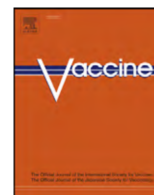
Vaccine 28 (2010) 6361–6366

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Adjuvant effect of Cliptox^{TM} on the protective immune response induced by an inactivated vaccine against foot and mouth disease virus in mice

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ARTICLE INFO

Article history:
Received 16 December 2009
Received in revised form 16 June 2010
Accepted 29 June 2010
Available online 14 July 2010

Keywords:
FMDV
Vaccine
Cliptox
Protection

ABSTRACT

Foot and Mouth Disease (FMD) is an acute disease caused by Foot and Mouth Disease Virus (FMDV) which causes important economy losses, this is why it is necessary to obtain a vaccine that stimulates a rapid and long-lasting protective immune response. Cliptox^{TM} is a mineral microparticle that in earlier studies has shown adjuvant activity against different antigens.

In this study we have examined the effects of Cliptox^{TM} on the magnitude and type of immunity elicited in response to inactivated FMDV (iFMDV) vaccine. It was demonstrated that iFMDV-Cliptox ^{TM} stimulates a specific antibody response detected in mucosal and in sera. The different isotype profiles elicited by inoculation with this vaccine indicate a Th1/Th2 response. Also, an increase in dendritic cells and macrophages in the spleen in comparison with the iFMDV vaccine iFMDV-Cliptox^{TM} was detected. The Cliptox-iFMDV formulation was non toxic by using egg embryos and yielded increased protection against challenge with FMDV in the murine model.

Our results show that the incorporation of Cliptox^{TM} into FMDVi vaccine induces an increase of the specific protective immune response in mice and clearly indicate that Cliptox TM exert an (important) up-regulation on DC and MΦ. Additionally, Cliptox TM adjuvant can be used in vaccines for induction of mucosal immune response.

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1. Introduction

Foot and Mouth disease (FMD) is an acute, highly contagious viral disease. It is economically important because of the international restrictions it imposes to cattle commercialization. Routine vaccination with inactivated FMD virus (FMDV) in endemic regions can significantly reduce the economical impact of the disease.

An experimental murine model which correlates with the humoral immune and protective response against FMDV in cattle was previously developed in our laboratory [1]. In addition, in the mouse model of FMDV infection, Borca et al. [2] demonstrated that B cells are mainly responsible for the primary response, and that the protective immune reaction is T-independent. Recently Juleff et al. [3] confirming these results obtained in mice, demonstrated that the foot-and-mouth disease virus can induce a specific and rapid CD4+ T-cell-independent neutralizing and isotype class-switched antibody response in naïve cattle.

Adult Balb/c mice are not susceptible to natural infection with FMDV O1, but they can be experimentally infected by i.p. inoculation. The virus replicates in pancreatic cells and viremia lasts for 72 h post inoculation without clinical symptoms. Then, when neutralizing antibody (Nab) titers increase, viremia ends. After 14 days post infection, mice show a long-lasting humoral response dominated by IgG2b, followed by IgG1, IgG2a and IgG3. As in the natural host, infection elicits high levels of circulating NAb which persist life-long and prevent re-infection [4].

Aerosol transmission is the main route of FMDV infection, since as in the case of most other pathogens, this virus enters the body through mucosal surfaces. It has been well documented that mucosal [5,6] and serological [7] IgA responses can be detected after infection of cattle with FMDV.

Eble et al. [8] reported that the induction of IgA responses correlates with complete protection against challenge in pigs immunized with a highly concentrated inactivated vaccine. Cubillos et al. [9] reported a correlation between solid protection and IgA induction conferred by a peptide containing one copy of a FMDV

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doi:10.1016/j.vaccine.2010.06.098

T-cell epitope and branching out into four copies of a B-cell epitope.

The lack of potent adjuvants which stimulate mucosal and systemic immunity and exert adequate *in vivo* delivery capabilities dramatically limits the development of effective vaccine formulations. Microparticles have been shown to be effective delivery systems for vaccine formulations inducing potent cellular and humoral immune responses. Furthermore, they can protect the antigens against the aggressive conditions such as low pH, biliary salts and enzymes. Zeolites are microparticles of silica, silicates, and aluminosilicates, which act as non-specific immunostimulators similarly to superantigens with the ability to activate a relatively large fraction (5–20%) of the T-cell population, as well as humoral immunity [10]. Specifically, the immunostimulator properties were demonstrated by Ryu and Shaey [11,12] who showed that inactivated *Trypanosoma gambiense* parasite suspensions formulated with zeolite induced a significant protection against challenge with pathogenic strains.

On the other hand, virus–dendritic cells (DCs) interactions have to be taken into account since DCs are the only ones able to activate the adaptative inmune response.

Our earlier results [13] have demonstrated the adjuvant effect of natural microparticles of the zeolite clinoptilolite (Cliptox™) using two classic T-cell-stimulating antigens (ovalbumin and sheep red blood cells). Subcutaneous injection of mice with these antigens formulated with Cliptox™ elicited high titers of specific antibodies, with only irrelevant side effects at the site of inoculation. In addition, Cliptox™ proved to have the capacity to generate a mucosal immune response against exogenous antigens [13,14].

This work was aimed at analyzing the efficacy of incoporating the zeolite clinoptilolite (Cliptox) as adjuvant in FMD vaccines consisting of inactivated virus (iFMDV).

In this report, we present important evidence regarding the immunological and adjuvant properties of Cliptox™ and the efficacy to induce protection against FMDV O1C in our murine model.

2. Materials and methods

2.1. Mice

Balb/c mice between 8 and 12 wk of age, from the School of Veterinary Sciences, La Plata, Argentina were used. Animal care was performed in accordance with institutional guidelines.

2.2. Virus

Binary ethylenimine (BEI)-inactivated FMDV (iFMDV) (kindly provided by Biogenesis, Buenos Aires) was used in the experimental vaccine formulation and in ELISA assays. Infectious virus, O1Campos serotype, was used for viral challenge and was provided by SENASA (Buenos Aires). All the experiments involving infectious virus were performed in the NBS 3A SENASA facilities.

2.3. Toxicity

In order to evaluate the safety of Cliptox, toxicity was assessed by the HET-CAM test at the Centro de Toxicología y Biomedicina (TOXIMED), Santiago de Cuba, Cuba. Vaccines were classified as safe.

Toxicological Test (HET-CAM): embryonated eggs of White Leghorn hens (50–60 g of weight) were incubated for a total of 10 days at $37.5 \pm 0.5\,^{\circ}$C, with a relative humidity of $62.5 \pm 7.5\%$. At day 9, eggs were placed with their air chamber up and were kept in this position for 24 h. Eggs were then checked under a lamp light and those that showed good embryo conditions and no scratches or extra-porosity in the shell were selected, and their air chamber marked with pencil. The shell was carefully cracked in the area of the air chamber, and the exposed white membrane was moisted with 0.9% NaCl. Eggs were immediately returned to the incubator where they were kept for 20–30 min, to avoid cooling. The membrane was removed with ophtalmic surgical instruments, exposing the corioallantoic membrane. Reference standard solutions were added (1% SDS and 0.1 N NaOH) and the time of appearance (in seconds) of the following reactions was recorded during 5 min: hemorrage (H) (bleeding), vascular lysis (L) (vessel disintegration) and coagulation (C) (denaturing of intra- and extra-vascular protein). The irritation score (IS) was calculated with these values using the formula:

$$IS = \left(\frac{301\text{-s H}}{300} \times 5\right) + \left(\frac{301\text{-s L}}{300} \times 7\right) + \left(\frac{301\text{-s C}}{300} \times 9\right)$$

Then, the adjuvant to be tested was placed on the corioalantoid membrane so that at least half of the surface was covered, and left to act for 5 min, after which it was carefully washed with 0.9% NaCl. The severity of the above-mentioned reactions (hemorrage, lysis and coagulation) was evaluated according to the procedure described in INVITTOX No 47 protocol, 1992. This procedure has been validated by the European Center for the Validation of Alternative Methods (ECVAM), Ispra, Italy.

2.4. Vaccine formulations and vaccination

Two types of vaccines were prepared: 0.5 μg inactivated FMDV in phosphate buffered saline (PBS) (iFMDV), and the same formulation to which 1 μg of Cliptox in PBS had been added (Cliptox-iFMDV) and gently mixed. Groups of 10 Balb/c mice were immunized once (at day 0), named: (iFMDV)a and (Cliptox-iFMDV)a or twice (at day 0 and 7) named: (iFMDV)b and (Cliptox-iFMDV)b by the subcutaneous (s.c.) route with 0.2 ml/dose. Other animals recieved: 1 μg Cliptox™ alone, or PBS.

2.5. Viral challenge

Protection against FMDV in the mouse model is defined by the presence or absence of viremia 24 h after infection [15–17]. Briefly, mice were i.p. inoculated at 21 days post vaccination (dpv) with $10^{4.5}$ $TCID_{50}$ infectious FMDV O1C serotype. After 24 h, animals were anesthetized and bled through the retro orbital vein. The heparinized blood was spread in 48 well-plates onto BHK-21 cell monolayers and incubated for 40 min at 37 °C in a 5% CO_2 atmosphere. Then, cell monolayers were washed three times with sterile PBS. Fresh DMEM supplemented with 2% fetal calf serum (FCS) was added and the cells were incubated for 48 h at 37 °C, 5% CO_2. It was considered that the animals were protected if the cell monolayer did not present cytopathic effects after a blind passage. In every viral challenge assay, animals inoculated with sterile PBS were included as positive infection controls. These controls always had detectable viremia. Percentages of protection were calculated as 100× (protected/challenged mice).

2.6. Measurement of total FMDV-specific antibodies

A liquid-phase ELISA test was used, according to Hambling et al (1986). Briefly, Immulon IIHB plates were coated overnight at 4 °C with rabbit anti-FMDV serum diluted to the optimal concentration in carbonate-bicarbonate buffer, pH 9.6. After washing with 0.05% Tween-20/phosphate buffered saline (PBST) plates were blocked with PBST/1% ovalbumin (blocking buffer) for 30 min at 37 °C. Mice sera were serially diluted (1:10) in blocking buffer and a fixed amount of antigen was added. After 1 h incubation at 37 °C with shaking, the virus-antibody mixtures were transferred to the blocked plates, and incubated for 1 h at 37 °C.

An optimal dilution of guinea pig anti-FMDV serum diluted in PBS/2% normal bovine serum/2% normal rabbit serum was added for detection, followed by 1 h incubation at 37 °C. Plates were washed and peroxidase-conjugated anti-guinea pig IgG serum diluted in the same buffer was added, followed by 1 h incubation at 37 °C. Ortho-phenylene-diamine (1,2-benzenediamine) dihydrochloride (SIGMA) (OPD)/H_2O_2 was used as peroxidase substrate and absorbance at 492 nm (A_{492}) was measured in a MR 5000 microplate reader. Adequate positive and negative controls were included in each test. Antibody titers were expressed as the negative logarithm of the highest dilution of serum that causes an inhibition of color development higher than 50% in the average values of the control samples.

2.7. Detection of IgA against FMDV in saliva by sandwich ELISA

Maxisorp plates were coated ON at 4 °C with anti-FMDV rabbit serum in carbonate–bicarbonate pH 9.6 buffer. After three washing steps, the plates were blocked with polyvinylpyrrolidone blocking solution (0.5 M NaCl; 0.01 M buffer fosfato; 0.05% Tween-20; 1 mM EDTA; 1% polyvinylpyrrolidone 30–40 K, pH 7.2) for 60 min at 37 °C and then the inactivated FMDV was added at optimal dilution in PBST. Plates were incubated at 37 °C for 30 min. Then saliva samples diluted 1:2 in blocking solution were added. After 1 h incubation at 37 °C, plates were washed and an optimal dilution of biotin-conjugated anti-mouse isotype IgA (Caltag) was added. Plates were incubated for 1 h at room temperature (RT) and then washed. Horse radish peroxidase (HRP)-conjugated streptavidin was added and after 1 h incubation at RT, OPD–H_2O_2 was used as peroxidase substrate. The reaction was stopped using 1,25 M H_2SO_4 and A_{492} was measured in a MR 5000 microplate reader (Labsystems). Positive and negative control salivas were included in every plate.

2.8. Isotype Ab measurement

Inmulon II plates were coated ON at 4 °C with anti-FMDV rabbit serum in carbonate–bicarbonate pH 9.6 buffer. After three washing steps, inactivated FMDV was added at an optimal dilution in PBST. Plates were incubated at 37 °C for 30 min, and then blocked with polyvinylpyrrolidone blocking solution (at 37 °C for 45 min). Serum samples (1:150) were added to the plates and incubated for 2 h at room temperature followed by washing. Then, biotin-conjugated anti-mouse isotype (Caltag) was added and incubation followed for 1 h at room temperature. After washing, plates were first incubated with HRP-conjugated streptavidin for 1 h at room temperature, and then with OPD–H_2O_2, used as peroxidase substrate. A_{492} was recorded in a MR 5000 microplate reader (Labsystems). Positive control serum samples were included in every plate. The samples from mock-vaccinated or vaccinated with Cliptox alone had similar results of A_{492} measurements in the ELISA test.

2.9. Isolation of spleen cells

Mice were killed by cervical dislocation and dissected. Spleens were carefully removed and placed in sterile Petri dishes. After cutting-off their bottom ends, about 2 ml PBS supplemented with 10 mM EDTA were injected into each spleen, in order to withdraw the cells from the capsules. Cells were centrifuged, counted in a Neubauer chamber and adjusted to optimal concentrations.

2.10. Flow cytometry

Single-spleen cell suspensions obtained two days post vaccination were incubated (15 min, 4 °C) with different FITC or Phycoerythrin-labeled antibodies (e-Bioscience) diluted in PBS, to evaluate the expression of cell surface molecules. The following antibodies were used: CD11c/IA^d(MHC class II) y F4/80/Mac3 and their correspondent isotype controls. After washing the cells twice with PBS, fluorescence patterns were analyzed using a FACScan flow cytometer and CellQuest software (BD Biosciences). The results are expressed as percentages of positive cells in the gate.

Table 1
Protection levels induced in (iFMDV)b, (Cliptox-iFMDV)b or control groups. Mice ($n = 10$) were s.c. immunized twice with (Cliptox-iFMDV), named (Cliptox-iFMDV)b or (iFMDV), named (iFMDV)b at days 0 and 7 and then challenged 21 days post first immunization with $10^{4.5}$ $TCID_{50}$/ml infectious FMDV O1C serotype by the i.p. route. Protection against FMDV in the mouse model is defined by the absence of viremia 24 h after infection, and is here shown as percentages of protected animals in each group (% protection).

Group	% protection ($n = 10$)
(Cliptox-iFMDV)b*	90
(Ifmdv)b	20
(Cliptox™)b	0
Negative control (PBS)	0

* $p < 0.05$ for (Cliptox-iFMDV)b versus (iFMDV)b group measured by the Fisher exact test.

2.11. Statistical analysis

The protective data were analyzed by Fisher exact tests. ANOVA test was used to compare population cells results between groups. The statistical significance of differences in the IgA levels in saliva samples between two groups were calculated using student *t*-test. *P* value <0.05 was considered as an indicator of significant differences.

3. Results

3.1. Cliptox is non toxic

The potential toxicity of the Cliptox-iFMDV formulation was tested on the corion-alantoidal membrane of embryonated eggs. Microscopic examination of these membranes showed no differences with their negative controls, indicating that no toxic effects could be associated with Cliptox (Fig. 1).

3.2. Immunization with Cliptox-iFMDV vaccine confers protection against FMDV in challenged mice

Balb/c mice were s.c. inoculated with either one or two doses of iFMDV or Cliptox-iFMDV and challenged after 21 dpv with FMDV. Surprisingly 90% of the mice ($n = 10$) that were immunized twice with Cliptox-iFMDV were protected against challenge, as measured by the presence or absence of viremia 24 h after infection. On the other hand, only 20% of the iFMVD-vaccinated group were protected, while all animals immunized only with Cliptox or PBS, as negative controls, were not protected (Table 1) ($p < 0,05$ for (Cliptox-iFMDV)b versus (iFMDV)b group measured by the Fisher exact test). None of the mice immunized with only one dose of any of the formulations were protected against viral challenge (data not shown).

3.3. FMDV antibody levels induced by vaccines in Balb/c mice

As shown in Fig. 2, mice s.c. immunized with (Cliptox-iFMDV)b or (iFMDV)b displayed enhanced titers of FMDV-specific antibodies ($p < 0.05$) as compared to mice that received a single inoculation with the same formulation (Cliptox-iFMDV)a or (iFMDV)a. The latter showed low but detectable levels of FMDV-specific antibod-

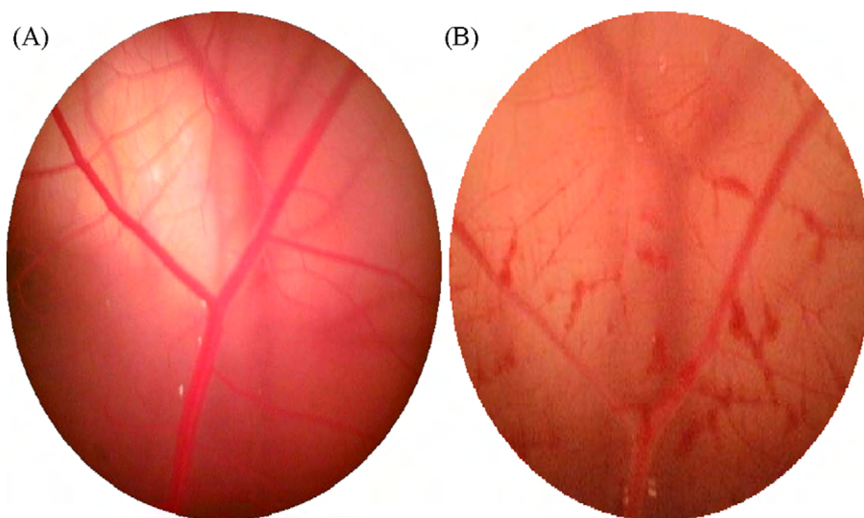

Fig. 1. Chicken's embryo corioallantoic membrane after the application of (A) Cliptox-iFMDV and (B) iFMDV. Similar results were observed in the negative control.

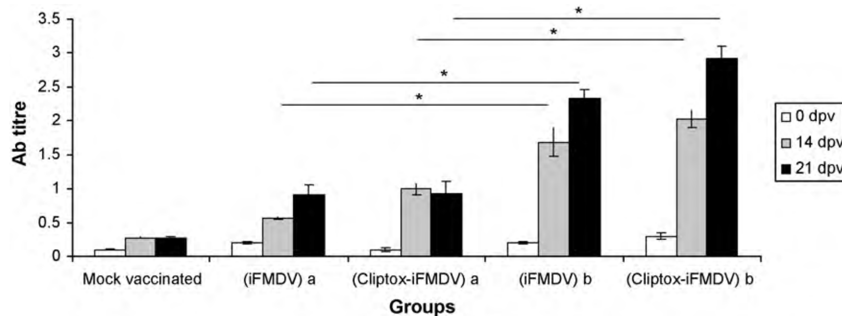

Fig. 2. Antibody titre response induced in mice at different days post vaccination. Mice were s.c. immunized once: (iFMDV)a and (Cliptox-iFMDV)a or twice (at 7 days post vaccination): (iFMDV)b and (Cliptox-iFMDV)b with 0.5 μg inactivated iFMDV mixed with 1 μg Cliptox/0,2 ml/mice. Anti-virus specific antibodies were detected by ELISA as described in Section 2. Each bar represents the mean Ab titre ± SE at 0, 14 and 21 dpv in each group of animals ($n = 10$). *Significant differences ($p < 0.05$) between (iFMDV)a and (iFMDV)b group at 14 and 21 dpv or (Cliptox-iFMDV)a and (Cliptox-iFMDV)b group at 14 and 21 dpv.

ies. No significant differences were observed between the groups (Cliptox-iFMDV)b or (iFMDV)b.

3.4. *Isotype of FMDV-specific antibodies in vaccinated mice*

The IgG isotypes produced in vaccinated animals were analyzed at 21 dpv. Sera from (Cliptox-iFMDV)b mice showed a significant enhancement of IgG2a and IgG2b isotypes ($p < 0.05$) as compared to sera from (iFMVD)b mice. IgG1 titers were also enhanced at day 21 of the experiment, but no significant differences between (Cliptox-iFMDV)b and (iFMVD)b groups were observed (Fig. 3). Interestingly, at 12 and 21 dpv, levels of FMDV-specific IgA in saliva samples were significantly increased ($p < 0.005$) in the (Cliptox-iFMDV)b group with respect to the (iFMDV)b group (Fig. 4). The latter showed

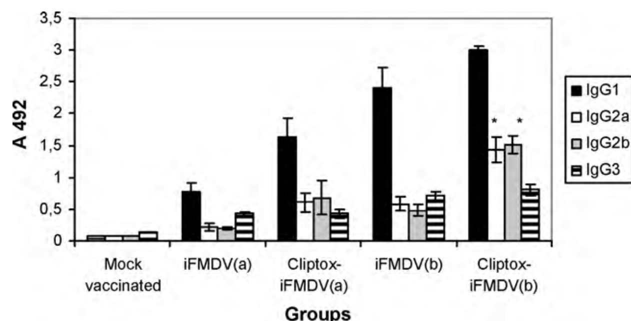

Fig. 3. Specific IgG isotypes induced by vaccination with the different formulations in Balb/c mice at 21 dpv. Mice were vaccinated once or twice as in Fig. 2. Each bar represents the mean A_{492} values ± SE of 8 sera (1:10 dilution) as measured by ELISA. *Significant differences ($p < 0.05$) with respect to the (iFMDV)a group, (Cliptox-iFMDV)a and the (iFMDV)b group.

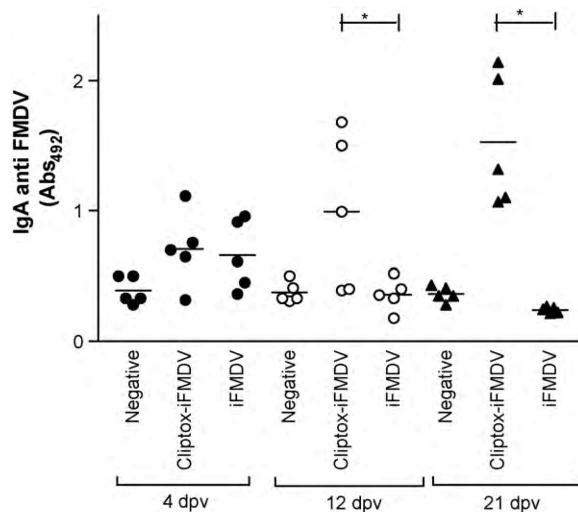

Fig. 4. Specific salivary IgA response to FMVD measured at different times in Balb/c mice s.c. immunized with (iFMVD)b, (Cliptox-iFMVD)b vaccine or PBS (mock-vaccinated). Each point represents mean A_{492} values ± SE of 5 murine saliva samples at days 4, 12 and 21 dpv. *Significant differences ($p < 0.05$) with respect to the (iFMDV)b group.

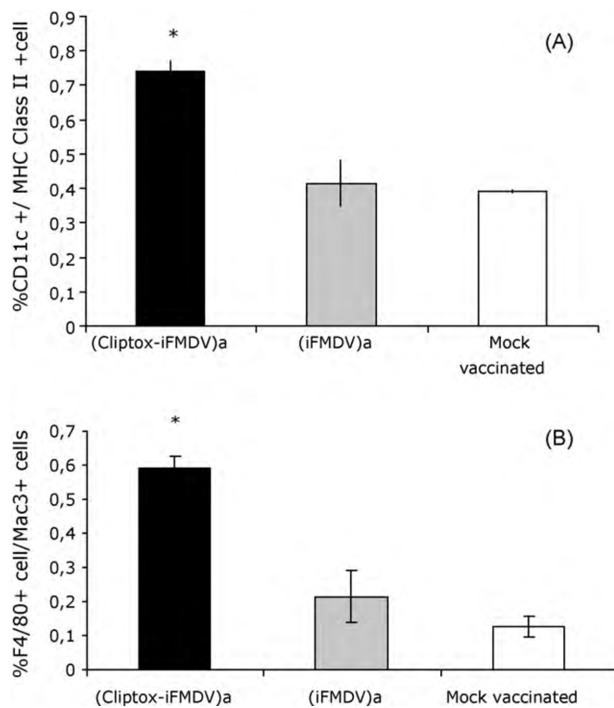

Fig. 5. Percentages of (A) CD11+/MHC II+ (dendritic cells) and (B) F4/80+/Mac 3+ cells (macrophages) from spleens of mice immunized with (Cliptox-iFMDV)a, (iFMDV)a or PBS (mock-vaccinated) ($n = 3$ in each group), measured at 2 dpv. *Significant differences ($p < 0.05$) with respect to the (iFMDV)a and mock-vaccinated group.

generation of specific IgA antibodies, but the levels of this Ig type rapidly decreased.

3.5. Effect of Cliptox-iFMDV vaccination on dendritic cells and macrophage subsets in the spleen

The early stimulation effects induced by the different formulations were analyzed in mice at 2 dpv. To this end, spleen cells from mock-vaccinated (Cliptox-iFMDV)a and (iFMDV)a, immunized mice were labeled with specific monoclonal antibodies and analyzed by flow cytometry to quantify the percentages of dendritic cells (CD11c, MHC class II positive cells) and macrophages (F4/80, Mac3+cells). Fig. 5 shows that Cliptox-iFMDV immunization yielded a significant ($p < 0.05$) expansion of the dendritic cells and macrophage populations in the spleen when compared to immunization with iFMDV alone.

4. Discussion

In this study we used a mouse model to evaluate the capacity of a new iFMDV vaccine containing a zeolite, Cliptox, as adjuvant, to induce a specific and protective immune response against FMDV.

The experimental murine model used was previously developed in our laboratory and correlates with the humoral immune and protective response against FMDV in cattle [1]. Recently, Juleff et al. [3] demonstrated in bovines the T independence immune response induced by infected virus, the same results were published by Borca et al. [2] using the mouse model.

The Cliptox-iFMDV formulation yielded increased protection against challenge with FMDV in the murine model. The different isotype profiles elicited by inoculation with this vaccine indicate a Th1/Th2 response. The total antibody titers against FMDV were similar in mice groups that received Cliptox-iFMDV or iFMDV alone, but in the first group, IgG2a and IgG2b were significantly elevated. It is likely that these antibody isotypes together with macrophages, are able, through opsonophagocytosis, to more efficiently protect mice from the virus than those elicited by iFMDV alone. The IgG1 levels, on the other hand, did not differ significantly in both groups.

It has been reported that IgG2b, followed by IgG1, are the predominant isotypes among total anti-FMDV IgG antibodies generated in FMDV infected mice between 14 and 60 d.p.i. According to [18,19], both IgG2a and IgG2b are the most effective isotypes in complement activation as well as in antibody-mediated cellular responses. Predominance of the IgG2b isotype in infected animals would agree with the hypothesis of McCullough et al. [20], who proposed that effective protection against FMDV is accomplished through an antibody-enhanced phagocytosis of viral particles by cells of the reticuloendothelial system. Augmented resistance to virus challenge at 210 d.p.i. was observed in animals vaccinated with oil formulations including inactivated virus and Avridine, with regard to those immunized with the conventional emulsion [21]. The same was reported when LPS and the water-soluble fraction of the Mycobacterium wall was included in the aluminum formulation [22]. Values of increased resistance to the viral challenge obtained with the inclusion of immunomodulators for both vehicles, did not show a very clear correlation with Seroneutralizing Index although for all experimental formulations an augmented production of IgG2b was registered. The results showed that the IgG2b isotype was predominant in sera from infected animals and from groups of immunized mice that presented a high percentage of resistance to viral challenge.

In this work, we also studied the generation of specific IgA antibodies in saliva against FMDV induced by vaccination. Since a vast majority of pathogens enter the body through mucosal surfaces, the use of adjuvants that induce mucosal immunity is highly desirable for effective prevention of infection. It is generally considered that an effective mucosal immunity will contribute to protection against infection with FMDV. This assumption underscores the relevance of the observed induction of mucosal immunity (IgA in saliva) by parental immunization to understand the protection observed in susceptible animals. The induction of IgA responses has been correlated with complete protection against challenge in pigs immunized with a highly concentrated inactivated vaccine [8]. Cubillos et al. [9] reported a correlation between solid protection and IgA induction conferred by a peptide vaccine against FMDV.

The observed induction of a specific IgA response after subcutaneous vaccination with Cliptox-iFMDV and its possible role in protection against challenge with FMDV merits further investigation and could be applied in the development of future FMDV vaccines. It is well known that both T-cell activation and release of specific cytokines are involved in the eventual process of B-cell activation, isotype switch, and specific integrin expression on antigen-sensitized B cells. Both Th1 and Th2 cells appear to contribute to the development of specific IgA responses [23]. It appears that the process of isotype switching of B cells to IgA-producing plasma cells begins in the mucosal inductive sites. Such switching requires specific signals by costimulating molecules, including cytokines and T helper cells. Following activation and acquisition of antigen specificity, the IgA-producing cell migrates to the lamina propia of the effector sites in the mucosal tissues, regardless of the site of initial antigen exposure.

In our experiments, we have detected increased levels of IL-2 and IFNγ cytokines in splenocytes from Cliptox-iFMDV-immunized mice that were *in vitro* stimulated with inactivated virus, when compared to the iFMDV group (data non shown). These data, together with the observed isotype profiles seem to indicate that the response to vaccination with Cliptox-iFMDV is mainly of type Th1, with some components of Th2.

The Cliptox-iFMDV formulation induced an increase in dendritic cells and macrophages in the spleen in comparison with the iFMDV

vaccine. Our hypothesis is that vaccine formulations containing the adjuvant could promote the presentation of the virus and in this way enhance the immune response generated and the protection obtained. On the other hand, Fc receptors efficiently promote the uptake of immune-complexed FMDV by macrophages [20,24,25], as well as by monocytes and plasmacytoid dendritic cells (pDC) [26]. Enhanced virus uptake has been observed in cell lines transfected to express Fc receptors [27]. The virus is not only efficiently taken up and internalized, but replication is also initiated, although this remains an abortive process in macrophages and dendritic cells [26].

In conclusion, Cliptox displays an important adjuvant activity for FMDV vaccines, increasing and modulating the humoral and cellular responses in vaccinated mice and yielding enhanced protection against challenge. Furthermore it is not toxic, making it an interesting adjuvant candidate for inclusion in commercial FMDV vaccines.

Acknowledgements

We thank Marta Radolci for technical assistance, N. Fondevila for statistic analysis and O. Zabal for kindly providing cells. This work was supported by SECYT(Argentina)-CITMA (Cuba) (Nro CU-PA03-SVIII/027) and from Project INTA AESA.

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