Development of Protective Immunity against Inactivated Iranian Isolate of Foot-and-Mouth Disease Virus Type O/IRN/2007 Using Gamma Ray-Irradiated Vaccine on BALB/c Mice and Guinea Pigs

Farahnaz Motamedi-Sedeh a  Hoorieh Soleimanjahi b  Amir Reza Jalilian a  Homayoon Mahravani c  Kamalodin Shafaee a  Masood Sotoodeh c  Hamdolah Taherkarami a  Faramarz Jairani c

a Nuclear Science and Technology Research Institute, Karaj, Tehran, Iran  b Virology Department, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran  c FMD Department, Razi Vaccine and Serum Research Institute, Karaj, Tehran, Iran

Abstract

Objectives: Foot-and-mouth disease virus (FMDV) causes a highly contagious disease in cloven-hoofed animals and is the most damaging disease of livestock worldwide, leading to great economic losses. The aim of this research was the inactivation of FMDV type O/IRN/1/2007 to produce a gamma ray-irradiated (GRI) vaccine in order to immunize mice and guinea pigs. Methods: In this research, the Iranian isolated FMDV type O/IRN/1/2007 was irradiated by gamma ray to prepare an inactivated whole virus antigen and formulated as a GRI vaccine with unaltered antigenic characteristics. Immune responses against this vaccine were evaluated on mice and guinea pigs. Results: The comparison of the immune responses between the GRI vaccine and conventional vaccine did not show any significant difference in neutralizing antibody titer, memory spleen T lymphocytes or IFN-γ, IL-4, IL-2 and IL-10 concentrations (p > 0.05). In contrast, there were significant differences in all of the evaluated immune factors between the two vaccinated groups of mice and negative control mice (p < 0.05). The protective dose 50 for the conventional and GRI vaccines obtained were 6.28 and 7.07, respectively, which indicated the high potency of both vaccines. Conclusion: GRI vaccine is suitable for both routine vaccination and control of FMDV in emergency outbreaks.

Introduction

Vaccination is a cost-effective way of controlling animal disease. In the case of viral diseases, it might be the only way to control them successfully in the absence of alternative therapies [1]. The joint FAO/IAEA program first promoted discussion and scientific exchange on the value of radiation attenuation to develop vaccines against animal diseases nearly 50 years ago [1, 2]. In contrast to chemical treatment with formalin or β-propiolactone, gamma rays have little impact on the antigenic structure and biological integrity of proteins [3].

Foot-and-mouth disease (FMD) virus (FMDV) is a highly infective agent of the Picornaviridae family that can affect all cloven-hoofed animals. FMDV contains a single-stranded, positive-sense RNA about 7.2–8.4 kb in...
size, encoding a single long open-reading frame protein. Seven major serotypes (A, Asia-1, C, O, SAT-1, SAT-2 and SAT-3) are known, and hundreds of isolates have already been described and partially sequenced [4]. A particular genetic lineage of FMDV serotype O, the Pan-Asia strain, is responsible for an explosive pandemic in Asia [5]. Outbreaks of FMD in various countries in recent years have had a severe economic impact on the agricultural industry worldwide [6]. The current FMDV vaccine, which successfully prevents disease, includes inactivated whole virus antigen. However, this vaccine has major drawbacks, such as its production requiring costly high containment facilities with permanent risks of virus escape. Moreover, difficulties can arise through insufficient chemical inactivation and chemical residues. Gamma rays can penetrate through all materials, excepting some such as lead (Pb), without any residues; therefore, there is no risk of an irradiated inactivated viral vaccine escaping. In addition, the duration of the viral inactivation process by gamma radiation is shorter than the time needed for chemical inactivation [7]. The inactivation of viruses by ionizing radiation shows that a single ionization is sufficient to inactivate a single virus particle. Evidently, the inactivation dose is related to the virus concentration, the size of the particles containing viral genomes, and the temperature of irradiation. The use of ionizing radiation has been developed in the production of effective antigens for vaccines as well as for diagnostic purposes. The aim of this research was the inactivation of FMDV type O/IRN/1/2007 and the formulation of a gamma ray-irradiated (GRI) vaccine to immunize mice and guinea pigs.

### Materials and Methods

#### Virus Multiplication and Titration

The strain IRN/1/2007 of FMDV type O was isolated from epithelial cells of cattle vesicles in Tehran-Ray, Iran, in June 2007, cultured on the IBRS2 cell line in the Razi Vaccine and Serum Research Institute of Iran. Its serotype was determined using polyclonal antibodies against the seven serotypes [8]. The nucleotide sequence data of the VP1 gene was deposited in GenBank [9, 10]. Virus titers were determined in tissue culture infection dose 50/ml (TCID50/ml) by endpoint dilution and by using the calculation method of Read and Münch [11]. The procedure was based on viral cytopathic effects (CPE) in tissue culture.

#### Virus Inactivation

Gamma radiation was used for the inactivation of FMDV. A Nordion model 220 gamma cell instrument was utilized at a dose rate of 4.8 Gy/s and activity of 20469 Ci to damage FMDV genomic RNA and to inactivate virus infectivity. Gamma ray doses of 10, 15, 20, 25, 30, 35, 40, and 50 kGy were administered for the frozen virus samples and 3 sample replicates were irradiated for each dose. The titers of irradiated virus samples were first calculated by the Read and Münch method, then a dose/survival curve was drawn using Origin software. The D10 value and optimum dose of gamma rays for viral inactivation were determined according to the dose/survival curve. Finally, 50 ml of the virus was irradiated by the optimum dose of gamma ray and formulated as the GRI vaccine. Some virus samples were inactivated by binary ethylene imines and formulated as conventional vaccine.

#### Safety Test and Complement Fixation Test

The infectivity of the irradiated inactivated virus sample by the optimum dose of gamma rays was determined by inoculating pig kidney cell line monolayers (IBRS2) at 37° for 24 h, which were then subcultured on fresh IBRS2 cells four times. In addition, the antigenic characteristics of irradiated and native virus samples were tested by the complement fixation (CF) test [12]. The irradiated inactivated virus was formulated as a GRI vaccine using aluminum hydroxide gel and saponin.

#### Animal Vaccination

Fifteen BALB/c mice were divided in three groups. The first group was vaccinated by GRI vaccine subcutaneously. The conventional vaccine was inoculated to the mice of the second group and the third group was injected with PBS as a negative control. The booster dose of vaccines was injected after 21 days, and then all mice were bled 10 and 70 days after the last injection. The sera of vaccinated mice were used to measure neutralizing antibodies. The splenic lymphocytes of vaccinated mice were stimulated by FMDV antigen and used to measure T cell proliferation and the concentration of IFN-γ, IL-4 and IL-10.

#### Specific Neutralizing Antibody Response

The sera were diluted in Eagle’s maintenance medium in a 2-fold dilution from 1:4 to 1:128. The serum neutralization test was carried out according to Karber’s protocol [13]. This test was performed on a monolayer of BHK21 cells in flat-bottom 96-well microplates. Any well in which the virus had been neutralized and for which the cells remained intact was tagged as a positive well, and the remaining wells in which the virus had not been neutralized and CPE could be shown were tagged as negative wells.

#### Spleen T Lymphocyte Proliferation Response

The splenic lymphocytes were removed and cultured using a T lymphocyte proliferation assay with MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide]. The spleens of the vaccinated mice were removed aseptically 14 days after boost immunization. The single splenic lymphocyte suspensions were prepared and incubated in 96-well plates at 5 x 10⁴ cells/well with RPMI 1640 plus 10% fetal calf serum at 37° in 5% CO₂. The cells were stimulated with 50 μl of phytohemagglutinin (50 μg/ml; positive control), 2.5 μg/ml of 146S antigen of FMDV type O/IRN/1/2007 (specific antigen stimulation) and no antigen (negative control), in triplicates. After 48 h, the MTT assay was performed by means of a Cell Proliferation Kit 1 (MTT; Roche), according to the manufacturer’s instructions. The absorbance was measured at 540 nm and the stimulation index (SI) was calculated as the mean OD value of triplicate wells corresponding to cells stimulated with an antigen, divided by the mean OD value of the triplicate wells corresponding to cells stimulated with the medium [14, 15].

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Immune Responses of GRI Vaccine against FMDV in an Animal Model

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Cytokines Assay
For the measurements of IFN-γ, IL-4, IL-2 and IL-10, splenic lymphocytes were incubated and stimulated with FMDV antigen as described above for the proliferation assay. After 48 h, the supernatants were collected and different dilutions were assayed in duplicate, using commercial ELISA kits for IFN-γ, IL-4, IL-2 and IL-10 (mouse IFN-γ, IL-4, IL-2 and IL-10 kits; eBioscience) [14, 15].

Statistical Analysis
Statistical analysis was done by the analysis of variance (one-way ANOVA) followed by Duncan’s multiple range test. Differences were considered to be statistically significant for p < 0.05.

Viral Challenge
Thirty-six guinea pigs (weighing 250–300 g) were divided into twelve groups (each group included 3 animals) for the viral challenge test. Five groups were vaccinated by five serial dilutions of GRI vaccine (1, 1/2, 1/4, 1/8 and 1/16), five other groups were immunized by the same serial dilution of conventional vaccine and two groups were selected as a negative control without immunization. The vaccination was given subcutaneously and the two booster doses followed 3-week intervals. On the tenth day after the last immunization, each guinea pig was subcutaneously challenged with 0.2 ml of 100 ID₅₀ of guinea pig-adapted live virus (seventh passage) injection into the footpad, and housed separately for a 7-day period of examination in a BSL-2 laboratory (Nuclear Agriculture Research School, Karaj, Iran). Total protection was defined as no lesions on the footpad. Partial protection was defined as lesions occurring on the injected foot only. No protection was defined when lesions were found on two or more feet and the tongue of the challenged animal. The observations were recorded and the protective dose 50 (PD₅₀) was calculated for both kinds of vaccines [16, 17].

Table 1. Virus titers for irradiated and native FMDV type O/IRN/2007 preparations

<table>
<thead>
<tr>
<th>Dose, kGy</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCID₅₀, ml</td>
<td>10⁸.⁵</td>
<td>10⁸.⁵³⁷</td>
<td>10⁴</td>
<td>10².³¹⁸</td>
<td>10¹.³⁷⁵</td>
<td>10⁰.⁷⁵</td>
<td>10⁰.⁵</td>
<td>10⁰.⁵</td>
<td>10⁰</td>
</tr>
</tbody>
</table>

Fig. 1. Dose/survival curve for the irradiated FMDV type O/IRN/2007.

Results

Virus Titration and Inactivation
Serotyping of the isolated FMDV was done by ELISA which subsequently showed serotype O Pan-Asia. The nucleotide sequence data of the FMDV VP1 gene were deposited in GenBank (accession No. JF288761). The FMD virus titration was calculated as being 10⁸.⁵ TCID₅₀/ ml. The virus titration of irradiated and native virus samples and the dose/survival curve for irradiated virus samples are shown in table 1 and figure 1, respectively. During the inactivation process, samples were collected and their infectious titers were determined in TCID₅₀. The inactivation rate was based on the calculation of the regression coefficient and the inactivation endpoint [18]. The endpoint is the most important parameter to be determined and is dependent on the inactivation rate, the dose of irradiation and the virus concentration per volume unit. The minimum endpoint is defined as being one logₐ₀ lower than the titer which gives 1 infectious unit in the total volume under inactivation [18]. For a successful inactivation the calculated endpoint has to be lower than the minimum endpoint. The difference between the calculated and the minimum endpoint (DIM) has to be positive. Determination of the DIM value for each successful inactivation process is very important. According to table 1 and figure 1, virus infectivity decreased gradually with an increase in gamma radiation doses; also the D₅₀ value factor (the dose of gamma radiation which could decrease 1 logₐ₀ of the virus population) obtained was 5 kGy. In this research, the virus titer was 10⁸.⁵ TCID₅₀ per ml, the inactivation endpoint was calculated as log 10⁸.⁵ per ml and the minimum inactivation endpoint for 1 ml was therefore log 10⁹.⁵. Since the D₅₀ value was 5 kGy, to reach the successful minimum inactivation endpoint it is necessary.
to use a dose of irradiation about 9.5 times that of the $D_{10}$ value ($9.5 \times 5 = 47.5$ kGy). After termination of the inactivation process, each virus suspension had to be tested by the inoculation of at least two cell culture vessels during two or three subsequent blind passages at 48-hour intervals. These blind passages were done as safety tests and the result of the safety test showed that 45 kGy was insufficient, whereas 50 kGy was suitable to reach the minimum inactivation endpoint of this virus antigen. Therefore, the optimum dose of gamma radiation for FMDV type O Pan-Asia (O/IRN/2007) inactivation with a virus titer of $10^{8.5}$ TCID$_{50}$/ml was 50 kGy, which did not alter the virus antigenicity.

**CF and Safety Tests**

The safety test after four blind passages on the IBRS2 cell line was successful when no CPE could be observed after inactivation by 50 kGy of gamma rays applied to the O/IRN/2007 strain of FMDV titrating with $10^{8.5}$ TCID$_{50}$/ml (table 2). The specific binding between the antiserum specific to FMD virus type O and irradiated virus samples was assessed by CF test (for irradiation doses ranging from 0 to 50 kGy). It showed that the antigenic characteristics of virus samples were not changed after irradiation (table 3). As the G-H loop in the VP$_1$ peptide of FMD capsid is the specific site for binding to neutralizing antibody, the CF test results suggest that this loop in irradiated FMDV was conserved from the native virus. Motamedi-Sedeh et al. [19] reported that the irradiated FMDV type A87/IRN antigen was bound specifically to antisera directed against both irradiated inactivated FMD vaccine and conventional vaccine (inactivated by binary ethylene imines) by liquid-phase blocking sandwich ELISA.

**Neutralizing Antibody Titer**

Antibody titers were expressed as the logarithm of the reciprocal value of the last serum dilution in the virus/serum mixture to neutralize 100 TCID$_{50}$ of homologous FMDV antigen at the 50% endpoint. The neutralizing antibody titers were calculated for the sera after the last vaccination (table 3). Antibody titration for the mice immunized subcutaneously using both the GRI vaccine and conventional vaccine showed significant differences when compared with the negative control ($p < 0.05$). The titers of neutralizing sera in both vaccinated groups were protective. There was no significant difference between the GRI vaccine and the conventional one ($p > 0.05$).

**T Lymphocyte Proliferation and Cytokine Results**

Induction of the specific immune response against FMDV involves B lymphocytes recognizing epitopes on the virus particles to produce specific antibody. In addition, recognition of T lymphocyte epitopes following antigen processing and presentation in the context of the

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**Table 2. Safety test outcome**

<table>
<thead>
<tr>
<th>Irradiated virus samples</th>
<th>CPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first passage</td>
</tr>
<tr>
<td>Irradiation dose 40 kGy (1)</td>
<td>weakly positive</td>
</tr>
<tr>
<td>Irradiation dose 40 kGy (2)</td>
<td>weakly positive</td>
</tr>
<tr>
<td>Irradiation dose 45 kGy (1)</td>
<td>weakly positive</td>
</tr>
<tr>
<td>Irradiation dose 45 kGy (2)</td>
<td>negative</td>
</tr>
<tr>
<td>Irradiation dose 50 kGy (1)</td>
<td>negative</td>
</tr>
<tr>
<td>Irradiation dose 50 kGy (2)</td>
<td>negative</td>
</tr>
</tbody>
</table>

**Table 3. Results of the CF test for irradiated and native FMDV type O/IRN/2007** (number of positive wells with red blood cell agglutination)

<table>
<thead>
<tr>
<th>Irradiation dose, kGy</th>
<th>Dilution of FMDV type O/IRN/2007 (10$^{8.5}$ TCID$_{50}$/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/2</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
</tr>
</tbody>
</table>

Tr = Trace (not quantifiable).
Major histocompatibility complex type II is essential. Likewise, stimulation of the helper (Th) lymphocytes to provide for the growth and differentiation factors necessary for developing the immune response is vitally important. Therefore, B lymphocytes can respond directly to the antigen, but T lymphocytes require the antigen to be processed into small peptides presented in association with MH molecules. The stimulation of T cells therefore requires a third cell type capable of this processing and of the presentation of antigens. These are the antigen-presenting cells which include monocytes, macrophages and dendritic cells. In addition to the cellular components and interactions required for the induction and development of the immune responses, a number of soluble factors are critical – these are the cytokines. T lymphocytes produce cytokines including IL-2, IL-4, IL-5, IL-10, TNFα and IFN-γ. These cytokines can be described as Th1 and Th2 cytokines. Th1 cytokines include IL-2, IFN-γ and TNFα; Th2 cytokines include IL-4, IL-5, IL-6 and IL-10 [20].

The results of T lymphocyte proliferation (MTT assay) and the concentrations of IFN-γ, IL-4, IL-2 and IL-10 in the three groups of mice 10 days after the last vaccination are shown in Table 4. Table 5 shows the results of the MTT assay and IFN-γ concentration after 70 days. The SI values of the vaccinated mice were significantly higher than the negative control group 10 and 70 days after the last vaccination (p < 0.05). By contrast, there is no significant difference between the two groups of vaccinated mice with each other after 10 and 70 days. Therefore, this indicates that T lymphocytes were stimulated against FMD antigens after 10 days and this stimulation continues for at least 70 days in the same way as for 10 days postvaccination for both of the vaccinated mice groups.

The concentration of IFN-γ and IL-2 in the two vaccinated groups were significantly different compared with the negative control group (p < 0.05), but the differences were not significant between the two groups after 10 days (p > 0.05). Th1 lymphocyte stimulation occurred for the two vaccinated groups as shown at 10 days postvaccination. However, after 70 days, the concentration of IFN-γ in the two vaccinated groups was significantly higher than that of the negative control group (p < 0.05), but the concentration decreased after 10 days postvaccination. It showed that the stimulation of Th1 lymphocytes had gradually decreased from 10 to 70 days postvaccination.

The concentrations of IL-4 and IL-10 in the two vaccinated groups of mice were significantly different compared with the negative control group (p < 0.05), but the differences were not significant between the two groups after 10 days (p > 0.05). Th1 lymphocyte stimulation occurred for the two vaccinated groups as shown at 10 days postvaccination. However, after 70 days, the concentration of IFN-γ in the two vaccinated groups was significantly higher than that of the negative control group (p < 0.05), but the concentration decreased after 10 days postvaccination. It showed that the stimulation of Th1 lymphocytes had gradually decreased from 10 to 70 days postvaccination.

Challenge Test

PD₅₀ indicates the number of protective doses in a vaccine estimated from the resistance to live virus challenge of animal groups receiving different amounts of vaccine and calculated by the Reed and Münch method. The PD₅₀ for the conventional vaccine and GRI vaccine obtained were 6.28 and 7.07, respectively.
Discussion

Vaccination is the most important control and prevention strategy against microbial diseases. Gamma irradiation is a highly reliable procedure to inactivate viruses, with the advantage of minimal molecular changes to viral proteins and viral structure [21]. Virus inactivation by gamma radiation follows physical laws, including the concept of an ‘exponential law’, which means that an organism will probably survive irrespective of the irradiation dose. The value of such a probability is called the ‘sterility assurance level’, or SAL. SAL is in general a value of 10^6 (a one in a million chance of having live microorganisms) [22]. For the preparation of inactivated vaccine, the minimum inactivation endpoint is the most important parameter and is dependent on the inactivation rate, dose of irradiation and virus titer per volume unit [18]. The virucidal effect of gamma rays is associated with damage to the viral nucleic acid molecular structure [23–25]. There is much advanced research on using ionizing radiation to inactivate some viruses, bacteria and parasites, for example Lombardo and Smolko [26] from Argentina studied gamma-radiated FMDV with unaltered antigenicity as an inactivated vaccine. Furthermore, Motamedi-Sedeh et al. [27, 28] used irradiated inactivated FMDV type A 87/IRN as a GRI vaccine to immunize guinea pigs, which induced increasing neutralizing antibody titers. In addition, the ability of gamma ray-inactivated influenza virus to induce both a strong humoral as well as a potent Tc cell response should encourage the evaluation of this technique in the search for a vaccine against other viral diseases [21]. The inactivation endpoint for influenza virus was achieved in the absence of viral protein denaturation and with retention of virion integrity, which induced Tc cell responses and excellent antiviral antibody response. This gamma-flu vaccine is potent in eliciting cross-protective immunity against heterotypic viral challenges in mice [29].

In this research, we showed that radiation can safety inactivate the Iranian isolated FMD virus type O/IRN/2007 with unaltered antigenic characteristics, and the inactivated viral whole-virus antigen is used as a good candidate for the immunization of mice and guinea pigs to induce a high level of neutralizing antibody titers, IFN-γ, IL-4, IL-2 and IL-10 concentrations, as well as T cell proliferation. Therefore, the results indicate that this GRI vaccine can stimulate B cells, Th class 1 and Th class 2 lymphocytes, as well as the conventional vaccine. The concentrations of IFN-γ, IL-4, IL-2 and IL-10 for the GRI vaccine are higher than that of the conventional vaccine, albeit with a negligible difference (p > 0.05). Moreover, the concentration of IFN-γ was decreased after 70 days as compared to 10 days postvaccination; however, neutralizing antibody titers after 70 days were similar to those exhibited after 10 days postvaccination for the last time. If more stimulation of Th cells is required, then one booster dose of vaccine is needed after 70 days. This is not necessary for the neutralizing antibody.

The recommended potency level of standard FMDV vaccines is 3 PD50 for the duration of the shelf life claimed by the manufacturer. In addition, this vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks, higher potency vaccines (e.g. >6 PD50 for the duration of the shelf life claimed by the manufacturer) are recommended for their wider spectrum of immunity as well as their rapid onset of protection [30–34]. According to the PD50 results of the GRI vaccine (which was manufactured at the Nuclear Science and Technology Research Institute of Iran) and the conventional vaccine (which was manufactured in the Razi Vaccine and Serum Research Institute of Iran), both deliver more than 6 PD50, so these vaccines are suitable for both routine vaccination and control of FMDV outbreaks in naïve calves.

Other advantages of GRI vaccines are: (i) the short time needed for the inactivation process, which takes just a few minutes; (ii) lower risk of the virus escaping, and (iii) no chemical residue in the final product. The results of this work indicate that the GRI vaccine against FMDV type O/IRN/2007 could be a good candidate to immunize cattle, obviously requiring future studies in the target species.

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References


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