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Original Article

Altered adjuvant of foot-and-mouth disease vaccine improves immune response and protection from virus challenge ☆

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ABSTRACT

Vaccines for foot-and-mouth disease (FMD) generally use oil adjuvants. For better immunization and safety, an adjuvant should be selected only after careful consideration. In this study, we produced vaccines for O, A, and Asia1 serotypes by mixing oil adjuvants, Emulsigen-D (ED), ISA 201, and ISA 206 with and without an aluminum hydroxide (AL) gel and measured their immunogenicity and safety to obtain information regarding critical differences (survival or weight loss) of vaccine quality in mice; the goal of this test was to overcome the difficulties associated with experiments large or medium-sized animals. The groups immunized with the vaccines containing only the oil adjuvants (ED, ISA 201, and ISA 206) had similar or higher levels of neutralizing antibodies and structural protein antibodies for the FMD virus (FMDV) than the groups immunized with the vaccines including the oil adjuvants mixed with the gel. However, in a challenge test using a mouse model, the protection rate showed the highest results in ISA 201 and ISA 206 mixed with AL. The mice immunized with vaccines containing ED showed temporary weight loss in the early postvaccination stages. Cell-mediated immunity was formed relatively strongly in the group vaccinated with vaccines including ISA 201 and ISA 206. We proposed that combinations of these adjuvants represent candidates for future FMD vaccines.

1. Introduction

Foot-and-mouth disease (FMD) is a viral infectious disease that forms vesicles in the mouth and hooves of artiodactyls, such as pigs, cattle, sheep, and goats, resulting in weight loss, reduced milk production, and growth delays. The disease can be spread rapidly not only by the excrement of infected animals, but also by contaminated feed, vehicles, and humans. Thus, the economic damage is substantial once an outbreak occurs. Therefore, FMD is subject to international regulations for the global trade of both livestock and their products [1,2]. The administration of vaccines is a highly effective method for preventing FMD. The selection of an appropriate adjuvant is the most important factor in determining the efficacy of these vaccines. To ensure a prompt and appropriate response to outbreaks of FMD, we previously investigated the immune response and protective effects to develop vaccines, with the aim of identifying the vaccine with the best immunogenicity and protection against the virus O/Andong/SKR/2010, which was isolated during FMD outbreaks in Korea in 2010 and 2011 [3]. In another study [3], we performed an experiment using ISA 201, ISA 206, Carbigen, Emulsigen-D (ED), and an aluminum hydroxide ($Al(OH)_3$; AL), gel in order to select adjuvants for pigs and dairy goats. The ED with AL gel resulted in stronger immunity and protective effects compared to ED only. Thus, it remains to be determined whether the oil-based adjuvants ISA 201 and ISA 206 mixed with the gel result in better immune responses.

In studies of vaccine developments for FMD, it is desirable that the adjuvants are applied directly to susceptible target animals. However, such experiments are time-consuming and costly in pigs and cattle. It is difficult to obtain precise decisive data like mortality and body weight variation in dealing with large or medium-sized animals because of subjective analysis of protection or safety. Hence, to establish a prompt and accurate comparison of the newly developed adjuvant using a mouse model, we measured the immunity on each serotype using the vaccines containing O, A, or Asia1

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serotypes, which have been used for disease control in Asian countries and examined the safety and protection capability of each adjuvant. The results were compared with those of pigs, the target animals.

2. Materials and methods

2.1. Virus purification and inactivation

The FMD viruses O/Andong/SKR/2010 and Asia1/MOG/05 were used for antigen preparation in a BHK21 cell line. For virus infection, the culture medium was replaced with serum-free Dulbecco's modified Eagle's medium (Cellgro, USA), and the cell was inoculated with the virus. After 1 h of incubation at 37 °C in an atmosphere of 5% CO_2, the extracellular virus was removed. Twenty-four hours after infection, the viruses were inactivated by 0.003 N of BEI for 24 h and concentrated with polyethylene glycol 6000 (81260; Sigma Aldrich, WI, USA). The virus was layered on 15–45% sucrose density gradients and centrifuged [4]. After ultracentrifugation, the bottom of the centrifuge tube was punctured, and 1 ml fractions were collected. The presence of FMD viral particles and the FMD viral protein in a sample of each fraction were tested with a lateral flow device (BioSign™ FMDV Ag, PBM, USA). The concentrated and inactivated FMDV, A22 Iraq antigen for type A supplied by Merial Co. Ltd (UK) was used to manufacture the vaccines for the immunity and safety tests.

2.2. Preparation of the vaccines

The concentrated O/Andong/SKR/2010, A22 Iraq and Asia1/MOG/05 antigens were diluted with a Tris-NaCl buffer (with a pH of 7.6) and then added to each adjuvant: Emulisgen-D® (ED; MVP Technologies, USA), Montanide™ ISA 201 VG (ISA 201; Seppic, France), Montanide ISA 206 VG (ISA 206; Seppic, France), and aluminum hydroxide gel (AL; Rehydragel® HPA; General Chemical, USA). The ratio of the adjuvant to the total volume was 20:80 for ED and 50:50 for both ISA 201 and ISA 206 (volume [v]/v). In the oil/gel adjuvant mixture, 10% of AL was added. The mixture was stirred at 300 rpm for 10 min at 30 °C in a water incubator to form a water-in-oil-in-water blend. The stability of the vaccines was tested using the dropping method [5]. To maintain the same amount of antigen per dose of vaccine, the same amount of antigen was prediluted to the same concentration before mixing it with the adjuvant.

2.3. Immunization and FMDV challenge in the mice

In the first study, eight-week-old female BALB/c mice were divided into seven test groups ($n = 4$ in each group), and a nonvaccinated control (NVC) group ($n = 2$). The test groups were as follows: Carbigen, ED, ED + AL, ISA 201, ISA 201 + AL, ISA 206, and ISA 206 + AL. With the exception of the control group, all the mice were inoculated intramuscularly with an experimental vaccine containing 1 μg of inactivated antigen (O/Andong/SKR/2010), and they were inoculated once more with the same method seven weeks later. The serum of each mouse was collected 0, 2, 4, 6, 7, and 9 weeks after vaccination. Nine weeks after the first vaccination, all the mice were stimulated with 1 μg of purified virion antigen (O/Andong/SKR/2010) for cell-mediated immune responses, and cytokine assays were performed on blood samples collected 24 h after the stimulation.

In the second study, eight-week-old female C57BL/6 mice ($n = 3$) were used to compare the seven vaccinated groups (ED, ED + AL, ISA 201, ISA 201 + AL, ISA 206, ISA 206 + AL, and FMD trivalent vaccine [Aftopor® Trivalent, Merial, France]) and the nonvaccinated group. All the mice were vaccinated intramuscularly with an experimental vaccine containing 1 μg of inactivated antigen (A22 Iraq). On the 28th day after vaccination (dpv), all the mice were challenged with $10^{6.0}$ of $TCID_{50}$/0.1 ml of A22 Iraq by the intraperitoneal route. Sera were collected 0, 2, 4, and 6 weeks after vaccination and 3 days after the challenge, and antibodies to the virus and the FMDV structural proteins (SPs) were measured. For the safety test, the safety of the ED, ED + AL, ISA 201, and ISA 201 + AL vaccines was tested in eight-week-old female C57BL/6 mice. The FMDV trivalent vaccine was used as a control. In the safety test, each group ($n = 8$) received a subcutaneous vaccination containing 50 μl of each adjuvant. Each mouse was weighed daily for 10 days after vaccination.

In the third study, eight-week-old female C57BL/6 mice were divided into the seven groups (ED, ED + AL, ISA 201, ISA 20 + AL, ISA 206, ISA 206 + AL, and FMD trivalent vaccine [Aftopor® Trivalent, Merial, France]) ($n = 8$) and the nonvaccinated group ($n = 4$). First, to observe early immune reaction, the mice were vaccinated with 0.2 μg of inactivated antigen (Asia1/MOG/05) and challenged with 100 median lethal dose (LD_{50}) of Asia1/Sha/89 (Asia1 Shamir) after one week fater vaccination. To observe mid-term immune reaction, the challenge was performed in an identical manner four weeks after vaccination. The survival rate was checked for 10 days after the challenge. To investigate long-term immune reaction, the mice were challenged 12 weeks after vaccination and were observed for 10 days after the challenge.

2.4. Immunization and FMDV challenge in pigs

For the pig experiment, the pig serum employed in the previous study was used [3]. The 22 female pigs (eight-week-old) were divided into five test groups ($n = 4$ each group; Carbigen, ED, ISA 201, ISA 206, ED with AL [ED + AL]) and an unvaccinated control group ($n = 2$). All the pigs were inoculated with an experimental vaccine containing 10 μg of antigen per dose (2 ml). The serum of the pigs was collected into serum-separating tubes 0, 7, 14, 21, and 28 days after vaccination, and neutralizing antibodies were measured. At 28 days after vaccination, test pigs were challenged with $10^{5.0}TCID_{50}$/0.1 ml of FMDV O/Andong/SKR/2010 from the vesicles of infected pigs by intradermally injecting it into the foot pad. The sera collected one day after the challenge and 10 days after the challenge were used to measure the SP antibodies for the FMD virus and IL-12 and IL-4 cytokine secretion, respectively. In the pig test, the sera of only the ED, ISA 201, and ISA 206 groups were used after excluding the Carbigen and ED + AL.

2.5. Structural protein (SP) ELISA and nonstructural protein (NSP) ELISA

For the detection of SP antibodies, PrioCHECK FMDV type A and type O (Prionics AG, Schlieren-Zurich, Switzerland) were used. Nonstructural protein (NSP) antibodies were detected with a PrioCHECK FMDV NSP (Prionics AG, Schlieren-Zurich, Switzerland) ELISA kit in serum samples of mice. The absorbance of the ELISA plate was converted to the percent inhibition (PI) value. When the PI was 50% or above, the mice were regarded as antibody positive.

2.6. Virus neutralization test

Titers of neutralizing antibodies in the serum were measured with a virus neutralization test. Serum samples were collected from the animals after the vaccinations and the virus challenge. The serum samples were heat inactivated at 56 °C for 30 min. Following incubation of the test serum with FMDV 100 $TCID_{50}$/0.1 ml for 1 h, LF-BK cells were added to the plate and incubated

for two–three days. The cytopathic effect was used to determine the titers that were calculated as the log10 of the reciprocal antibody dilution to neutralize 100 $TCID_{50}$ of the virus [6].

2.7. Analysis of the replication of the FMDV in the mice

Real-time RT-PCR was performed for heparinized blood and swab samples from the test animals. The swab samples were gathered from the mouth and nose using cotton swabs. Total cellular RNA was extracted using the MagNa pure 96 system (Roche, Germany) according to the manufacturer's protocol. Real-time RT-PCR was conducted using the one-step Primescript RT-PCR kit (TAKARA, Otsu, Japan). These procedures were conducted according to the manufacturer's instructions. Primers targeting the FMDV 3D region were sense 5′-GGAACYGGGTTTTAYAAACCTGTRAT-3′ and antisense 5′-CCTCTCCTTTGCACGCCGTGGGA-3′. The probe was 5′-CCCADCGCAGGTAAAGYGATCTGTA-3′. Its 5′ end was labeled with 6-FAM, and the 3′ end was labeled with TAMRA. The CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, USA) was used [7].

2.8. Determination of cell-mediated immunity

Th1 cytokine and Th2 cytokine secretion were identified with Bio-Plex Pro™ cytokine assays (Bio-Rad, USA). After the second immunization with the vaccines, all mice were stimulated with the inactivated antigen O/Andong/SKR/2010 (1 μg/mouse). One day later, the secretion of Th1 (IL-2, IL-12, GM-CSF, and IFN-gamma, and TNF-alpha) and Th2 cytokines (IL-4, IL-5, and IL-10) was measured.

For the pig experiment, a porcine IL-4 ELISA kit (R&D Systems, MN, USA) and a porcine IL-12/IL-23 p40 Quantikine ELISA kit (R&D Systems, MN, USA) were used.

2.9. Statistical analysis

Student's *t*-test was performed with GraphPad Instat® v. 3.05 (GraphPad Software, CA, USA) to examine the immunogenicity and protective effects of the vaccines.

3. Results

3.1. Immune responses after vaccination with oil or gel-mixed adjuvants in mice

Seven weeks (49 days) after the first vaccination with the type O antigen, boosting was performed with the same vaccines, and changes in the antibody titers were observed until the ninth week (64 days). High levels of antibodies after the first vaccination were detected in the ED, ED + AL, and ISA 201 vaccination groups (>1:100 of virus neutralizing [VN] titer) (Fig. 1A, B). The other vaccination groups produced relatively high levels of neutralizing

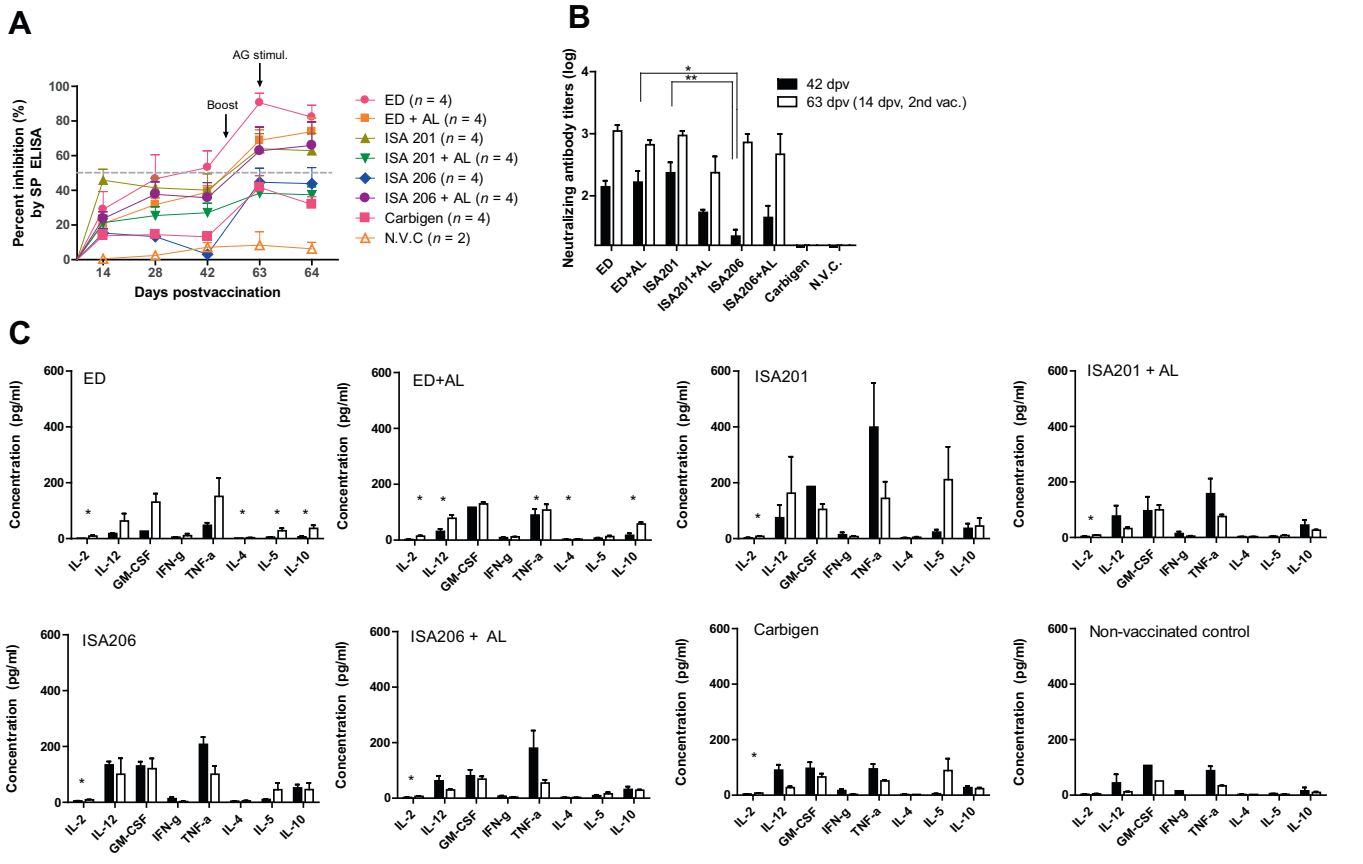

Fig. 1. Immune responses in the mice vaccinated with the various adjuvants for the experimental type O foot-and-mouth disease (FMD) vaccine. All BALB/c mice were vaccinated with the experimental type O (O/Andong/SKR/2010) FMD vaccine mixed with the various adjuvants and boosted 49 days later with the same vaccine. The vaccination dose was 1/10 (1 dose = dose for one cow or pig). (A) Antibody titers of structural protein (SP) against the FMD virus (FMDV) in the serum were measured by ELISA 14, 28, 42, 63, and 64 days postvaccination (dpv). All the mice were stimulated with 2 μg of purified antigen 63 dpv. Percent inhibition (PI)>50 was considered the cutoff of a positive reaction. (B) Serum-neutralizing antibody responses (log 10) to the various adjuvanted FMD vaccines. NVC: nonvaccinated control. $^{*}p < 0.05$, $^{**}p < 0.01$. The error bar represents the standard error of the means. (C) Th1 (IL-2, IL-12, GM-CSF, IFN-gamma, and TNF-alpha) and Th2 (IL-4, IL-5, and IL-10) cytokines in the adjuvanted vaccination groups were identified. Black-filled rectangle: 14 days after the second vaccination. White-filled rectangle: 1 day after stimulation (15 days after the second vaccination) with purified virion antigen (O/Andong/SKR/2010) of 1 μg/head. $^{*}p < 0.05$. The error bar represents the standard error of the means.

antibodies (>1:100) only after the second vaccination (Fig. 1B). Six weeks (42 days) after the first vaccination, ISA 201 exhibited the highest potential for the production of neutralizing antibodies, followed by ED + AL and ED. The ED and ISA 206 adjuvants mixed with AL did not result in statistically significant increases compared to unmixed oil adjuvants. The high level of neutralizing antibodies in the ED + AL or ISA 201 groups exhibited a statistically significant difference from that in the ISA206 group ($p < 0.05$). Nine weeks (63 days) after the vaccination, the highest level of neutralizing antibodies was detected in the ED group. The neutralizing antibodies of the ED, ISA 201, and ISA 206 groups exhibited no statistically significant differences from those of the AL-mixed groups with AL ($p > 0.05$) (Fig. 1B).

In the analysis of cell-mediated immunity through Th1 (IL-12, GM-CSF, and TNF-α) and Th2 (IL-5, and IL-10) cytokines within the sera of mice stimulated with inactivated antigen after the second vaccination, ISA 201 and ISA 206 groups exhibited the strongest cytokine secretion (Fig. 1C).

3.2. Safety and viremia after vaccination and challenge

To identify the protective effects and the safety of the adjuvants, the type A (A22 Iraq) antigen was selected and used in a challenge experiment with the various adjuvanted vaccines in the C57BL/6 mouse [8]. SP ELISA antibodies to the FMDV were detected in the serum two weeks (14 dpv) and six weeks (42 days) after the first vaccination (Fig. 2A). High levels of neutralizing antibodies were detected in sera of the ED, ED + AL, and ISA 201 vaccination groups two weeks (14 days) post vaccination. The level of decline in neutralizing antibodies was lower in the ISA 201 + AL group than in the ISA 201 group. It was also lower in the ISA 206 group than in the ISA 201 and ED groups. Only a low level of neutralizing antibodies was detected in the commercial trivalent vaccine vaccination group (Fig. 2B).

Fig. 2C shows the body weight changes in mice resulting from the ED and ISA 201 adjuvants mixed with AL inoculation, and all tested mice were alive during the safety test. However, most of the mice in the ED and ED + AL groups underwent weight loss until the second day after vaccination but exhibited a pattern of recovery from the fourth day after vaccination (Fig. 2C). The ISA 201 and ISA 201 + AL vaccination groups exhibited no pattern of weight loss compared to the ED or ED + AL groups ($p < 0.05$ in the second days after vaccination). The currently used commercial trivalent vaccine was associated with about a 2% decline in weight until the second days after vaccination. The body weight recovered from the fourth day and increased gradually thereafter. Three days after the virus challenge, low-titer viruses were detected only in the sera of the groups inoculated with the trivalent vaccine and ED + AL (Fig. 2D).

3.3. Comparison of vaccination experiments in mice

When the results of many adjuvants in the first and second experiments were compared (Table 1), the ED, ED + AL, and ISA 201 vaccination groups showed high levels of immunogenicity (>1:100 of VN titer). The groups immunized with ED exhibited a weight loss. In terms of cell-mediated immune responses, the ED and ED + AL vaccination groups exhibited statistically significant increases after antigen stimulation in both Th1 and Th2 cytokines, although they exhibited a low level of cytokines. Th1 reactivity was

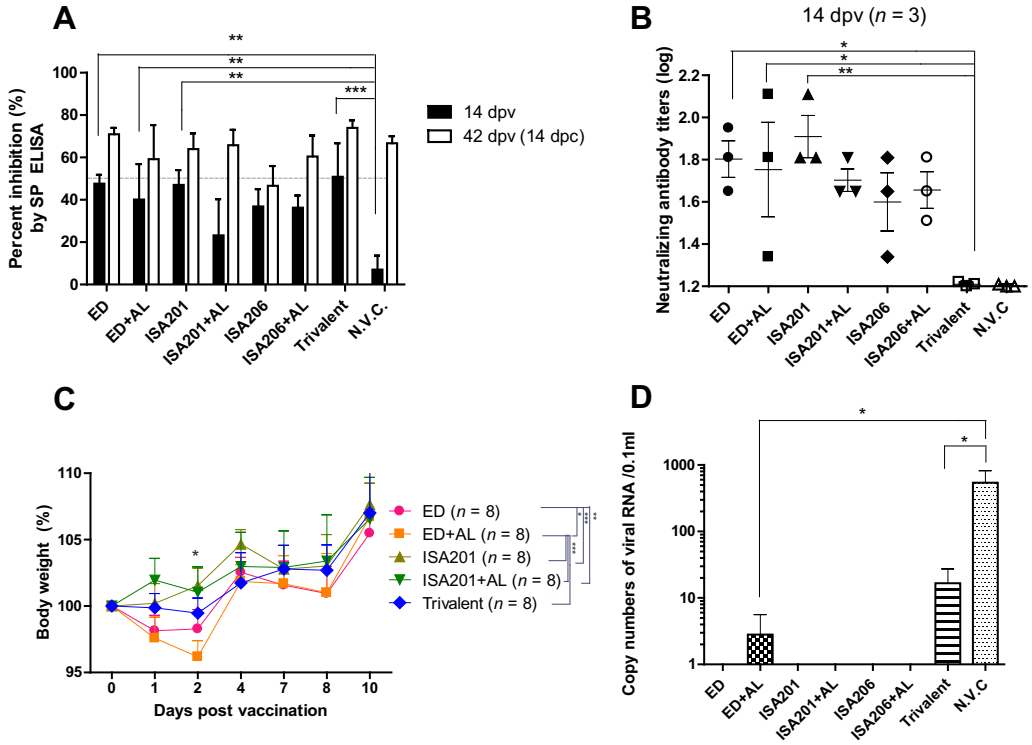

Fig. 2. Protective profile and virulence in mice of the various adjuvants for the experimental type A FMD vaccine in the mice. (A) Antibody titers of structural protein (SP) against the FMDV in the serum were measured by ELISA 14 and 42 dpv. (B) Serum-neutralizing antibody responses (log 10) for the various adjuvanted type A (A22 Iraq) FMD vaccines. All the C57BL/6 mice were vaccinated with the indicated adjuvanted vaccine, and each serum sample was collected 14 dpv. (C) Body weight change after vaccination with the various adjuvants. All the C57BL/6 mice were vaccinated via the intraperitoneal route. (D) All the mice were vaccinated with the various adjuvants and challenged with the A22 FMDV 28 dpv. Three days after the challenge, serum was collected, and virus titers in the serum were determined by real-time PCR. The error bar represents the standard error of the means. Trivalent: trivalent vaccine (Merial), NVC: nonvaccinated control. The error bar represents the standard error of the means. Student-*t* test was performed and the values were expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1
Summary of the protective effect of the various adjuvants and the immune responses after vaccination in mice.

Groups vaccinated with the various adjuvants	Vaccination twice[a]						Vaccination once[b]			
	Rate (%) of animals with a high VN titer[c]		No. of cytokines of high level [d] (after 2nd vac./after antigen stimul./total)		No. of cytokines showing a significant changed ($p < 0.05$) after antigen stimul. (reacted/total)		Rate (%) of animals with a high VN titer	Rate (%) of animals with viremia after challenge	Rate(%) of animal with body weight loss	
	42dpv[j]	63dpv	Th1[e]	Th2[f]	Th1[e]	Th2[f]	14dpv		2dpv	7dpv
ED [g]	75	100	0/1/5	0/0/3	1/5	3/3	0	0	88	13
ED + AL[h]	50	100	0/2/5	0/0/3	3/5	2/3	33	33	100	25
ISA 201	75	100	1/3/5	0/1/3	1/5	0/3	33	0	13	0
ISA 201 + AL	0	75	1/1/5	0/0/3	1/5	0/3	0	0	25	25
ISA 206	0	100	3/3/5	0/0/3	1/5	0/3	0	0	n.d.	n.d.
ISA 206 + AL	25	100	1/0/5	0/0/3	1/5	0/3	0	0	n.d.	n.d.
Carbigen	0	0	0/0/5	0/0/3	1/5	0/3	n.d	n.d	n.d	n.d
Trivalent vaccine	n.d. [k]	n.d.	n.d.	n.d.	n.d.	n.d.	0	67	63	0
NVC [i]	0.0	0.0	0/0/5	0/0/3	0/5	0/3	0	100	n.d	n.d

[a] BALB/c mice vaccinated twice with the experimental O/Andong/SKR/2010 vaccine.
[b] C57BL/6 mice vaccinated once with the experimental A22 Iraq vaccine or commercial vaccine (types O, A, Asia1).
[c] A high virus neutralizing (VN) antibody titer was defined as a value of >1:100.
[d] A high level of cytokines in sera was defined as a value of >100 pg/ml.
[e] Th1 type includes five cytokines; IL-2, IL-12, GM-CSF, IFN-gamma, and TNF-alpha.
[f] Th2 type includes three cytokines; IL-4, IL-5, and IL-10.
[g] ED: Emulsigen-D.
[h] AL: aluminum hydroxide.
[i] NVC: nonvaccinated control.
[j] dpv: days postvaccination.
[k] n.d.: not done.

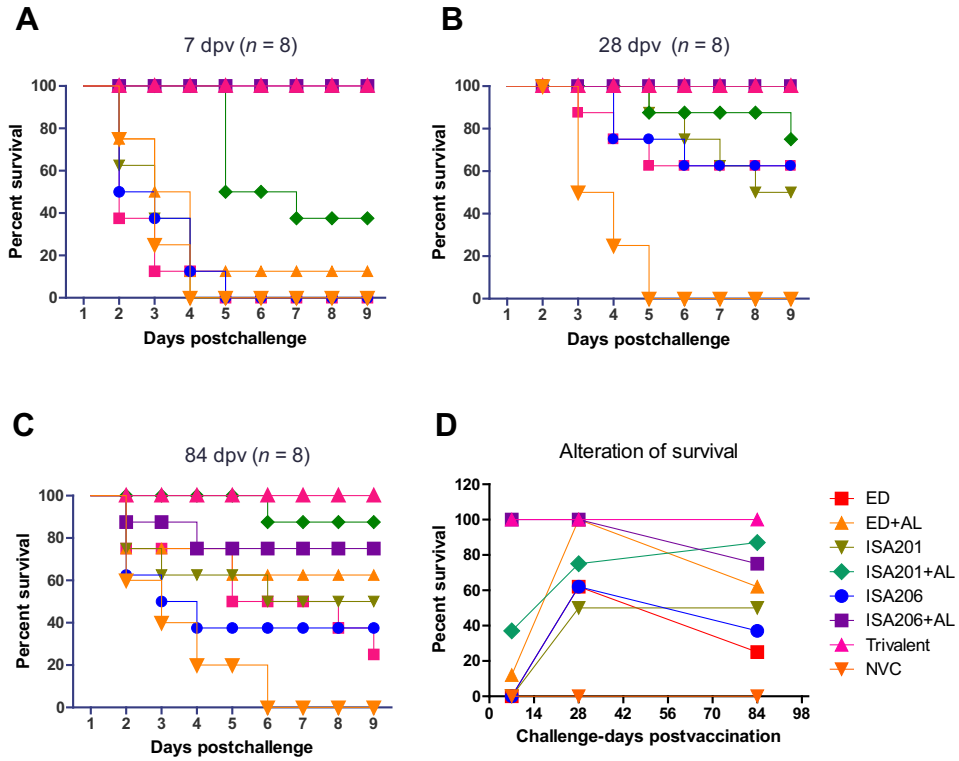

Fig. 3. Variation of mouse survival after type Asia1 vaccination according to the various adjuvants and various challenge points. (A) Survival rate of C57BL/6 mice after vaccination (7 dpv) and challenge with the various adjuvants. All the mice were vaccinated via the intramuscular route. One week after vaccination, all the mice were challenged with 100 LD_{50} of Asia1 Shamir FMDV. (B) Survival rate of C57BL/6 mice after vaccination (28 dpv) and challenge with the various adjuvants. (C) Survival rate of C57BL/6 mice after vaccination (84 dpv) and challenge with the various adjuvants. (D) Variation of survival rate according to different adjuvants and various challenge points.

stronger in the ED + AL vaccination group than the ED-only vaccination group. The ISA 201 group had a high level (>100 pg/ml) of IL-12, GM-CSF, and TNF-alpha both days on days 63 and 64 after the second vaccination and antigen stimulation (Table 1 and Fig. 1C).

In the safety test, 88% of the mice in the ED group exhibited weight loss on the second day after vaccination. Although weight loss was observed in all the mice in the ED + AL vaccination group, on the seventh day after vaccination, the level of weight loss was low in the ED and ED + AL vaccination groups (13% and 25%, respectively). The weight loss in the ISA 201 and ISA 201 + AL vaccination groups was 13% and 25%, respectively on the second day after vaccination. However, all the animals in the ISA 201 vaccination group showed weight loss recovery, whereas those in the ISA 201 + AL vaccination group did not, with the weight loss continuing subsequent to the fourth day after vaccination (Table 1).

3.4. Variation of mouse survival by various challenge times

To compare the protection ability after vaccination, the survival rate was observed following a vaccination and challenge of Asia1 Shamir to a challenge model of C57BL/6 mice. First, an early immunity reaction was observed during the first week after vaccination (Fig. 3A). As shown in Fig. 3A, the ISA 206 + AL group had the strongest protection and early protection (seven days after vaccination) was also possible in the ISA 201 + AL group. The virus challenge was performed four weeks after vaccination to observe the mid-term immunity (28 days after vaccination), and the survival rate improved overall compared to the short-term (seven days after vaccination) group (Fig. 3B). In particular, all vaccinated mice survived in the case of ISA 206 + AL and ED + AL groups, and the ISA 201 + AL group showed the second highest survival rate. In the long-term (84 days after vaccination) group (Fig. 3C), the survival rate was highest in the ISA 201 + AL group, and second highest in the ISA 206 + AL group. In the ISA201 + AL, and ISA206 + AL groups, the survival rate was highly maintained until day 84 after vaccination (Fig. 3D).

3.5. Comparison with the immunity results in pigs

We tested the immunogenicity and cytokine secretion in pigs, the target animals, when either ED, ISA 201, or ISA 206 was used as the adjuvant. Fig. 4A shows the measurement of neutralizing antibodies after vaccination and challenge. Whereas the highest concentration of neutralizing antibodies was detected in the ED group in the early stage (7 day postvaccination [dpv]), the highest concentration was detected in the ISA 206 group 14dpv and in ISA 201 21dpv and 28dpv. The antibody against NSPs of FMDV in the sera was measured ten days after the challenge. According to the results, the lowest concentration of the NSP antibody was detected in the ISA 201-adjuvanted vaccine group, indicating no replication of virus in the animals. A similar level of the NSP antibody was detected in the ED and ISA 206 groups (Fig. 4B). The Th1-type cytokine of the IL-12 and the Th2-type cytokine of IL-4 were measured, and a higher level of the Th1-type cytokine was detected in the ED and ISA 201 groups (Fig. 4C). However, a similar level of the Th2-type cytokine of similar level was detected in four groups (Fig. 4D).

4. Discussion and conclusions

The selection of adjuvants in FMD vaccine formulation is important for both early and long-lasting immunity and protection. We previously investigated the immunogenicity and antigenicity of vaccine adjuvants using pigs and dairy goats as target animals

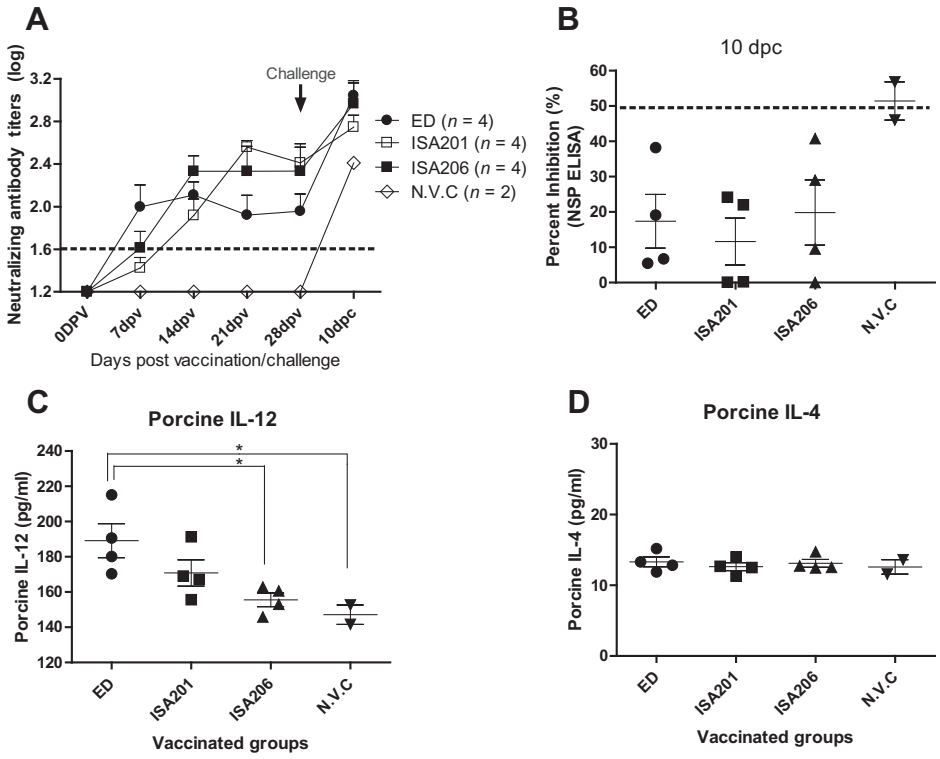

Fig. 4. Protective profile in pigs of the various adjuvants for the experimental type O FMD vaccine. (A) Neutralizing antibody titers in the sera by various experimental FMD vaccines until four weeks after vaccination and 10 days postchallenge (dpc). Neutralizing antibody titers of >1:45 (1.653 log10) was considered the cutoff of protectable value. (B) Antibodies against non-structural protein (NSP) of FMDV from challenged animals in the porcine sera were measured by NSP-ELISA 10 dpc, PI > 50 was considered the cutoff of a positive reaction. (C) IL-12 cytokine secretion in porcine serum was detected 1 dpc. All the pigs were inoculated with an experimental vaccine containing 10 μg of antigen per dose (2 ml). At 28 dpv, test pigs were challenged with FMDV O/Andong/SKR/2010, (D) IL-4 cytokine secretion in porcine serum was detected at 1 dpc. Student's *t*-test was performed and the values were expressed as $^{*}p < 0.05$.

for the FMD vaccine [3]. The $Al(OH)_3$ gel (AL) is the most commonly used adjuvant in commercial vaccines [15]. A previous report showed that AL induces Th2-type responses in animal models, facilitating the dissemination of antibodies from the injected region [9,10]. In addition, the gel was shown to play an important role in memory responses by inducing the differentiation of macrophages. Gel-adjuvanted FMD vaccines are currently used only in cattle, because they offer only a short period of immunity, making them unsuitable for use in pigs [11]. Moreover, the immune responses in sheep and goats are poorer than those of oil-based vaccines [12,13]. The combined components of oil and AL have been used to protect against rabies in bovines [16]. However, the components for FMD have not been studied. We confirmed that vaccine's stability, which means no separating as oil-layer and water layer in the contents of experimental vaccine, had maintained for three months.

In a previous study [3], we tested a combination of oil with a gel to enhance the immune responses. We found that the inoculation with ED + AL produced superior effects in pigs or goats, target animals. We tentatively concluded that AL improves the effects of ED adjuvants; in present study, we thus attempted to demonstrate that mixing with various oil-adjuvant and gel would induces similar protection in mice. We confirmed slight increases in the level of humoral immune response in the ED + AL and ISA 206 + AL groups and a reduced level of immunity in the ISA 201 + AL group. Therefore, not all types of oil vaccines are likely to induce the same immune reactions. We also examined the reactivity of the Th1/Th2 paradigm, which is a new indicator of immune response [14]. Although most of the vaccination groups showed Th1 reactivity (IL-2 cytokine responses) after antigen stimulation following the second vaccination, only the ED and ED + AL vaccination groups exhibited statistically significant changes in Th2-type cytokine responses. Furthermore, the ISA 201 and ISA 206 vaccination groups showed a high level of Th1 and Th2 cytokines in the days following the second vaccination or antigen stimulation. Through the formation of strong immune responses for both Th1 and Th2 type, we confirmed the immune stimulation effects of the adjuvants. The addition of AL did not induce a substantial change in the formation of antibodies. Moreover, the associated weight loss in the vaccination groups with the AL gel added to the vaccination-related stress. According to the protection results using the challenge model, ISA 201 + AL and ISA 206 + AL showed the best performance in short-, mid-, and long-term immune reactions. The protection rate of the oil vaccine was not high in the case of oil-only use, but it improved remarkably when mixed with gel. Th1-type cytokine in the pigs was relatively strongly detected in the ED and ISA 201 groups, indicating a potential impact on protection ability. In NSP ELISA or virus neutralization antibody tests in pigs, ISA 201 had the best results. Summarizing the evaluation results on the immunity, safety, and protection of the test animal and target animal, we suggest that ISA 201 and ISA 206 are effective. In the case of ED + AL, survival effects after the mid-term period (28 dpv) were observed. In a previous study, it was confirmed that the mixed use of ED and gel can improve the protection ability in the target animal [3]. However, no research has examined the direct improving potential of the mixed use of ISA 201 or ISA206 and AL gel, and hence additional experiments in target animals is necessary. The current findings differ slightly from those of our previous experiment using pigs [3], in which increased immunity was detected. Differences in the immunity and injected dose according to species, small laboratory animals (mice) and target animals (pigs), may explain the discordant findings.

In conclusion, this study showed increases in the protective effects of adding AL to ED, ISA 206, and ISA201. In the case of ISA 201, this mixture produced reduced levels of humoral immunity. However, ISA 201 and ISA201 + AL resulted in better cell-mediated immune response and safety in mice. Weight loss was observed for a short period after vaccination in the ED and ED + AL groups post vaccinations. Because this study used mice, although the safety and weight loss can be expected to be different in large and medium-sized animals susceptible to FMD when vaccinated in the field, prior to the application of ED, ISA 206 or ISA 201 with gel in the target animals in the field, the experiments designed to determine its safety in the small laboratory animal would be needed. Oil-adjuvanted vaccines can result in the formation of local lesions in the injected areas. Thus, to avoid granuloma, long-term studies with a reduced volume of vaccines should be carried out to identify whether such lesions increase or decrease when AL gel is added to the vaccines. In addition, comparative studies on long-lasting immunity in experimental and target animals may be required for the development of new vaccines.

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References

[1] R.P. Kitching, Foot-and-mouth disease: current world situation, Vaccine 17 (1999) 1772–1774.
[2] R.F. Meyer, R.C. Knudsen, Foot-and-mouth disease: a review of the virus and the symptoms, J. Environ. Health 64 (2001) 21–23.
[3] M.E. Park, S.Y. Lee, R.H. Kim, M.K. Ko, K.N. Lee, S.M. Kim, et al., Enhanced immune responses of foot-and-mouth disease vaccine using new oil/gel adjuvant mixtures in pigs and goats, Vaccine (2014).
[4] J.N. Park, S.Y. Lee, J.Q. Chu, Y.J. Lee, R.H. Kim, K.N. Lee, et al., Protection to homologous and heterologous challenge in pigs immunized with vaccine against foot-and-mouth disease type O caused an epidemic in East Asia during 2010/2011, Vaccine 32 (2014) 1882–1889.
[5] P.D. McKercher, J.H. Graves, A review of the current status of oil adjuvants in foot-and-mouth disease vaccines, Dev. Biol. Stand. 35 (1976) 107–112.
[6] V.L. Fowler, N.J. Knowles, D.J. Paton, P.V. Barnett, Marker vaccine potential of a foot-and-mouth disease virus with a partial VP1 G-H loop deletion, Vaccine 28 (2010) 3428–3434.
[7] S.M. Kim, J.H. Park, K.N. Lee, S.K. Kim, Y.J. Ko, H.S. Lee, et al., Enhanced inhibition of foot-and-mouth disease virus by combinations of porcine interferon-alpha and antiviral agents, Antiviral Res. 96 (2012) 213–220.
[8] F.J. Salguero, M.A. Sanchez-Martin, F. Diaz-San Segundo, A. de Avila, N. Sevilla, Foot-and-mouth disease virus (FMDV) causes an acute disease that can be lethal for adult laboratory mice, Virology 332 (2005) 384–396.
[9] R.K. Gupta, B.E. Rost, E. Relyveld, G.R. Siber, Adjuvant properties of aluminum and calcium compounds, Pharm. Biotechnol. 6 (1995) 229–248.
[10] J.M. Brewer, M. Conacher, A. Satoskar, H. Bluethmann, J. Alexander, In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production, Eur. J. Immunol. 26 (1996) 2062–2066.
[11] J.H. Park, Requirements for improved vaccines against foot-and-mouth disease epidemics, Clin. Exp. Vaccine Res. 2 (2013) 8–18.
[12] P.K. Patil, J. Bayry, C. Ramakrishna, B. Hugar, L.D. Misra, C. Natarajan, Immune responses of goats against foot-and-mouth disease quadrivalent vaccine: comparison of double oil emulsion and aluminium hydroxide gel vaccines in eliciting immunity, Vaccine 20 (2002) 2781–2789.
[13] P.K. Patil, J. Bayry, C. Ramakrishna, B. Hugar, L.D. Misra, K. Prabhudas, et al., Immune responses of sheep to quadrivalent double emulsion foot-and-mouth disease vaccines: rate of development of immunity and variations among other ruminants, J. Clin. Microbiol. 40 (2002) 4367–4371.
[14] S. Romagnani, T-cell subsets (Th1 versus Th2), Ann. Allergy Asthma Immunol. 85 (2000) 9–18. quiz, 21..
[15] A.C. Rimaniol, G. Gras, et al., Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type, Vaccine 13 (22) (2004) 3127–3135.
[16] G.S. Reddy, V.A. Srinivasan, Performance of aluminium hydroxide gel and oil adjuvant rabies vaccines in bovines, Zentralbl Bakteriol 286 (1997) 523–526.