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Short communication

Baculovirus treatment fully protects mice against a lethal challenge of FMDV

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ABSTRACT

Foot-and-mouth disease virus (FMDV) causes a highly contagious and economically devastating disease that affects cattle, swine, goat and sheep among others. FMDV is able to overcome the initial host innate immune response by inhibiting the induction of antiviral molecules at both the transcriptional and the translational levels. It has been demonstrated that FMDV A/Arg/2001 causes the death of adult C57Bl/6 mice within 72 h. We evaluated the capacity of *Autographa californica* nuclear polyhedrosis virus (AcNPV), an insect virus with potent innate immunostimulating effects, to promote early protection against FMDV A/Arg/2001 challenge in C57Bl/6 mice. Groups of 8–9 weeks old female mice were injected intravenously with AcNPV and challenged with a lethal dose of FMDV at different times post-administration. Our results showed that pretreatment of mice with a single injection of AcNPV 3 h or 3 days before FMDV challenge resulted in complete abrogation of mortality and complete or partial suppression of viremia, respectively. Furthermore, no signs of disease were observed. AcNPV could be a valuable tool to improve the design of a novel vaccine that protects as an adjuvant at early times post-vaccination.

Foot-and-mouth disease (FMD) is one of the most contagious and economically devastating diseases of cloven-hoofed animals, including cattle, swine, goat and sheep. The disease is caused by foot-and-mouth disease virus (FMDV), a single-stranded, positive sense RNA virus, member of the family *Picornaviridae*, that exists in the form of seven serotypes: A, O, C, Asia 1, SAT1, SAT2 and SAT3 (Bachrach, 1968; Knowles and Samuel, 2003).

The short incubation times along with the high contagiousness and the high antigenic variation of the virus contribute to the difficulty for rapidly controlling the disease when an outbreak occurs. Although current vaccines can induce an effective protective response, it is only by about 7 days post-vaccination when protection levels are reached (Golde et al., 2005). Therefore, it is crucial to develop new strategies such as antiviral approaches and novel vaccines or adjuvants that can rapidly control the disease.

An adult C57Bl/6 mice model for FMDV infection has been characterized by Salguero et al. (2005). They described that FMDV injected intraperitoneally (IP) is lethal for C57Bl/6 mice, although 50% lethal doses vary among serotypes and even among strains of the same serotype. The infected animals present ruffled fur, humped posture, apathy and ataxia prior to death, which occurs within 48–72 h post-infection. The virus spreads to almost all organs, causing a systemic infection. In addition, mice show a severe lymphopenia as well as microscopic lesions in several organs similar to those described in the natural hosts (Salguero et al., 2005).

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has a double-stranded circular DNA genome of approximately 130 kbp that contains more than 150 open reading frames (Ayres et al., 1994). The ability of AcNPV to infect insect cells has led to its use as a protein expression system (Matsuura et al., 1987; O'Reilly et al., 1994) and as a plant insecticide. AcNPV also infects a variety of mammalian cell types, although its genome does not replicate or integrate into mammalian chromosomes (Tjia et al., 1983; Volkman and Goldsmith, 1983). It has been demonstrated that AcNPV strongly activates innate immune responses in animals within few hours post-injection by inducing inflammatory cytokines (IL-6 and IL-12) and type I and II IFN. However, by 24 h post-injection all of these cytokines return to basal levels (Abe et al., 2003; Kitajima et al., 2006, 2008).

The purpose of this study was to evaluate the protective potential of AcNPV stimulation against a serotype A FMDV challenge. AcNPV was produced in a *Spodoptera frugiperda* cell line (Sf9) and cultured in SF900 medium with 2% of fetal bovine serum (FBS) at 27 °C. Virus titers were calculated by end point dilution assay and converted to PFU/ml as described by O'Reilly et al. (1994). Two hundred microliters of wild type AcNPV containing 5×10^7 PFU were administered to groups of five C57Bl/6 (H-2b) 8–9 weeks old female

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mice by intravenous injection in the retro orbital plexus. At different times post-administration (3 h and 3, 6 and 14 days) mice were challenged IP with 8.5×10^3 PFU of FMDV A/Arg/2001 contained in 0.1 ml of DMEM–Hepes (DMEM, HyClone, 25 mM Hepes, pH 7.5). FMDV A/Arg/2001 kills adult C57Bl/6 mice in doses as low as 10^2 PFU within 48 h (García-Nuñez et al., 2010). Twenty-four hours post-challenge, two mice per group were bled and euthanized. Blood samples were collected in 5 μM heparin, centrifuged at $3000 \times g$ at 4 °C for 15 min and plasma was isolated. Levels of IFN-γ were measured by ELISA (BD Opt EIA AN-18, BD Biosciences) and 50% Cell Culture Infectious Doses ($CCID_{50}$) were determined by the method of Reed and Muench (1938). Brain, pancreas, liver and spleen were harvested and homogenized in DMEM–Hepes and a peritoneal lavage was collected using DMEM–Hepes. Viral particles were released by three successive cycles of freezing and thawing, and a tenfold dilution of each sample was added in quadruplicate to 96-well plates containing 90% confluent BHK-21 cells. The presence of cytopathic effect was determined after 48 h. In parallel, 200 μl of pancreas homogenates were added to 900 μl of TRIzol© (Invitrogen) and total RNA was obtained following the manufacturer's instructions. Total RNA in each sample was measured using a NanoDrop nd-1000 spectrophotometer and cDNA was synthesized in the presence of MMLV reverse transcriptase (Promega) using 0.5 μg of Random Primers (Biodynamics) and 1 μg of total RNA. Finally, a real-time PCR assay was performed in an ABI Prism 7000 sequence detection system as previously described (García-Nuñez et al., 2010). The three remaining mice of each group were daily observed for signs of disease and death, and all surviving mice were tested for seroconversion by liquid phase blocking ELISA to whole virus on day 18 post-challenge (Hamblin et al., 1987). The results are representative of three independent experiments.

Mice inoculated with supernatants of non-infected Sf9 cells (SN) 3 h before challenge (bc) or with AcNPV 14 days bc died within 72 h post-challenge (Fig. 1), and the average virus load in plasma was 8.4 ($\log CCID_{50}$/ml) (Table 1). On the contrary, pretreatment of mice with a single injection of AcNPV 3 h or 3 days bc resulted in complete abrogation of mortality and complete or partial suppression of viremia, respectively (Table 1). Furthermore, no signs of disease were observed and no seroconversion was detected in either group (data not shown). Pretreatment of mice with AcNPV 6 days bc reduced the viremia and gradually delayed the death of two out of three mice (Fig. 1). Basal levels of IFN-γ were detected in plasma of all groups by 24 h after challenge (data not shown).

To evaluate protection by AcNPV between 3 and 6 days bc, groups of 4 adult C57Bl/6 female mice were inoculated with AcNPV 3, 4, 5 and 6 days bc (data not shown). Although the death was delayed, 50% of the mice inoculated with AcNPV 4 days bc died. All the animals inoculated with AcNPV 5 or 6 days bc died. All the mice inoculated with AcNPV 3 days bc survived.

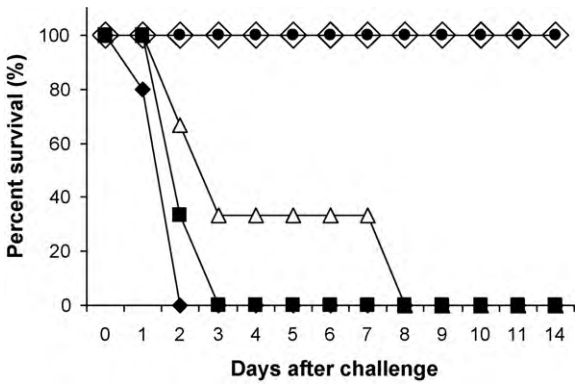

Fig. 1. Baculovirus injection protects mice from FMDV infection. Survival curves of adult C57Bl/6 mice infected with 8.5×10^3 PFU of FMDV A/Arg/01 that had been previously inoculated with AcNPV (5×10^7 PFU) at different times before challenge (bc). ♦, mice inoculated with supernatant of non-infected Sf9 cells (SN) 3 h bc; ●, mice inoculated with AcNPV 3 h bc; ◊, mice inoculated with AcNPV 3 days bc; △, mice inoculated with AcNPV 6 days bc; ■, mice inoculated with AcNPV 14 days bc.

As can be seen in Fig. 1 (SN vs 3 h bc), the antiviral effect of AcNPV was not due to other factors derived from the insect cells. Moreover, it has been shown that specific inactivation of AcNPV completely abrogates the inmunopotentiation effect (Hervas-Stubbs et al., 2007). Based on these data, the integrity of the virions and no other factors contained in the supernatants are responsible for the antiviral activity.

As shown in Table 1, FMDV spread to almost all the organs analyzed in every group of challenged mice. However, only mice treated with AcNPV 3 h or 3 days bc did not show the FMDV presence in brain and significant lower quantities of FMDV RNA were detected in pancreas ($p < 0.05$, Kruskal–Wallis), the most affected organ of FMDV infected C57Bl/6 mice (Sanz-Ramos et al., 2008). As anti-FMDV neutralizing antibodies were not detected on day 18 post-challenge in these latter groups (data not shown), these data would suggest that the overall viral load in mice treated with AcNPV 3 h or 3 days bc was not sufficient to elicit a humoral response. Moreover, it has been previously reported that a replication threshold for FMDV is required for seroconvertion (Kamstrup et al., 2006; Rodríguez Pulido et al., 2009).

The possibility that AcNPV could have a direct inhibitory effect on FMDV replication was studied *in vitro*. BHK-21 cells are transduced by AcNPV (Chiang et al., 2006; Ojala et al., 2004); therefore, we infected BHK-21 cells with FMDV in the presence or absence

Table 1
The presence of FMDV 24 h post-challenge.

Mouse	Time of AcNPV inoculation[a]	Viremia ($\log CCID_{50}$/ml)[b]	Viral RNA[c]	Cytopathic effect[d]				
				Pancreas	Brain	Peritoneal cells	Liver	Spleen
1	None	8.5	4.75×10^5	+	+	+	+	+
2	None	8.5	8.75×10^4	+	+	+	+	+
3	3 h	0	5.06×10^2	+	–	+	+	+
4	3 h	0	1.39×10^2	+	–	–	+	+
5	3 days	0	2.04×10^3	+	–	–	+	–
6	3 days	3.83	2.9×10^3	+	–	+	+	+
7	6 days	7.83	1.29×10^5	+	+	+	+	+
8	6 days	8.17	6.51×10^4	+	+	+	+	+
9	14 days	8.17	1.46×10^5	+	+	+	+	+
10	14 days	8.5	2.6×10^4	+	+	+	+	+

[a] Time points are given before challenge with FMDV.
[b] FMDV viremia titers: mice were bled 24 h after challenge and $CCID_{50}$ was calculated for each mouse.
[c] Total RNA was extracted from pancreas and a real-time PCR was performed to detect FMDV as previously described (García-Nuñez et al., 2010). FMDV RNA titers are expressed as the number of molecules per μg of total RNA.
[d] A tenfold dilution of each sample was inoculated in quadruplicate to BHK-21 cells in 96-well plates. Cytopathic effect was determined after 48 h. +, presence; –, absence.

of 200 μl of wild type AcNPV containing 5×10^7 PFU. Plaque assays were performed as previously described (Tami et al., 2003) and cell monolayers were fixed and stained at 48 h post-infection. No differences were observed between both treatments, suggesting that the effect on virus replication *in vivo* was not mediated by any interaction between FMDV and AcNPV. Since AcNPV is known to induce a complex state of activation of innate immune mechanisms, these are most likely responsible for the protection observed (Abe et al., 2005; Gronowski et al., 1999). Induction of cytokines could be a plausible explanation, as IFN-α has been reported to protect pigs against infection with FMDV (Chinsangaram et al., 2003; Moraes et al., 2003). AcNPV induces high levels of IFN-α and IFN-β as fast as 3 h post-inoculation, but also other cytokines of relevance, most notably IFN-γ (Abe et al., 2003; Hervas-Stubbs et al., 2007; Kitajima et al., 2008; Kitajima and Takaku, 2008).

It has been demonstrated that pretreatment of cells with IFN-α/β dramatically inhibits FMDV replication (Ahl and Rump, 1976; Chinsangaram et al., 2001), and at least two IFN-α/β-stimulated gene products (ISGs), double-stranded-RNA-dependent protein kinase and 2′,5′-oligoadenylate synthetase/RNase L are involved in this process (Chinsangaram et al., 2001; de Los Santos et al., 2006; Moraes et al., 2007). At the same time, type II IFN (IFN-γ) has an antiviral activity against FMDV in cell culture and displays a synergistic antiviral effect in combination with IFN-α (Moraes et al., 2007).

It is known that FMDV is able to overcome the initial host innate response by inhibiting the induction of antiviral molecules at both the transcriptional and the translational levels (Grubman et al., 2008). Although the mechanisms of protection generated by AcNPV are not well understood, the inoculation of AcNPV before FMDV challenge may be generating an antiviral status probably due to the presence of IFN and ISGs among other factors, thus bypassing the cap-dependent translation inhibition caused by FMDV. The protection observed in animals inoculated with AcNPV 3 h bc is probably a consequence of the high levels of circulating IFN. On the other hand, although IFN secretion returns to basal levels 24 h post-inoculation, other factors induced by IFN, most probably ISGs, may remain in sufficient levels so as to generate the protection observed in animals challenged 3 days after AcNPV inoculation. However, this protection decreases dramatically 4 days after AcNPV inoculation, when only 50% of the animals were protected. It is known that AcNPV is inactivated by complement. Some reports have shown that AcNPV displaying the decay-accelerating factor or coated with polyethylenimine protected from the inactivation mediated by complement (Kaikkonen et al., 2006; Kaname et al., 2010; Yang et al., 2009). Thus, it would be interesting to evaluate a modified AcNPV with prolonged middle life in blood in order to extend the antiviral protection.

Kamstrup et al. (2006) using Balb/c mice and CpG ODN as an antiviral approach discussed that innate immune cells such as monocytes or natural killer cells (NK) could favor the immune control of infection of FMDV. Activation of monocytes could help to control the infection since monocytes are known to play a role in transporting FMDV to other sites (Rigden et al., 2002). AcNPV can activate these cells (Abe et al., 2003). Recently, it has been demonstrated that AcNPV activates NK cells, thus, FMDV-infected cells would become targets for NK-mediated killing (Suzuki et al., 2010). However, if activation of monocytes and NK cells occurred in our model, it was not sufficient to limit FMDV spread, as a systemic dissemination of FMDV occurred, indicating that the infection was not controlled at the site of inoculation.

Previous results have demonstrated that AcNPV protects mice against lethal challenges of encephalomyocarditis virus, a picornavirus (Gronowski et al., 1999), and influenza H1N1 (Abe et al., 2003).

We have shown here that AcNPV injection completely abrogates the development of signs of FMD when is injected as far as 3 days bc and as soon as 3 h bc, presumably based on the induction of a complex state of activation of innate immune mechanisms (Abe et al., 2009; Hervas-Stubbs et al., 2007). AcNPV could be a valuable tool to improve the design of a novel vaccine that protects susceptible hosts at early times post-vaccination as an adjuvant with antiviral properties.

As in the case of CpG treatment, that protects mice but not pigs (Alves et al., 2009; Kamstrup et al., 2006), this finding may be relevant if the results can be reproduced in natural hosts. AcNPV seems to activate more than one immune innate pathway as shown by Abe et al. (2009), so it could be most probably effective in natural hosts.

Further studies are needed to clarify the precise mechanisms underlying the antiviral responses that confer a protective immunity against FMDV challenge induced by AcNPV injection *in vivo*.

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