMDV subtype O2016 of the VP1 protein. Peptide 2 (VP1 198-211, EARHKQKIVAPVKQ) is embedded in a B-cell epitope in positions 141-161 of the FMDV subtype O2016 of the VP1 protein. Predictions for peptides 1 and 2 were made using IEDB-AR
(http://tools.iedb.org/), and NetMHCpan-2.0 (http://www.cbs.dtu.dk/services/NetMHCpan) and were synthesized by Dg Peptides Co., Ltd (Hangzhou, China). Process immunization taken on seven ginea pig groups. The indirect Elisa was used to determine the effect of the vaccine on the animal model.

Material and method

Dimiristoylphosphatidylcholine (DMPC), Dimiristoylphosphoglycerol (DMPG), Distearoylphosphorylcholine (DSPC), 1,2-distearoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) (DSPG) and Cholesterol (CHOL), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethane sulfonic acid) (HEPES). Methanol, ethanol, and isopropanol (Emsure®), dimethyl sulfoxide (DMSO), acrylamide. The peptides used in our study are given in Table 1 that purchased from Dg peptidesCo.

Peptide synthesis

Two different synthesized amino acid sequences corresponding to epitopes in the VP1 proteins as the predominant epitope that elicits the production of neutralizing antibodies by B cells. Peptides stock prepared by dissolving in DMSO (10mg/ml).

Table 1: two synthetic peptides

<table>
<thead>
<tr>
<th>peptide</th>
<th>Serotype</th>
<th>amino acid position</th>
<th>amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>O2016</td>
<td>141-161</td>
<td>ATNVRGDLQVLAQKAARTLP</td>
</tr>
<tr>
<td>P2</td>
<td>O2016</td>
<td>198-211</td>
<td>EARHKQKIVAPVKQ</td>
</tr>
</tbody>
</table>

Formation of nanoliposome and characterization

Nano-Liposomes prepared by dehydration-rehydration method. Lipids were used at a molar ratio of 16:4:5 (DMPC, DMPG, Chol) and dissolved in chloroform. This method involves remove organic solvent by rotary evaporator (IKA, Germany) and freeze dryer device (Christ, Germany) and formation of a thin film lipid layer. Then the lipid film was rehydrated by 10mM HEPES buffer containing 10% sucrose and 10µl peptide(containing peptide was dissolved in DMSO 10 mg/ml ) per 1 ml liposomal solution. Liposomes were prepared to contain 60 mM of total lipid. The hydrated lipids were shaken by rotary evaporator (without vacuum condition) at 35°C (10°C above the transition temperature) or pipetting. Then the LUV liposomes were sonicated (the 160-W output power, Elmasonic, Germany) 10 minute afterward, the Nano liposomes containing peptide were extruded through 400 nm, 200 nm and 100 nm polycarbonate membranes employing a mini extruder (Avanti, USA) to form small unilamellar vesicles (SUVs). Liposomes were stored at 4 ºC under argon. UN encapsulated peptides removed by dialysis bag (10 kD)(12). Quality of liposome formation was assessed by Dynamic light scattering (DLS) and Transmission electron microscopy (TEM).

Determination encapsulation efficiency
To evaluate the encapsulation efficiency of the peptides 10 KDa dialysis bag used to separate the peptides which have not been encapsulated in liposomes. The peptide contents measured by Lowry method with some modification(13).

Encapsulation Efficiency (EE %) of peptides calculated as following:

\[
EE\% = \frac{\text{peptide concentration in liposome after dialysis}}{\text{peptide concentration in liposome before dialysis}} \times 100
\]

Dynamic light scattering (DLS)
The average size of the liposome diameter, polydispersity index (PI) and zeta potential was determined by dynamic light scattering (DLS), Zeta Sizer 1000 HSA (Malvern, UK). This technique is based on the Brownian movement which is depended on the size of the particles(14).

Transmission electron microscopy (TEM)
Nanoliposomes have been described by transmission electron microscopy (TEM) (Eindhoven, NL). Samples were prepared using the process reported by Moscho et al., 1996. A drop of the liposome suspension was floating on a formvar coated copper grid that was treated with a poly-l-lysine solution. After three minutes, the nanoliposome suspension was replaced by a decrease in negative stain (phosphotungstic acid 2%, w/v, pH 6.5 in distilled water)(15).

Peptide Release
Nanoliposomes containing peptides dialyzed to isolate peptides which have not been encapsulated. Liposome formulations containing peptides formulated under a freeze-thawing protocol were stored in a dialysis bag (10KDa) for 14 days in a shaker incubator at 37 ° C. Sampling of the nanoliposomes by removing 0.05ml of the content and replacing it with HEPES / Sucrose buffer performed at predetermined time intervals(16).

The release percentage calculated as following:

\[
\text{Release}\% = \left( \frac{\text{peptide concentration after first dialysis} - \text{peptide concentration after second dialysis}}{\text{peptide concentration after first dialysis}} \right) \times 100
\]

Animals
Male guinea pigs weighing 250 to 300 g were provided from the Razi Vaccine and Serum Research Institute; they were kept under clean-air condition and the sera titer was checked by ELISA to be free from antibodies against FMD virus. They were allotted into seven groups of five each.

Immunization groups
Thirty five guinea pigs were randomly divided into seven groups of five animals each. Seven groups of vaccine recipients described as follows: group 1: liposome containing Peptide; group 2: Combined liposome with inactivated virus; group 3: combined Alum with peptide; group 4: combined Freund with peptide; group 5: combined Alum with inactivated virus; group 6 combined Freund with combined inactivated virus; group 7 PBS buffer. In Table2, the group division of vaccinations is shown.
All groups have been immunized by an intramuscular injection of 0.5 mL of different vaccine formulations. According to the formulations 100µg of each peptide, 10µg of inactivated FMD virus (O2016) inoculated. All groups injected three times with intervals of two weeks. Bleeding was performed and serum was collected at 2, 3, 5 and 6 weeks after the first injection from all guinea pigs in each group and then the sera was subjected to indirect ELISA.

Table 2: Division of animal groups for vaccine recipients

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine Formulation</td>
<td>Lip*-pep**</td>
<td>Lip-virus</td>
<td>Alum-pep</td>
<td>Freund-pep</td>
<td>Alum-virus</td>
<td>Freund-virus</td>
<td>buffer</td>
</tr>
</tbody>
</table>

*Lip: Liposome  
**pep: peptide 1 and 2

Indirect ELISA detection of IgG response in guinea pigs

An indirect ELISA was used to detect FMDV-specific IgG response in vaccinated guinea pigs. Blood samples were collected from vaccinated guinea pigs. Micro titer plates with 100 µl/well of 10µg/ml FMD virus in Carbonate buffer were coated and incubated overnight at 4°C. After three washes with PBST (PBS containing 0/05% tween 20) the coated plate blocked by adding 250 µ1 per well of 5% of skim milk in PBST and incubated at 37°C for 1:30 h. After three times washing, the plates incubated with serum samples diluted in 1% skim milk (1:50) at 37°C for 1:15 h. After washing with PBST, the plates were treated with rabbit anti-guinea pig horseradish peroxidase conjugated whole IgG antibody for 1:15 h at 37°C. After a final washing step, 100µl BM blue Roche substrate (Sigma, St. Louis, MO, USA) was added and kept for 20 minutes in darkness at room temperature. By adding 0.1 M sulfuric acid the reaction was terminated, and FMDV reactivity was read by a microplate spectrophotometer (BioTek Instruments Inc., USA) based on optical density (OD) at 450nm.

Result

Liposome characterization:
The size and zeta potential of Nano-liposomal formulations are shown in figure 1. Results show that the zeta potential of Nano-liposomal formulation is negative and the size of formulations are in the range of 120-130 nm. The final encapsulation efficiency was measured and result shows in table 1.

Table 3: size, zeta and Encapsulation Efficiency of Nano-liposomal vaccine

<table>
<thead>
<tr>
<th>DMPC/DMPG/CHOL 60 mM</th>
<th>SIZE Concentration 60mM</th>
<th>ZETA</th>
<th>Encapsulation Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>129.6±.9</td>
<td>-27.7</td>
<td>67%</td>
<td></td>
</tr>
</tbody>
</table>

Peptides release profile

The peptide release value for DMPC: DMPG: Chol can be found in Figure 1. Peptide release of DMPC: DMPG: Chol nanoliposome is shown to be less than 2% after 24 hours. After 168-hr, the liposomal formulation released all the peptide content in the release process.
Figure 1: Diagram released peptide from DMPC:DMPG:Chol liposomes for 7 days.

Transition Electron Microscopy

Figure 2: TEM images of nanoliposomes containing peptides, panel A: Nanoliposome with the size of 100 nm is shown, panel B population distribution outlook for nanoliposomes is reported

Antibody response
In current study two synthetic peptides vaccine with different adjuvant such as Nano-liposome, Alum and Freund were tested intramuscularly in guinea pigs. During all the tests animals were kept in isolation with no direct or indirect contact with other animals. Anti-FMDV antibodies have been tested with indirect ELISA in vaccinated animals. The result of IgG titer level is shown in Figures 1, 2 and 3. The result shows that anti-FMDV-serotype-O antibodies were detected in all peptide-vaccinated groups two weeks post vaccination (WPV), but the amount of anti-body titer was not significant. As shown in figure 3 when comparing peptide groups such as liposome peptide, alum peptide and Freund peptide, the highest IgG response is related to combined freund adjuvant with peptide, and then the liposome containing peptides then combined Alum with peptide have the highest antibody titer. Anti-FMDV-serotype-O antibodies have been detected in 2 WPVs, as shown in Figure 2 in virus-vaccinated groups
such as virus freund, virus alum and virus Nano- liposome, the highest level of IgG response was detected in the freund virus, followed by the alum virus and the liposome virus. Figure 3 shows that the freund virus has the highest level of IgG response then alum virus, liposome virus, freund peptide, alum peptide and liposome peptide have a high level of IgG respectively.

![Image](image-url)

**Figure 3**: Levels of anti-FMDV specific IgG in pooled sera of different groups of guinea pigs immunized by IM injection three times in two week intervals, by liposome containing peptide, combined Alum with peptide, combined Freund with peptide and negative control group, have a highest IgG titer respectively.

![Image](image-url)

**Figure 4**: Levels of anti-FMDV specific IgG in pooled sera of different groups of guinea pigs immunized by IM injection three times in two week intervals by Freund with virus, Alum with virus, liposome with Virus, and negative control, have a highest IgG titer respectively.
Figure 5: Levels of anti-FMDV specific IgG in pooled sera of the different groups of guinea pigs immunized by IM injection three times in two week intervals by Freund virus, Alum virus, liposome with Virus, Freund with peptide, liposome with peptide, Alum with peptide and negative control, have a highest IgG titer respectively.

Discussion

Our priority is to use novel adjuvant containing immunodominant peptide as an FMD vaccine to improve long-term immune response and safety. The selective suitable adjuvant can be beneficially programmed for humoral antibody response, which plays an important role in protecting against FMD. Lipid-based adjuvants, such as liposomes, are thought to induce a powerful immune response (17). The composition of the liposome plays a major role in protecting the cargo against body clearance (18). Phospholipids which are used to make liposomes are (DMPC), (DMPG) and (CHOL). As previously reported liposomes with intermediate transition temperature (T_m 25 -40° C) can induce immune response better than liposomes with high transition temperature(T_m>50° C)(19). The transition temperature for DMPC and DMPG is 25°C, making this composition suitable for induce immune response. Cholesterol was applied to the liposomal formulations, causing significant humoral immune responses (19). The head groups of phospholipids affect the charge of liposomes. Due to the presence of neutral PC (DMPC) and negative PG (DMPG) phospholipid head groups, the liposome charge revealed a negative charge that could be affected by antigen charges. In the current study, the zeta potential of liposome formulation is -27 which is more stable and appropirate for positive peptides encapsulation (Table 3).

The capillary absorption depends on size, charge and hydrophobicity. Particle sizes between 10 and 100 nm are eliminated by lymphatic vessels; particles greater than 100 nm remain as a reservoir at the injection site and particles smaller than 10 nm are extracted by the blood vessels (20). The average size of liposome formulations is approximately 129 nm (Table 3).
The peptide sequence P1 as an antigen corresponding to the amino acid positions 141 to 161 and the peptide sequence P2 to the amino acid position 198-211 of VP1 in the strain O/2016 were used as a B cell epitope. The Nano-liposomes containing peptides are a good candidate for safer and more effective FMD vaccines.

Results showed that after three injections of liposomal vaccine containing peptides, although antibody titer was observed, the amount was much lower compared to the groups that received inactivated virus-containing liposomes. The results are inconsistent with other reported results, emphasizing that amino acid positions 140-160 peptides of VP1 protein have the ability to cause high level of humoral immunity(10). Further studies of virus-inactivated groups combined with alum, freund, and Nano-liposome showed that the virus-freund group reached the highest level of antibody titer after 2 weeks of injection. Six weeks after the first injection, the antibody titer reached the highest level in all three groups containing the inactivated virus.

In order to improve the effectiveness of the vaccine delivery mechanism, the stability of the liposome formulation as a carrier of the vaccine is very significant. Determining the amount and time of release of the trapped portion is important. This in vitro stability is important for a nanoliposomal vaccine containing peptide that can operate as a depot of antigens, display the antigen for a longer period of time and trigger the full immune response before the RES cleared antigen is released. A slow release was shown in the release study (Figure 1) and showed that the antigen was not released as a trigger release and shown the liposomal formulation containing peptide release all content over seven days. This result is consistent with other studies(15).

In 2014, Saravan and colleagues injected liposomes (VacciMax®) containing inactivated virus and achieved 25 to 63 percent protection in the cows being tested(21) similar to other study as shown in Figure 4, the liposome formulation used in the current study is a good adjuvant and may induce an immune response. It appears that the liposome has a long-term immune response compared to the freund adjuvant and the alum adjuvant. Results show that by injecting 10µl (10mg/ml) peptide in 1 ml liposome in liposome containing peptide group, the antibody titer is lower than the inactivated virus groups. It seems that if the amount of injectable peptide increased 2 or 3 times, the IgG antibody is expected to increase.

Comparison of all vaccinated groups in figure 3 shows that the freund virus has the highest level of IgG response then alum virus, liposome virus, freund peptide, alum peptide and liposome peptide have a high level of IgG respectively.

Long-term immune response in liposomal adjuvant compared to alum and freund adjuvants is recommended for further study.

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