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Synergistic effect of ribavirin and vaccine for protection during early infection stage of foot-and-mouth disease

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In many countries, vaccines are used for the prevention of foot-and-mouth disease (FMD). However, because there is no protection against FMD immediately after vaccination, research and development on antiviral agents is being conducted to induce protection until immunological competence is produced. This study tested whether well-known chemicals used as RNA virus treatment agents had inhibitory effects on FMD viruses (FMDVs) and demonstrated that ribavirin showed antiviral effects against FMDV *in vitro*/*in vivo*. In addition, it was observed that combining the administration of the antiviral agents orally and complementary therapy with vaccines synergistically enhanced antiviral activity and preserved the survival rate and body weight in the experimental animals. Antiviral agents mixed with an adjuvant were inoculated intramuscularly along with the vaccines, thereby inhibiting virus replication after injection and verifying that it was possible to induce early protection against viral infection prior to immunity being achieved through the vaccine. Finally, pigs treated with antiviral agents and vaccines showed no clinical signs and had low virus excretion. Based on these results, it is expected that this combined approach could be a therapeutic and preventive treatment for early protection against FMD.

Keywords: foot-and-mouth disease, ribavirin, vaccine

Introduction

Foot-and-mouth disease (FMD) occurs in animals with cloven hooves. FMD viruses (FMDVs) are a positive-sense single-stranded RNA virus [16] and belong to the genus *Aphthovirus*, family *Picornaviridae* [4]; the size of the virus genome is approximately 8.5 kb. These viruses are classified into seven serotypes—A, O, Asia1, C, SAT1, SAT2, and SAT3— and more than 60 subtypes [16]. FMD outbreaks cause a reduction in the productivity of infected animals (*e.g.*, pigs, cows, lambs, and goats) [2,3]. The culling of infected animals and events such as environmental contamination and loss of revenue from the livestock have emerged as important socioeconomic issues [21,23].

Thus far, vaccination is the preferred method for prevention of FMDV infection. However, the immune response takes 7 to 30 days [10]. Antiviral agents using chemical compounds are easily synthesized and scaled up in bulk, and they can be stored for a long period because chemical compounds are more stable than biologics. In addition, it was reported that such agents act directly on viruses as RNA inhibitors and are not specific to certain serotypes; thus, they may be administered to animals for emergency use when FMD occurs. FMD has never been eradicated and occurs in many countries in the world; regardless, research on antiviral agents to prevent and treat FMDVs is still limited.

During the past two decades, research on mutagenic nucleoside analogs has been conducted on compound agents such as ribavirin, 5-fluorouracil, 5-azacytidine, 2'-C-methylcytidine, and T1105 to protect against RNA viruses. T1105, a favipiravir derivative, exhibited antiviral activity at a high dose (200−400 mg/kg/day) in pigs [26]. It was also reported that pyrimidylthiophene and 2-aminothiazole compounds showed antiviral activity against FMDV-infected cells [9]. Another study reported that

ribavirin may be helpful in inhibiting the replication of FMDVs in cells and protecting against their transmission in mice [33]. The antiviral agent interferon alpha (IFN-α) was expressed using a recombinant adenovirus to verify its protective effect against FMDV in pigs [8,12]. A high concentration of IFN-α was measured in serum from pigs [6,19] to verify the effects of the adenovirus. By utilizing IFN-α, IFN-γ, and a recombinant adenovirus in combination, inhibition of the replication of FMDV was induced, delaying clinical symptoms [14,15]. Moreover, protection against FMDV in adult mice using IFN inducers such as polyinosinic (poly C) [28] and CpG [13] has been verified [31], and it was reported that protection against FMDV was possible in pigs by using IFN combinations [20]. It was also reported that antiviral agents (compounds, IFN, small interfering RNA) were helpful in protecting against FMD [1,5,6,22].

Antiviral agents have emerged to complement the early immunity of vaccines or to replace vaccines when emergency inoculation is required. For such reasons, the development of more appropriate antiviral agents is important for protection against FMD and prevention of its transmission. In particular, one report stated that treatment with ribavirin along with chemical agents provided an excellent inhibitory effect against RNA virus replication in cells [25] and guinea pigs [30]. However, there have been no reports of the ribavirin effect against FMD in pigs.

In this study, to examine the ability of antiviral agents to inhibit FMDV replication and to determine whether they provide protection in animals, a series of experiments was conducted to determine an appropriate dosage and method of administration. Even though there is a limitation of field application of ribavirin as a dose-dependent side effect: hemolytic anemia [7,18], it was evaluated for its antiviral effect with a proper tool to overcome disadvantage on FMDV-infected cells and model animals. It was demonstrated that complementary treatment with ribavirin and vaccines are synergistically effective in sustaining survival rates and body weights of infected animals, with no deaths during the experimental period. In this study, we intended to verify whether ribavirin was appropriate for inhibiting FMDV replication in cells and preventing clinical disease in laboratory or target animals, without side effects, by co-administering the agent with emergency vaccines for early FMD protection.

Materials and Methods

Cells and viruses

Swine kidney cells (IBRS-2) were cultured for 1 day in a 37°C, 5% CO_2 incubator with 10% fetal bovine serum (FBS; pH 7.4) and 1% antibiotics added to minimum essential medium-alpha. Virus titers were determined in IBRS-2 for FMDV. Three days later, the 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Reed and Muench method. FMDV O/Andong/SKR/2010 (GenBank accession No. KC503937) was used for challenging the pigs.

In vitro evaluation of antiviral agents

IBRS-2 cells were cultured for 1 day in a 37°C, 5% CO_2 incubator in 96-well plates that were inoculated with FMDV O/SKR/2002 of 100 $TCID_{50}$. After 1 h, the antiviral agents ribavirin (Sigma, USA), 6-azauridine (Sigma), or T1105 (Alfa Aesar; Johnson Matthey, United Kingdom) were serially diluted (3.25−400 μM) and added. In a 5% CO_2 incubator at 37°C, cells were treated for 3 h with MTS (CellTiter 96 Aqueous One Solution Replication Assay; Promega, USA) after 48 h of incubation. Absorbance was measured with a microplate reader at 490 nm (Molecular Device, USA), and the 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) were calculated using GraphPad 5.0 (GraphPad Software, USA). EC_{50} is the effective concentration where the cytopathic effect is 50% with the control value as the standard; the CC_{50} is the cytotoxicity concentration at which normal cells account for 50% of the total cells based on the control value (Table 1).
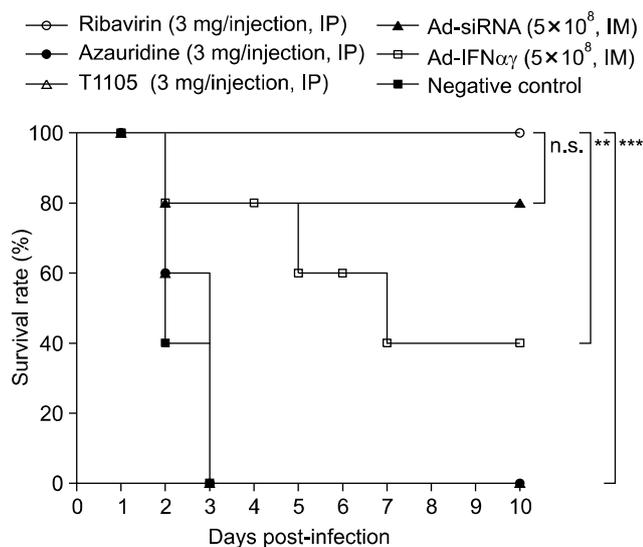
In vivo evaluation of anti-FMDV activity of antiviral agents

The inhibitory effect on FMDV replication was tested in mice using ribavirin, 6-azauridine, T1105, and recombinant adenoviruses, which are antiviral agents known to inhibit RNA virus replication (Fig. 1). Seven-week-old, 17 to 19 g C57BL/6 mice were purchased from the Orient (Korea). Intraperitoneal (IP) inoculations of 3 mg of ribavirin, 6-azauridine, and T1105 were performed at zero to three days. A 3 mg/dose of ribavirin or 6-azauridine was injected IP twice per day from day 0 to day 3. A 3 mg/dose of T1105 was injected IP at 0, 8, 13, 24, and 30 h. Six hours after the agent was first administered, the mice (n = 5 per group) were IP inoculated with 50 LD_{50} (50% lethal dose) Asia1 Shamir [24]. In addition, as a positive control, intramuscular inoculation of recombinant adenovirus Ad-3siRNA expressing

Table 1. Cytotoxic responses and effectiveness of antiviral agents in IBRS-2 cells against foot-and-mouth disease virus (FMDV)

Agents[*]	CC_{50} (μM)	EC_{50} (μM)	Selectivity index (CC_{50}/EC_{50})
Ribavirin	1,898.50	28.09	67.59
T1105	341.05	68.65	4.97
Azauridine	1,893.00	343.70	5.51

Data are presented as mean ± SD. CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration. [*]IBRS-2 cells were inoculated with 100 $TCID_{50}$ ($TCID_{50}$, 50% tissue culture infective dose) FMDV, O/SKR/2002 for 1 h, and then treated with antiviral agents after removing the supernatants.

Fig. 1. *In vivo* evaluation of anti–foot-and-mouth disease virus (FMDV) activity of antiviral agents. Mice (n = 5 per group) were injected through the intraperitoneal (IP) or intramuscular (IM) routes with several antiviral candidates for protection against FMD. Six hours after the mice were injected with the first antiviral agents, 50 LD_{50} (LD_{50}, 50% lethal dose) of Asia1/Shamir was challenged IP. A 3 mg/dose of ribavirin or 6-azauridine was injected IP twice per day from day 0 to day 3. A 3 mg/dose of T1105 was injected IP at 0, 8, 13, 24, and 30 h. One day before inoculation with FMDV, 5×10^8 $TCID_{50}$ (50% tissue culture infective dose) of Ad-3siRNA or Ad-IFNαγ was injected IM. The *p* value is < 0.0001 (repeated measures ANOVA). n.s., no significant difference; IFN, interferon. **p < 0.01, ***p < 0.001.

three siRNAs against FMDV and Ad-IFN-αγ of 5×10^8 $TCID_{50}$ expressing concurrently IFN-α and IFN-γ [14], these were injected 1 day before the mouse challenge. At day 0, the challenge virus was injected IP. The survival rates for all mice were monitored for 10 days. The animals were treated in accordance with the ethical guidelines of the Animal Welfare Committee of the Animal and Plant Quarantine Agency (APQA). All animal procedures were approved by the Institutional Animal Care and Use Committee of the APQA (approval No. 2015-04). All efforts were made to minimize animal suffering.

Comparison of administration routes of antiviral agents in mice

To examine the antiviral activities against FMDV using various inoculation methods, ribavirin was IP or intramuscularly (IM) injected from day 0 to day 3 (panel A in Fig. 2). Six hours after the agents were first provided, 50 LD_{50} Asia1 Shamir were injected IP in all groups (n = 5 per group). Weight changes and survival rates of all mice were monitored for 10 days.

Six, 10, and 15 mg per day of ribavirin were supplied in pellet

feed that had absorbed the reagents and were given to mice (n = 5 per group) from day 0 to 6 (panel B in Fig. 2). Six hours after the first provision of reagents, 50 LD_{50} Asia1 Shamir were injected IP. Weight changes and survival rates of the mice were monitored for 10 days. In addition, at days 0, 2, 4, 6, and 8, blood was collected, serum was prepared, and RNA was extracted to measure the amount of virus present. PrioCHECK FMDV SP (Prionics, Switzerland), an enzyme-linked immunosorbent assay (ELISA) kit for the detection of FMDV structural protein (SP) antibodies in pig serum, was employed to detect SP antibodies against FMDV.

## Responses to repeat challenge in mice treated with antiviral agents/vaccine

At day 0, trivalent vaccinations were administered, and 6 mg or 10 mg ribavirin absorbed in feed were provided from day 0 to day 6 to each mouse (Fig. 3). Six hours after vaccine administration and the provision of feed in which reagents had been absorbed, mice (n = 5 per group) were injected IP with 50 LD_{50} Asia1 Shamir. Then, at day 10, a re-challenge was performed with 50 LD_{50} Asia1 Shamir. Changes in weights and survival rates of the mice were monitored for 15 days.

Adult mice were divided into seven groups including a negative control group. As parenteral model for the animals with poor appetite during FMD infection, the experimental groups were inoculated IP with 15 mg ribavirin, or oil/gel adjuvant, or with/without commercial vaccine (trivalent vaccine containing O1 Manisa, A22 Iraq, and Asia1 Shamir; Boehringer Ingelheim Animal Health, France) at day 0 (Fig. 4). Oil adjuvant (water in oil in water type, Montanide ISA 201 VG; SEPPIC, France) or Rehydragel HPA (General Chemical, USA) were employed as adjuvants. Six hours after the drugs were injected; the mice were IP inoculated with 50 LD_{50} Asia1 Shamir. Another group with the same conditions was established and 6 hours after inoculation of the drug and at day 3, the mice were IP inoculated with 50 LD_{50} Asia1 Shamir. Changes in weight and survival rates of the mice were monitored for 10 days.

In addition, blood was collected, serum was separated, and RNA was extracted using the MagNaPure LC 96 System (Roche, Switzerland) and real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) was conducted to quantify the amount of FMDV.

Applicability of antiviral agents/vaccine in SPF pigs as a target animal model

To determine the potential for protection from FMDV challenge with IM injection of ribavirin and vaccine, specific pathogen-free (SPF) minipigs were used as a target animal model (Table 2). Yucatan SPF mini pigs that weighed 10 to 15 kg and verified to be negative for FMDV SP antibodies were supplied by Opti-Farm Solution Medi-Pig (Korea). The animals
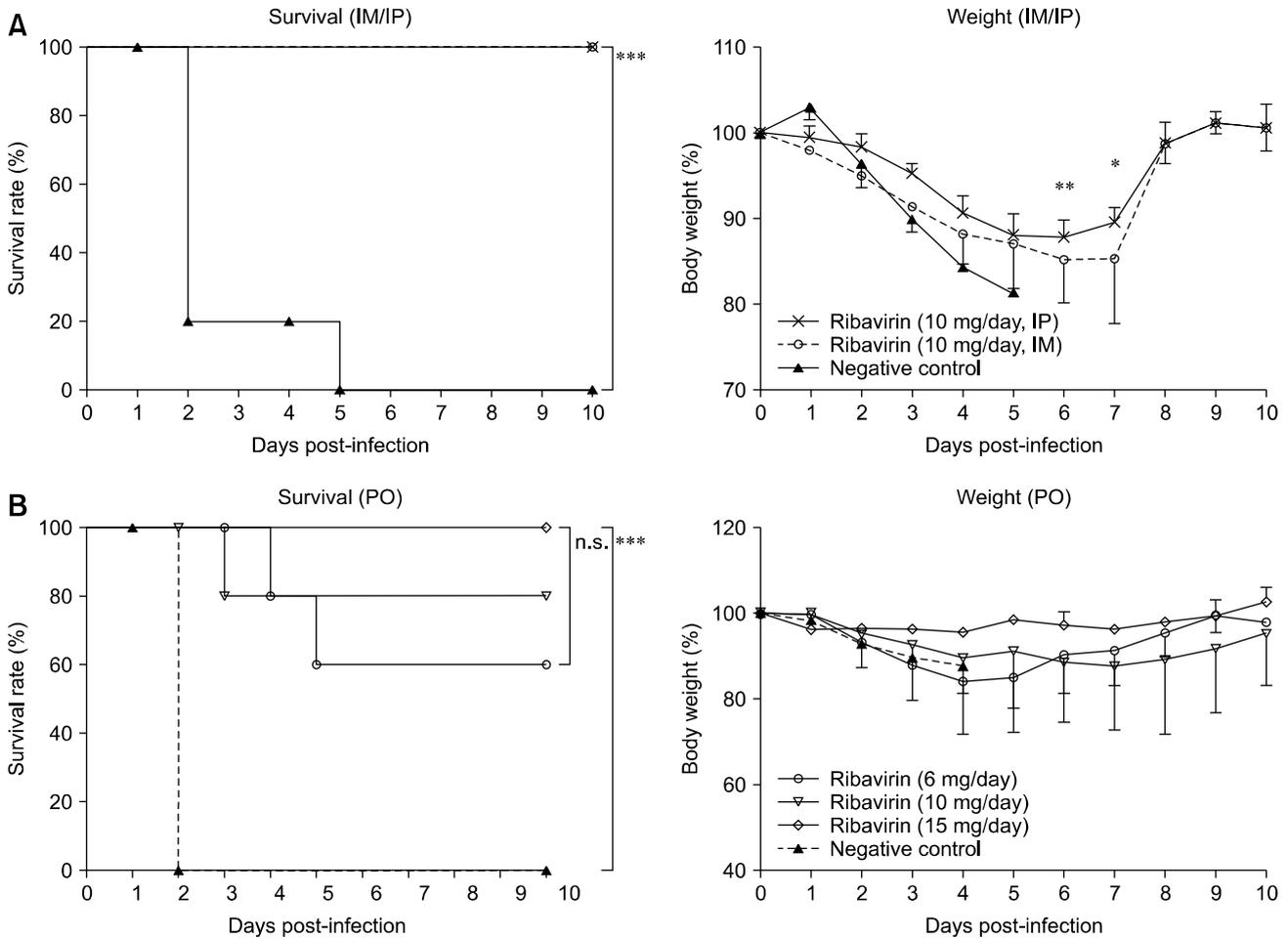
Fig. 2. Comparison of administration routes of antiviral agents in mice. (A) Ribavirin (10 mg/day) was injected intraperitoneally (IP) or intramuscularly (IM) in mice (n = 5 per group) from day 0 to day 3. Six hours after being administered with the first antiviral agent, mice were injected IP with 50 LD_{50} (LD_{50}, 50% lethal dose) of Asia1/Shamir. Survival rate of mice by IP or IM ($p < 0.001$ in ANOVA) and average weight for live mice were determined for 10 days. (B) The mice were administered per oral route (PO) (6, 10, and 15 mg/days, mixed form with feed) from day 0 to day 6. Survival rate of mice ($p < 0.0001$ in ANOVA) and average weight of live mice were determined for 10 days. Six hours after being administered with the first antiviral agent, mice were injected IP with 50 LD_{50} of Asia1/Shamir.

were kept and cared for at the APQA and were used after approval from the Animal Care and Use Committee was obtained. There were three experimental groups of SPF pigs including a negative control group used in the field target animal test. At 0 days, SPF pigs (n = 2 per group) were administered ribavirin (1,800 mg/pig; the dosage was determined in a preliminary dose-dependent survival/efficacy test in the pigs) and oil adjuvant (Montanide ISA 206 VG; SEPPIC), which was mixed in a weight ratio 1:1, or injected IM with the ribavirin-oil components along with a commercial vaccine (trivalent vaccine containing O1 Manisa, A22 and Asia1 Shamir; Merial), and immediately afterward, the pigs were challenged with 10^5 $TCID_{50}$ Asia1 Shamir by needle challenge to the heel-bulb. Blood and oral swabs were collected, serum

was separated, and RNA was extracted using the MagNaPure LC 96 System (Roche) and real-time RT-PCR was conducted to quantify the amount of FMDV. Each clinical observation was given a score (maximum, 16 points) using the following criteria: (a) lameness (1 point), (b) vesicles in the hoof or foot (1 or 2 points for each affected hoof and foot), and (c) vesicles in the snout, lips, or tongue (1 point for each affected area) [15].

Statistical analysis

Repeated measures analysis of variance (ANOVA) was performed using GraphPad Instat (ver. 3.05; GraphPad Software) and GraphPad Prism 5 (GraphPad Software) to statistically assess differences in survival rates and weight changes. Student's *t*-test was also used for statistical significance
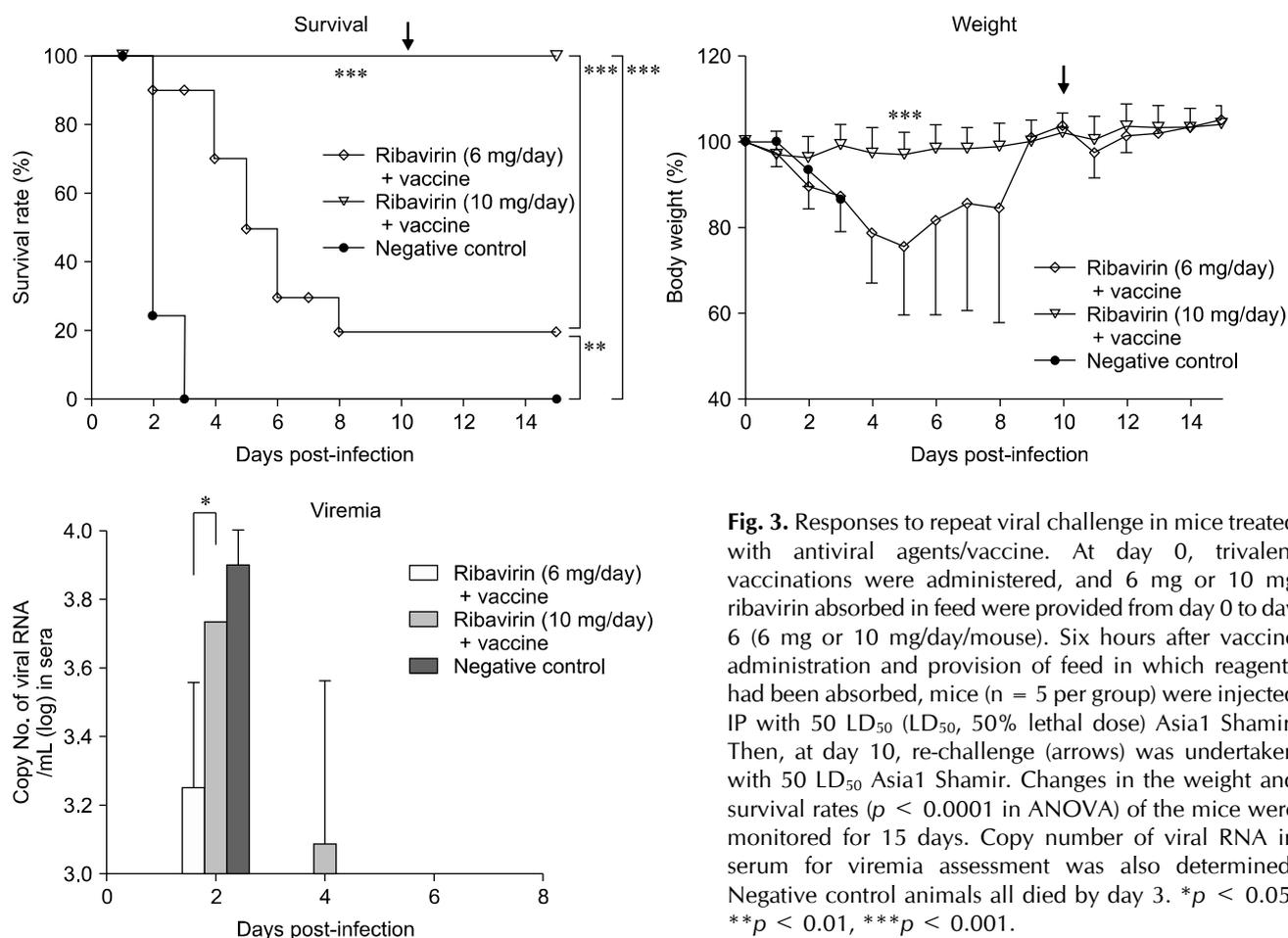
Fig. 3. Responses to repeat viral challenge in mice treated with antiviral agents/vaccine. At day 0, trivalent vaccinations were administered, and 6 mg or 10 mg ribavirin absorbed in feed were provided from day 0 to day 6 (6 mg or 10 mg/day/mouse). Six hours after vaccine administration and provision of feed in which reagents had been absorbed, mice (n = 5 per group) were injected IP with 50 LD_{50} (LD_{50}, 50% lethal dose) Asia1 Shamir. Then, at day 10, re-challenge (arrows) was undertaken with 50 LD_{50} Asia1 Shamir. Changes in the weight and survival rates ($p < 0.0001$ in ANOVA) of the mice were monitored for 15 days. Copy number of viral RNA in serum for viremia assessment was also determined. Negative control animals all died by day 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

analysis. *P* values smaller than 0.05 indicated a significant difference.

Results

Cellular toxicity and virus growth inhibition by the different antiviral agents

The growth inhibitory effects of the various antiviral agents against FMDVs were measured in IBRS-2 cells. The selectivity index of ribavirin had the highest value (Table 1). Regarding cell toxicity (CC_{50}), ribavirin and azauridine had similar values but T1105 produced the lowest reaction by the cells. For the degree of resistance (EC_{50}) against FMDV, ribavirin was able to provide an antiviral effect at the lowest concentration.

We examine whether the chemical agents, ribavirin, azauridine, T1105, and adenovirus, known to protect against RNA virus replication, could treat and protect against FMDVs (Fig. 1). Azauridine and T1105 had tendencies similar to the negative control group and did not protect against FMDV compared to treatment with the same quantity of the other agents. Adult mice were inoculated with Ad-IFN-αγ and their survival rate was

40% while their Ad-3siRNA survival rate was 80%. However, when 3 mg of ribavirin was administered twice a day from day 0 to day 3, all mice survived (Fig. 1).

Response to administration routes of ribavirin

We examined whether there was a difference in the effects of ribavirin in protecting against FMDVs that depended on the administration routes (Fig. 2). Both IP and IM inoculation resulted in 100% survival rates in the mice (panel A in Fig. 2). However, when the weight change rates of the mice were compared, weight change was greatest from day 5 to day 7 in the case of IP inoculation, and from day 6 to day 7 in the case of IM inoculation. Thus, IP inoculation was more effective in the protection against FMDVs than IM inoculation.

We examined whether oral administration of the agent that had been absorbed into the feed provided protection against FMDV (panel B in Fig. 2). As the daily dose increased, the survival rates of the mice increased. When 15 mg of ribavirin was orally administered in the feed, the survival rate of the mice was 100% for 10 days. When ribavirin was orally administered in the feed, there were less than 10% weight changes in the
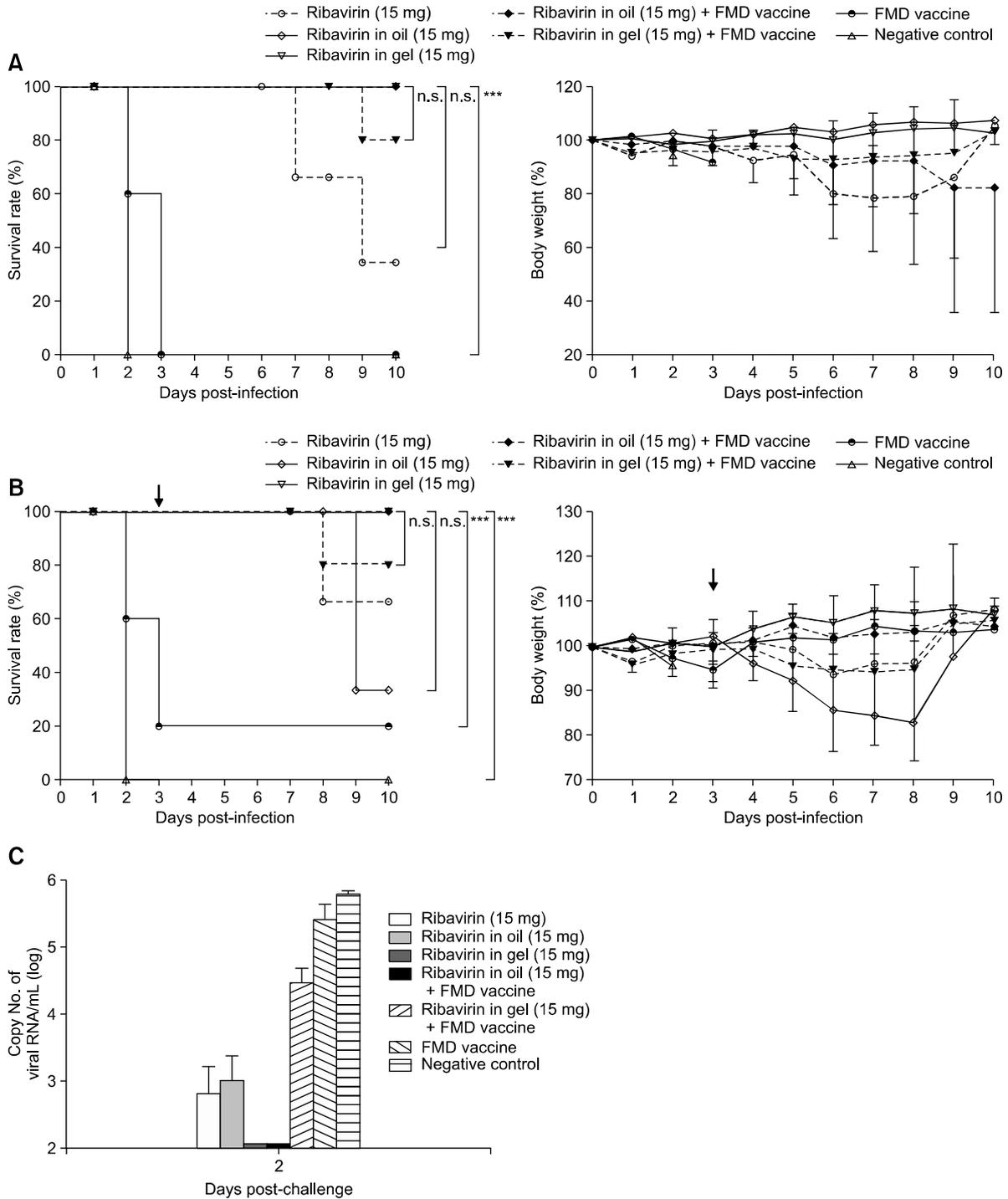
Fig. 4. Anti-foot-and-mouth disease (FMD) virus responses in mice administrated with antiviral agents containing adjuvants and vaccine. Mice (n = 5 per group) were injected intramuscular with vaccine or with 15 mg of antiviral agent on day 0. Six hours after injecting the first antiviral agent or commercial vaccine trivalent vaccine containing O1 Manisa, A 22 Iraq and Asia1 Shamir; Merial), the mice were inoculated intraperitoneal (IP) with 50 LD_{50} (LD_{50}, 50% lethal dose) of Asia1/Shamir. (A) Survival rate of mice for 10 days ($p < 0.001$ in ANOVA) and average weight of live mice for 10 days. On six hours and the third day after being inoculated with the first antiviral agent, mice were twice injected IP with 50 LD_{50} of Asia1/Shamir. The mice were monitored for 10 days. (B) Survival rate of mice for 10 days ($p < 0.0001$ in ANOVA) and average weight of live mice for 10 days. The arrows indicate the day of the second challenge (3 dpc). (C) Copy number of viral RNA in serum. Negative control animals all died by day 3. The arrows indicate the day of the second challenge. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2. Evaluation of foot-and-mouth disease protection by virus challenge after administration of a combination of vaccine and antiviral components in a SPF pig model for assessment of applicability to target animals

Clinical indexes for 7 dpc	Experiment groups of SPF pigs (n = 2)		
	Ribavirin in oil adjuvant*	Ribavirin in oil adjuvant* + vaccine[†]	Negative control
First day of appearance of clinical signs	2.5 ± 0.3	NA	2.0 ± 0.0
Highest clinical score in a day (point)	4.0 ± 1.0	0	6.5 ± 0.5
Accumulate clinical score (point)	12.0 ± 2.0	0	34.5 ± 9.5
Total days of apparent clinical signs	5.5 ± 0.5	0	6.0 ± 0.0
First day of detected virus in swab sample	2.5 ± 0.5	1.5 ± 0.5	2.5 ± 0.5
Highest detected virus in a day in swab sample (copy No.)	5,928.8 ± 2,759.5	93.4 ± 7.2	2,421.5 ± 232.3
Total days of virus detection in swab sample	4.0 ± 0.0	2.5 ± 0.5	3.0 ± 1.0
Protection after challenge (No. of protected/No. of tested)	No (0/2)	Yes (2/2)	No (0/2)

Data are presented as mean ± SD. SPF, specific pathogen-free; dpc, days post-challenge; NA, not available. *Vaccine oil adjuvant (W/O/W type; SEPPIC). [†]Commercial vaccine (trivalent vaccine containing O1 Manisa, A22 and Asia1 Shamir; Merial).

mice. In the case of administration by parenteral injection, there was efficacy in protection against FMD with only 3 mg/day, but with oral administration, 100% survival rates resulted only when more than 15 mg/day was administered.

Synergistic effects of combined application of vaccines and antiviral agents

The group treated with a combined application was administered both vaccines and ribavirin (Fig. 3). When the vaccines were administered at day 0 and 10 mg of ribavirin in feed was orally administered from day 0 to day 6, the adult mice had a significantly high 100% survival rate ($p < 0.0001$). The adult mice weight change rate (Fig. 3) for each day was less than 5%, with no significant daily differences. When 6 mg/day of ribavirin was orally administered in feed or when vaccines only were given, all groups had low survival rates, or all mice died. On the day of challenge inoculation with FMDVs, ribavirin was orally administered and vaccines were injected; the higher the concentration of the antiviral agent, the higher the survival rate of the adult mice, with a significant difference from survival in the group that was inoculated with virus only. In addition, when 10 mg or 6 mg of ribavirin per day was orally administered after vaccines were given, daily weight changes for the adult mice did not significantly differ from that before the inoculation. At day 10, the adult mice were challenged with the same concentration of FMDV; survival rates and weights of the mice did not change or they showed a decreased then increased pattern of change.

The group to which ribavirin was orally administered had FMDV detection levels in sera similar to or lower than those in the control group at day 2 (Fig. 3), while at day 4, the detection of FMDV decreased compared to that at day 2 in all groups.

Synergistic effect of concomitant administration of antiviral agents with adjuvants and/or vaccine

ISA 201 or gel (Rehydragel HPA) used as an adjuvant was concomitantly administered with ribavirin (Fig. 4). When the vaccine alone was given, there was no significant difference in survival from the negative control ($p > 0.5$), but when ribavirin was concomitantly inoculated with adjuvants or adjuvants and vaccines, there were significant differences in survival compared to the control group ($p < 0.01$). When adult mice were inoculated with FMDVs once (day 0) (panel A in Fig. 4), the adult mice's survival rates were higher than or similar to that when the mice were inoculated with FMDVs twice (on day 0 and day 3) (panel B in Fig. 4). When the amount of FMDV was measured in the serum of the mice at 2 days post-challenge (dpc; panel C in Fig. 4), the amounts of virus in all groups were lower than that of the negative control, or no FMDVs were detected.

Regarding the antiviral ability of ribavirin, the SP antibody of FMDV was detected at 16 days post-infection (dpi) in the 10 or 15 mg/day groups in which ribavirin was orally administered. In addition, in the 6 mg/day group in which ribavirin was orally administered along with vaccines, the antibody was not detected at 10 or 16 dpi. The SP antibody by SP ELISA in vaccine-only injected group was not detected at 16 dpi.

Synergistic effect of combined administration of antiviral agents with adjuvants and vaccine in the SPF pig model

Pigs that were administered ribavirin with oil adjuvant (water in oil in water) were observed to have the highest clinical score (5 points) within 3 dpc (Table 2). Another group that was injected with ribavirin with oil and commercial vaccine did not exhibit any clinical signs during all experimental days, even though the pigs were exposed to approximately 10^{2-4} RNA copy numbers from 2 to 3 dpc. The negative control was found to

have high RNA copy numbers of viremia (Table 2).

Discussion

A great deal of recent research has aimed at the use of antiviral agents to prevent the spread of RNA viruses responsible for a variety of diseases. Compounds such as ribavirin, guanidine-HCl, and 6-azauridine may inhibit the replication of RNA viruses such as the Chikugunya virus, Semliki Forest virus, and coronavirus *in vitro* [1,22,27]. IFN-α and gamma provide protection against RNA viruses such as hepatitis C virus, herpes simplex virus, and cytomegalovirus [29,32]. Among the different antiviruses, oral treatment of hepatitis by ribavirin and aerosolized ribavirin for respiratory syncytial virus have been approved by the Food and Drug Administration [11]. In the present study, although safe doses of ribavirin in mice or pigs were lower 40 or 2,000 mg (unpublished), ribavirin provided the best protective effect against FMDVs among the different antiviral agents known to defend against and treat RNA viruses.

Given that the mice exhibited high survival rates when ribavirin was administered, it is considered to have protection and transmission delay effects on FMDV, an RNA virus. With regard to the administration pathway, systemic application by an IP route was more effective than a local application by an IM route. This observation is similar to the results in a study by Salazar *et al.* [30], which reported that IP administration is effective.

Simple oral administration, as opposed to inoculation through injection into adult mice, was shown to have a protective effect against FMDVs. The provision of agents absorbed into feed more effectively treated FMD than the oral administration of ribavirin. When the agents were absorbed into feed, there can be almost continuous oral administration, maintaining the *in vivo* concentration. However, there is a disadvantage in that the agents are administered only when the animals voluntarily consume the feed; thus, a lack of appetite, which may accompany infection with FMDV, may result in lower effectiveness.

Although different serotypes of FMDV for tests of antiviral activity were used, the effects on type Asia1 (*in vivo*) and type O (*in vitro*) experiments were shown to be similar (Table 1, Fig. 1). We think that the antiviral agents may be commonly effective in different serotypes. In prior research, guinea pigs were IP inoculated with ribavirin (45 mg/kg) for two weeks, and their survival after exposure to Argentine hemorrhagic fever (Junin virus) was extended from 15 to 25 days; similarly, when the animals were IP inoculated with 60 mg/kg of ribavirin for 25 days their survival was extended from 14 to 37 days [30]. These results show that life extension is possible but the protection is incomplete. In the present study, the measured amount of FMDV in the serum of the mice showed that 15 mg/day of ribavirin may inhibit the replication of FMDVs within approximately 4 days. When the mice were inoculated with agents along with vaccines, or when adjuvants were used, the concentration of ribavirin was reduced to 6 mg/day, resulting in the survival of all adult mice. Considering the research result in which survival rates were heightened when adult mice were inoculated with oil or gel as the adjuvant along with an antiviral reagent, the use of adjuvant with antiviral agents might have extended the antiviral reactivity in the mice. This outcome proves that the dose of the injection, rather than the method of administration, more strongly affects the reagent's protective effect against FMDVs. Assuming that target animals in farms are typically re-infected by FMDVs, a second round of challenge was performed; under those conditions, there was a slight weight change but no change in survival rates. Thus, immunity generated through the first infection may protect against pathogenic re-infection. SP antibodies against FMDV were not detected because virus replication was inhibited by vaccines or ribavirin until day 10. The antiviral agent, ribavirin, helped to delay clinical signs when pigs were challenged with FMDV. The pig group treated with ribavirin and vaccine did not show any clinical signs or measured RNA copy numbers in test swabs. Therefore, we think that ribavirin is an excellent antiviral agent to protect against FMDV, and it can provide early immunity.

Importantly, there are side effects to be considered before undertaking clinical application of ribavirin. It has been reported that all ribavirin-treated pigs show significant decreases in body weight and red-blood-cell counts until 8 dpi [17]. Moreover, in ribavirin-treated FMDV-uninfected pigs, clinical signs of dyspnea, anorexia, weakness, and depression were present until 5 days [17].

In conclusion, lowering the administrated amount of antivirals being co-administered with an adjuvant (oil or gel) used in FMD vaccine can enable protection from lethal challenge. Furthermore, administration of the ribavirin (1,800 mg/pig) along with an FMD vaccine synergistically enhances antiviral activity during early viral infection. For a future application, the determination of safe and effective administration amounts of ribavirin in the field animals, such as cattle and pigs, should be a priority. In addition, there should be a thorough consideration of the method of administering antiviral reagents to target animals as well as the number of administrations.

Acknowledgments

Conflict of Interest

The authors declare no conflicts of interest.

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