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Co-inoculation of baculovirus and FMDV vaccine in mice, elicits very early protection against foot and mouth disease virus without interfering with long lasting immunity

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ABSTRACT

Baculoviruses (Bvs) potentiate the immune response against soluble antigens. We investigated whether Bv could be used as immunoactivator in foot-and-mouth disease (FMD) vaccines using the BALB/c mouse model.

Mice were vaccinated with a single dose of inactivated FMDV (iFMDV), iFMDV + Bv, Bv, or culture medium. Humoral and cellular immune responses were higher in animals immunized with iFMDV + Bv than in mice vaccinated with iFMDV alone. Animals receiving iFMDV + Bv had significantly lower viremia at 2, 4 and 7 dpv, than those immunized with iFMDV alone.

In order to prolong the immune response, iFMDV oil vaccine was co-inoculated with Bv. Animals receiving iFMDV oil vaccine + Bv were protected two days earlier than those receiving the iFMDV oil vaccine alone. Both formulations protected until 14 dpv, the last day of the experiment.

This is the first report in which Bv is used as an adjuvant in a FMDV vaccine.

1. Introduction

Foot and mouth disease virus (FMDV) infects cloven-hoofed animals, causing a highly contagious illness [1] that bears high relevance in terms of international trade [2] thus causing huge economic losses.

Current FMDV vaccines consist of inactivated whole-virus preparations formulated with adjuvants. These vaccines have been highly successful in reducing the number of outbreaks in many parts of the world where the disease is enzootic [3]. In recent years, considerable research efforts have been aimed at the development of emergency vaccines that can reduce the time between vaccination and the elicitation of a protective immune response.

Autographa californica nuclear polyhedrosis virus (AcMNPV) is a double-stranded circular DNA baculovirus (Bv) able to transduce a plethora of mammalian cell types, although it is unable to replicate and integrate its genome into mammalian chromosomes [4,5]. It has been reported that Bv has adjuvant properties that promote humoral and cytotoxic T lymphocyte (CTL) responses against co-administered antigens (Ag) [6] and that activates innate immune responses by inducing type I and II IFNs. Bv has been shown to protect mice from a lethal challenge with encephalomyocarditis virus (ECMV) or influenza virus [7,8]. A recent report showed that Bv injection 3 h to 3 days before challenge can abrogate the development of FMD clinical signs in a C57BL/6 murine model [9]. Thus, because of its adjuvant and antiviral properties, Bv could be a valuable tool to improve vaccines.

Knowledge on the immunity against FMDV in natural hosts is limited due to the impossibility of using inbred animals, the lack of specific reagents and the high costs involved in the use of large experimental animals such as cattle and swine. In response to these limitations, an experimental BALB/c murine model for FMDV was developed in our laboratory [10]. Despite the differences between mice and natural hosts, many similarities have been checked by comparing studies performed in the murine model with others conducted in natural hosts.

In the present report, the Bv adjuvant effect on the iFMDV antigen was studied. Our data show that co-inoculation of mice with Bv and iFMDV induces an immune response that is strong enough to protect against viral challenge at very early times post vaccination and that such protection can be maintained for up to two weeks if the iFMDV antigen is formulated in oil adjuvant.

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2. Materials and methods

2.1. Mice

Eight to 12-week-old male BALB/c mice (University of La Plata, Argentina) were used. Experiments with mice were performed in accordance with the institutional committee for the care and use of experimentation animals.

2.2. Virus

Binary ethylenimine (BEI)-inactivated FMDV serotype O1 Campos (iFMDV) was used to formulate vaccines and for ELISA assays. Infectious virus (same serotype) was used for viral challenge and was provided by the National Service of Animal Health, Argentina (SENASA). All experiments involving infectious virus were performed at INTA Biosafety Level 3A facilities.

Bvs were propagated by infecting Sf9 cells in SF900 medium culture (2% FCS, 27 °C). Supernatants were harvested and cell debris removed by centrifugation (4000 × *g*, 15 min, 8 °C). Endotoxin levels in Bv suspensions were measured using the Limulus amebocyte lysate test (E-TOXATE, Sigma). Virus stocks were considered free of endotoxins (<0.01 endotoxin U/ml).

2.3. Vaccine formulations and vaccination

A dose of 0.1 μg iFMDV per mouse (which elicited protection in less than 60% of vaccinated animals at 4 or 7 dpv), was chosen. iFMDV was inoculated formulated with PBS (iFMDV) or formulated in an oil emulsion (iFMDV oil vaccine). The iFMDV oil vaccine was formulated in a company that produces commercial vaccines in Argentina, using commercial adjuvant.

A dose of 5×10^7 pfu of Bv was used in combination with iFMDV in PBS (Bv + iFMDV), iFMDV oil vaccine (Bv + iFMDV oil vaccine) or alone (Bv). Mice were intraperitoneally (i.p.) vaccinated with 0.2 ml of one of these formulations in two separate sites. The negative control group was i.p. inoculated with insect cell growth medium ("medium" group).

2.4. Viral challenge

Protection against FMDV was assessed as described previously [11–13]. Briefly, mice were i.p. inoculated with 10^4 $TCID_{50}$ infectious FMDV. Animals were anesthetized and bled from the retro orbital plexus 24 h later. Heparinized blood was serially diluted in DMEM and dilutions were spread onto BHK-21 cell monolayers. After virus adsorption, monolayers were washed with sterile phosphate-buffered saline (PBS). Fresh DMEM with 2% fetal calf serum (FCS) was added and cells were kept at 37 °C in a 5% CO_2 incubator and after 72 h incubation, cytopathic effects were recorded. Viremia titers were calculated by the method of Reed and Muench [14]. To calculate the percentage of protected animals of each group, mice with undetectable viremia levels after a blind passage, were considered as fully protected. Percentages of protected animals were calculated as (number of protected mice/number of challenged mice) × 100.

2.5. Anti-FMDV Ab measurements

Total antibodies against FMDV were assessed by ELISA as described previously [11–13]. Briefly, Immulon II plates were coated with anti-FMDV rabbit serum in 0.05 M carbonate–bicarbonate buffer, pH 9.6. After washing, iFMDV was added. Plates were blocked with polyvinylpirrolidone buffer (0.5 M NaCl/0.01 M phosphate buffer/0.05% Tween-20/1 mM EDTA/1% polyvinylpirrolidone 30–40 K, pH 7.2). Serial dilutions of serum samples were added, followed by incubation with biotin-conjugated anti mouse IgG plus anti mouse IgM (ebioscience, San Diego, USA). Horseradish peroxidase (HRP)-conjugated streptavidin was added and o-phenylenediamine-H_2O_2 was used as peroxidase substrate. Absorbance was recorded at 490 nm (A_{490}) in a MR 5000 microplate reader (Labsystems, Minnesota, USA). The cut-off was established as the mean A_{490} of the negative sera plus two standard deviations and Titers were calculated according to Reed and Muench [14].

Seraneutralizing antibodies (SN Abs) were measured as previously described [15]. Briefly: sera were serially diluted and dilutions were incubated with 100 TCID50/well of infectious FMDV. The FMDV–serum mixtures were transferred onto BHK-21 cell monolayers and incubated. The appearance of cytopathic effects was recorded after 48 h of incubation at 37 °C.

2.6. Spleen cell preparations

Mice were killed by cervical dislocation and spleens were removed. Splenocytes were withdrawn with serum-free 10 mM EDTA/PBS. Cells were plated in 96-well flat-bottom plates (1×10^6 cells/well) with RPMI 1640 supplemented with 10% FCS and 50 mM 2-mercaptoethanol.

2.7. Cytokine measurements by ELISA

Splenocytes were incubated with RPMI 1640 supplemented with 10% FCS and 50 mM 2-mercaptoethanol for 3 days at 37 °C in a 5% CO_2 incubator. Cytokine concentrations (pg/ml) were determined in cell culture supernatants by commercial sandwich ELISA kits using standard curves (BD-pharmingen and ebioscience).

2.8. Cytokine measurements by intracellular staining

Splenocytes were incubated with RPMI 1640 supplemented with 10% FCS and 50 mM 2-mercaptoethanol for 18 h in the presence of brefeldin A (5 μg/ml). After washing, cells were fixed in 0.5% paraformaldehyde and permeated with saponin (0.1% in PBS). Permeated cells were incubated for 20 min with PE-conjugated Abs against TNF-α (BD Pharmingen) or isotype-matched control Abs. Stained cells were analyzed by flow cytometry using a FACScan flow cytometer and CellQuest software (BD Biosciences, San José, CA, USA).

2.9. Generation of bone marrow-derived dendritic cells (DCs)

Bone marrow-derived DCs were obtained as previously described [16] with minor modifications. Briefly, the marrows were flushed out of femurs and tibias of BALB/c mice. Red blood cells were lysed using 0.083% ammonium chloride. After washing, cells were suspended in RPMI 1640 supplemented with 10% FCS and 30% conditioned medium from GM-CSF-producing NIH-3T3 fibroblasts, and cultured for 7 days. Every 2 days, 50% of the medium was replaced with fresh medium of the same composition.

2.10. Flow cytometry analysis of cell surface molecules

Cell staining was performed using: anti-CD11c, anti-CD86 and anti-MHC II conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) (BD Pharmingen, San Diego, CA). Data were collected using a FACSCalibur flow cytometer and analyzed using the CellQuest software (BD Biosciences).

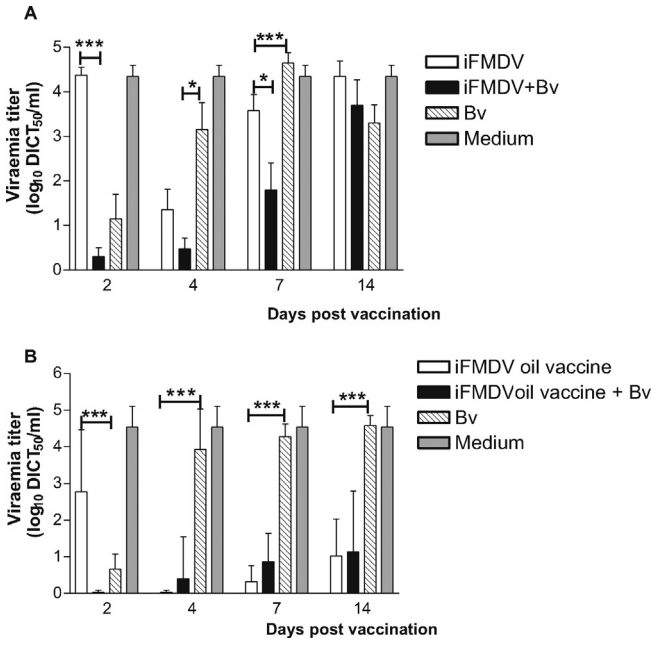

Fig. 1. Protective response in BALB/c mice. (A) Mice were vaccinated with: 0.1 μg of iFMDV (iFMDV); 0.1 μg of iFMDV and 5×10^7 pfu of Bv (iFMDV + Bv); 5×10^7 pfu of Bv alone (Bv) or with SF900 medium culture alone (medium). Viremia titers in blood collected from mice at 2, 4, 7 and 14 dpv after challenge with infective FMDV. (B) Vaccines are the same as in Fig. 1A but iFMDV antigen in formulated as an oil vaccine. Bars represent mean viremia titers ± SD. *and *** mark significant differences of $p < 0.05$ and $p < 0.001$, respectively.

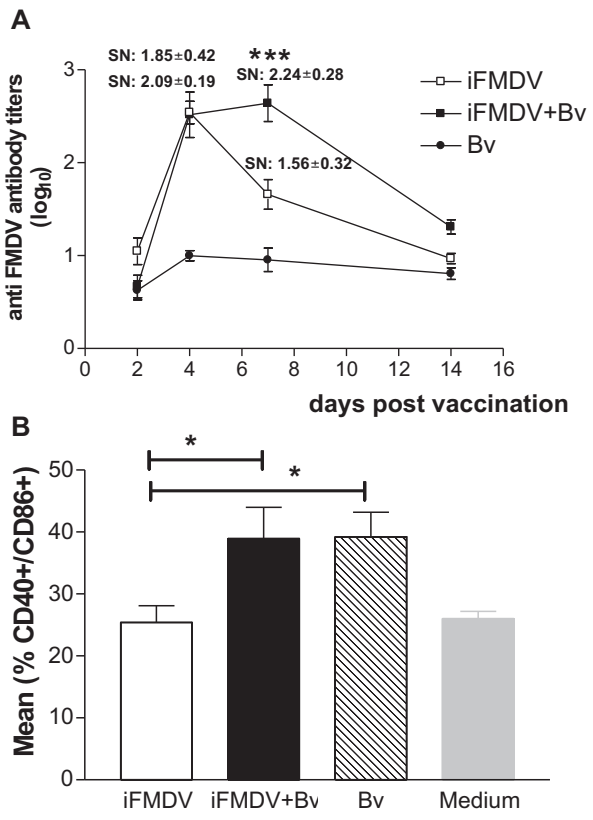

Fig. 2. (A) Antibodies against FMDV elicited by vaccination with iFMDV, iFMDV + Bv or Bv measured by ELISA. Each point represents the mean anti FMDV Ab titers ± SD of 5 sera, as measured by ELISA. Numbers over the symbols are the mean neutralizing antibodies titers ± SD elicited in each point. (B) Bone marrow-derived DCs were stimulated *in vitro* with iFMDV, iFMDV + Bv, Bv alone or medium during 24 h and then CD40 and CD86 expression was measured. *and *** mark significant differences of $p < 0.05$ and $p < 0.001$, respectively.

2.11. Statistical analysis

ANOVA and Bonferroni post ANOVA tests were used to compare data among groups. A $p < 0.05$ was considered as an indicator of significant differences.

3. Results

3.1. Co-inoculation of iFMDV and Bv confer protection against viral challenge at 2, 4 and 7 dpv, but not at 14 dpv

Mice were vaccinated with iFMDV, iFMDV + Bv, Bv or medium alone and challenged at 2, 4, 7 and 14 dpv.

At 2 dpv, viremia titers were significantly lower ($p < 0.001$) in the iFMDV + Bv group than in the iFMDV group. At 4 dpv, viremia titers were significantly lower ($p < 0.05$) in iFMDV + Bv than in the Bv group. At 7 dpv viremia was significantly lower in iFMDV + Bv than in groups iFMDV ($p < 0.05$) and Bv ($p < 0.001$). At 14 dpv, viremia titers in all assayed groups were similar to those in the medium control group (Fig. 1A). Formulations containing iFMDV alone protected 60% and 12% of the animals at 4 and 7 dpv, respectively, while no animals in this group were protected at 2 dpv. Co-inoculation of Bv with iFMDV increased the percentages of protected animals to 85% at 2 dpv, 80% at 4 dpv and 72% at 7 dpv. It is worth to note that at 2 dpv, 65% of animals in the Bv group were protected. Protection was not observed in any group at 14 dpv, or at any time point in the medium control group (Table 1 upper panel).

3.2. Co-inoculation of iFMDV and Bv induces an increase in Ab titers at 7 dpv

At 7 dpv both, total and SN Ab titers, were significantly higher in the iFMDV + Bv ($p < 0.01$ and $p < 0.001$, respectively) as compared to the iFMDV group, indicating a synergistic effect between the antigen and Bv (Fig. 2A). At 4 dpv Abs were detected, with similar levels, in the iFMDV and iFMDV + Bv groups. At 2 dpv, Ab titers were considered negative in all groups. At 14 dpv Ab titers start to dropp both in the iFMDV + Bv and the iFMDV groups (Fig. 2A). Animals inoculated with Bv did not produce Abs against the virus.

3.3. Co-inoculation of iFMDV and Bv or Bv alone induces an inflammatory response

At 4 dpv IL6 (Fig. 3A) and IFNγ (Fig. 3B) were significantly increased ($p < 0.05$) in supernatants of spleen cell cultures from iFMDV + Bv vaccinated animals, as compared to the iFMDV or medium groups. Both the iFMDV + Bv and Bv groups presented a significative increase ($p < 0.05$) in the number of spleen TNFα+ cells, as compared to the iFMDV group (Fig. 3C and D).

These results indicate that an inflammatory process is taking place in mice inoculated with iFMDV + Bv and Bv.

3.4. In vitro incubation of DCs with iFMDV plus Bv or Bv alone leads to up-regulation of co-stimulatory molecules

Bone marrow-derived DCs were incubated with medium, Bv, iFMDV and iFMDV + Bv, and activation was evaluated by the increase of cell surface markers CD40 and CD86 at 24 h.

As shown in Fig. 2C, these molecules were significantly increased ($p < 0.05$) when DCs were incubated with Bv or iFMDV + Bv in comparison with those incubated with iFMDV, indicating that Bv is capable of activating DCs.

Table 1
Percentages of protected animals against viral challenge at 2, 4, 7 and 14 dpv. Protection was established as undetectable viremia 24 h post inoculation of infective FMDV and after one blind passage. Then the percentage of protected mice was calculated as (number of animals with undetectable viremia levels/number of vaccinated animals) × 100. The numbers in brackets represents the percentage of protection of each group. Upper panel: 0.1 μg of iFMDV (iFMDV); 0.1 μg of iFMDV and 5×10^7 pfu of Bv (iFMDV + Bv); 5×10^7 pfu of Bv alone (Bv) or with SF900 medium culture alone (medium). Lower panel: same vaccines as upper panel but iFMDV antigen in formulated as an oil vaccine.

	2 dpv[a]	4 dpv	7 dpv	14 dpv
iFMDV	0/10 (0%)[b]	5/10 (50%)	0/10 (0%)	0/10 (0%)
iFMDV + Bv	8/10 (80%)	7/10 (70%)	7/10 (70%)	0/10 (0%)
Bv	6/10 (60%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Medium	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
iFMDV oil vaccine	2/10 (20%)[b]	8/10 (80%)	8/10 (80%)	8/10 (80%)
iFMDV oil vaccine + Bv	9/10 (90%)	8/10 (80%)	9/10 (90%)	8/10 (80%)
Bv	9/10 (90%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Medium	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

[a] Days post vaccination.
[b] Number of mice with undetectable viremia levels/number of mice challenged × 100.

3.5. Co-inoculation of iFMDV oil vaccine and Bv confer protection against viral challenge from 2 to 14 dpv

Since iFMDV and Bv elicited a high protective response at 2 dpv, which decreased at 7 dpv, the capacity of a combination of Bv and an iFMDV oil vaccine to maintain the immune response was evaluated.

As shown in Fig. 1B, when vaccinated mice were challenged at 2 dpv, there were significantly lower ($p < 0.001$) viremia titers in the groups iFMDV oil vaccine co-inoculated with Bv than in iFMDV oil vaccine alone. At 4, 7 and 14 dpv, there were significantly lower ($p < 0.001$) viremia titers in groups iFMDV oil vaccine than in Bv group, though no significant differences were found between iFMDV oil vaccine with or without Bv at days 4, 7 or 14.

At 2 dpv, the iFMDV oil vaccine did not induce protection, while the iFMDV oil vaccine plus Bv, protected 92% of the vaccinated mice, which is similar to the protection rate induced at this time point by Bv alone (Table 1 lower panel). It is worth to mention that the high levels of protection with iFMDV oil vaccine were maintained until 21 dpv (data not shown).

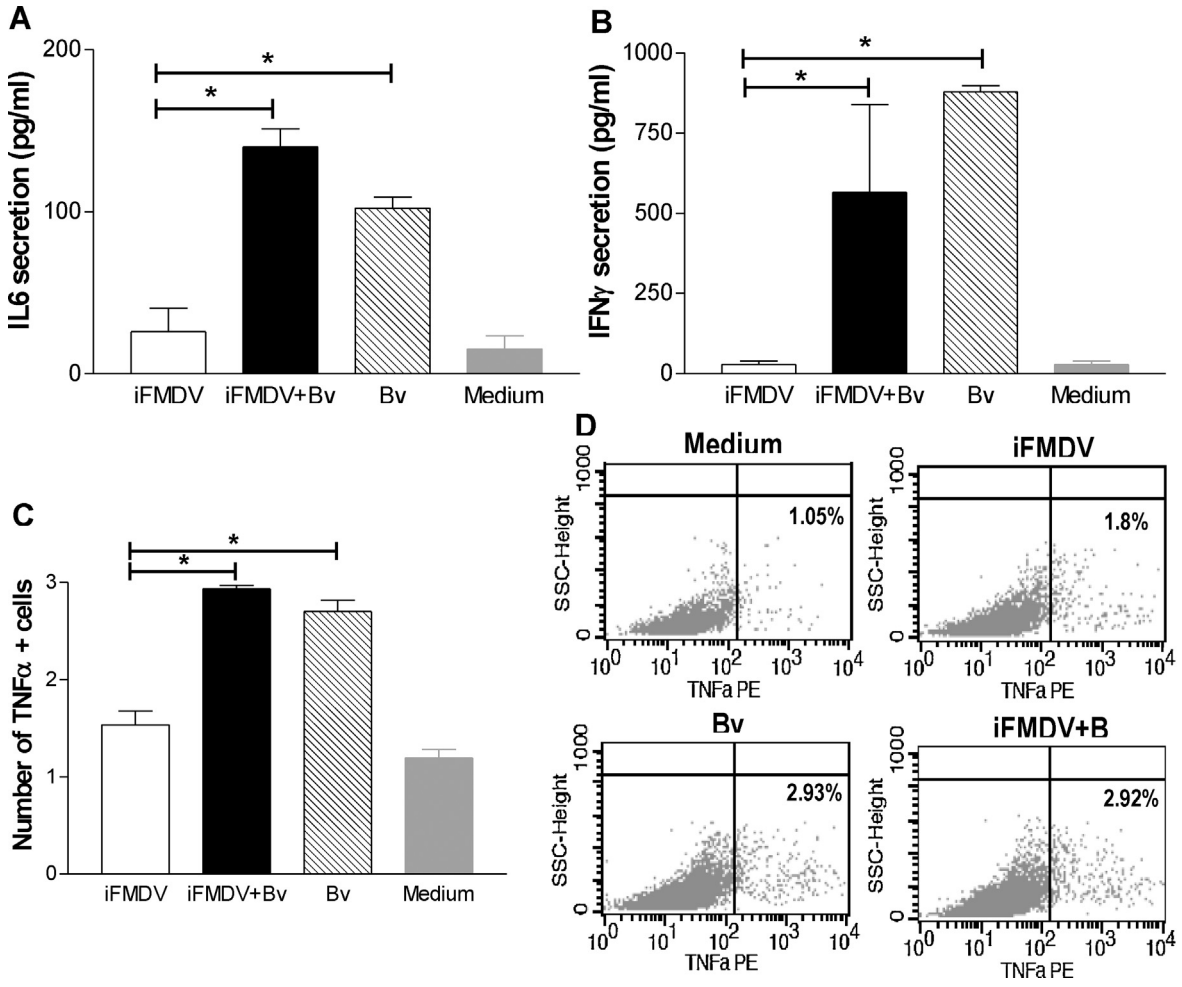

Fig. 3. Cytokines secreted by spleen cells at 4 dpv, from 5 mice vaccinated with iFMDV, iFMDV + Bv or Bv. The bars show mean concentrations (pg/ml) ± SD of IL6 (A) or IFNγ (B); .or mean numbers ± SD of TNFα staining positive cells, as measured by intracytoplasmatic flow cytometry (C). Dot plots of one representative experiment of intracytoplasmatic staining of TNFα (D). *Significant differences ($p < 0.05$) with respect to the iFMDV group.

Table 2
Antibodies against FMDV elicited by vaccination with iFMDV oil vaccine, iFMDV oil vaccine plus Bv or Bv. Upper panel: Total anti FMDV antibodies measured by ELISA. Lower panel: Seroneutralizing antibodies anti FMDV. Each number represents the mean anti FMDV Ab titers ± SD of 5 sera at 2, 4, 7 and 14 dpv, as measured by ELISA or fixed virus–variable serum neutralizing assay.

	2 dpv[a]	4 dpv	7 dpv	14 dpv
iFMDV oil vaccine	1.03 ± 0.34[b]	2.87 ± 1.17	2.98 ± 0.89	3.39 ± 0.91
iFMDV oil vaccine + Bv	0.88 ± 0.14	2.13 ± 0.87	2.24 ± 0.62	2.46 ± 0.90
Bv	0.82 ± 0.16	0.81 ± 0.14**	1.15 ± 0.50**	1.30 ± 0.30**
iFMDV oil vaccine	0.81 ± 0.18	2.19 ± 0.59	2.58 ± 0.38	2.21 ± 0.29
iFMDV oil vaccine + Bv	0.85 ± 0.18	2.31 ± 0.61	2.19 ± 0.19	2.28 ± 0.33
Bv	0.41 ± 0.17	0.42 ± 0.17**	0.41 ± 0.16**	0.43 ± 0.18**

** Significant differences ($p < 0.001$).
[a] Days post vaccination.
[b] Mean of antibody titers ± standard deviation.

3.6. Co-inoculation of iFMDV oil vaccine and Bv do not affect the Ab titers elicited by oil vaccine at 4, 7 or 14 dpv

iFMDV oil vaccine + Bv induced specific Ab titers against FMDV, that are not significantly different from those induced by iFMDV oil vaccine, at 4, 7 and 14 dpv (Table 2 upper panel). Both groups induced significantly higher titers of Abs than Bv group. The same trend was observed when SN Ab titers were evaluated (Table 2 lower panel).

4. Discussion

Recently, Bv has become a subject of great interest as immunopotentiators in mammals [8,17]. Since baculoviruses are able to transduce in mammalian cells but do not transcribe most of their genes and are unable to replicate, they are considered safe [5]. Hervas-Stubbs and collaborators [6] demonstrated that Bv have strong adjuvant properties, thereby promoting humoral and CTL responses against co-administered antigens, DC maturation and production of inflammatory mediators through mechanisms primarily mediated by IFNα and β.

Adult mice are not susceptible to natural infection with FMDV O1C, nevertheless they can be experimentally infected if the virus is inoculated by ip route and they have been widely used as model for FMDV. The infection is well characterized and includes viral replication in the pancreas, without clinical symptoms [10,18]. Despite the differences regarding infection and symptomatology between mice and natural hosts, a number of similarities have been established, for example: the main role of Abs in resolving the infection [10,19–21]; the life-long immunity after infection [22]; the T-independence of the immune response [19,23]; the key role for DC-secreted IL10 in FMDV-mediated immunosuppression [24–26]; *etc.* In addition, we have obtained preliminary data indicating that protection against viral challenge in adult BALB/c mice inoculated with commercial vaccines correlates with protection in cattle [13].

Molinari et al. [9] have demonstrated that the inoculation of Bv in C57bl/6 adult mice shortly before viral challenge with FMDV protects animals from infection. Nevertheless, this protective effect lasts only for 3 days. In order to increase the duration of immunity, we tested the effect of co-inoculation of Bv together with iFMDV antigen in our BALB/c murine model.

Our results showed that Bv enhanced the percentages of animals protected against viral challenge from 2 dpv up to 7 dpv. Levels of protection between iFMDV + Bv and Bv groups at 2 dpv were very similar, indicating that protection is directly related with immune responses elicited by Bv. The possibility that Bv could have an inhibitory effect on FMDV replication has been studied *in vitro*. When BHK-21 cells infected with FMDV were incubated in the presence or absence of wild type Bv, no differences were observed between treatments, suggesting that the effect on *in vivo* virus replication was not mediated by any interaction between FMDV and Bv [9]. We hypothesized that protection at 2 dpv is due to a complex state of activation of innate immune mechanisms induced by Bv that leads to an antiviral response. It is known that pre-treatment of cells with IFN-α/β dramatically inhibits FMDV replication [27,28] and IFN-α, has been reported to protect pigs against infection with FMDV [29,30]. In addition, although it has been described that IFNα/β secretion returns to basal levels 24 h post inoculation, IFN-stimulated genes (ISGs) may remain sufficiently stimulated to generate the protection observed in animals challenged 2 days after Bv inoculation. Nevertheless, more research is necessary in order to establish the actual cause of protection at 2 dpv.

Protection induced by Bv alone decreases dramatically 4 days after inoculation, while the percentage of protected animals in the iFMDV + Bv group is still high. However, at this time point, anti-FMDV Ab titers are increased in both groups. The Abs present in iFMDV + Bv vaccinated animals plus the inflammatory status produced by Bv could be responsible for the protection observed at 4 dpv.

On the other hand, it is well known for FMDV that Abs correlate with protection [10,19], and, consistently, at 7 dpv total and SN Ab titers in the iFMDV + Bv group are significantly higher than those in the iFMDV group, likely due to DC activation induced by Bv. The presence of iFMDV and activated DCs is probably the cause of the increased Ab titers seen at 4 and 7 dpv. Studies using murine and human DCs, showed that Bv are internalized by DCs and induce their maturation and the production of the pro-inflammatory cytokines IL-6 and IL-12 [9,31].

In the present report we found that Bv is able to enhance the very early immunity against FMDV and, in addition, iFMDV co-inoculated with Bv, protected against viral challenge until 7 dpv, but not at 14 dpv. With the aim to induce not only a very early but also a long lasting immunity, we studied the effect of co-administering Bv and an iFMDV oil vaccine, which is known as a good inducer of immunity from 7 dpv.

The excellent protective immunity induced by the iFMDV oil vaccine with or without Bv at 7 and 14 dpv could be related to anti-FMDV Abs, since they were detected in both animal groups at these time points. It is worth to mention that inoculation of Bv did not interfere with the development of a protective immune response induced by vaccination with oil vaccine and that the oil vaccine is capable of elicit high levels of protection in this murine model until 21 dpv (data not shown).

In summary, it is the first time that Bv is proposed as an adjuvant co-administered with a FMDV oil vaccine to induce a very early and long lasting immunity. Taking into account the promising results observed in this murine model, the adjuvants properties of Bv against FMDV, could be tested on natural hosts.

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