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Evaluation of Different Adjuvants Formulations for Bluetongue Vaccine

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ABSTRACT

This study investigated the adjuvant potential of W/O/W multiple emulsions and microemulsions, comparing them with traditional aluminum hydroxide and oil-in-water emulsion adjuvants against bluetongue vaccine (BTV). Local inflammatory reactions were assessed in rabbits by measuring the temperature of the animals and the skin thickness at the site of application. Antibodies titers were determined by serum-neutralization test. Histological analyses of lesions at the site of adjuvants application were done. Results showed that multiple emulsion and microemulsion maintained their stability even in the presence of complex components and presented adequate characteristics for subcutaneous administration. They were able to induce immune response against BTV, but it was smaller than the traditional adjuvants. Despite microemulsion adjuvant showed lower antibodies titre, it was easier to prepare more stable at 4°C and it was the only one that did not induce any local reaction.

Key words: Emulsion, microemulsion, vaccine adjuvants, immunology, biocompatibility

INTRODUCTION

Bluetongue is an infectious, noncontagious and hemorrhagic disease caused by an *Orbivirus*, family *Reoviridae*, which is transmitted to ruminants and camelids by *Culicoides* species of biting midges. Accordind to the World Organisation for Animal Health, 24 different Bluetongue virus (BTV) serotypes are spread worldwide, with little crossreactivity and crossprotection. It naturally infects the domestic and wild ruminants, particularly affecting sheep causing severe clinical disease (Maclachlan et al. 2009; Noad and Roy 2009; Bréard et al. 2011; García-Bocanegra et al. 2011). The use of vaccines containing inactivated or attenuated virus, mono or polivalents, has been the most efficient method of controlling the disease in outbreaks and endemic regions (Gorchs and Lager 2001; OIE 2011). Due to several risks associated with the use of live vaccines, including teratogenicity, reversion of virulence, immunosuppression and genetic assortment of gene segments, inactivated vaccines are considered safer and have been used in many European countries to control the outbreaks and to reduce viremia and virus circulation (Umeshappa et al. 2010).

In order to offer more efficient and safe inactivate vaccines against BTV, many studies have been conducted, searching for adjuvants able to improve the humoral and cellular immune response. Aluminum salts, which are colloidal dispersions of

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aluminum hydroxide (AH) or aluminum phosphate, are one of the most frequently used adjuvant in the vaccines due to their safety and low cost (Bowersock and Martin 1999). Aluminun salts have been used in veterinary and human vaccines since 1930's. However, they present, as a main drawback, the incapability to elicit cell mediated immune responses, particularly cytotoxic T cell response (Gupta 1998; Alving 2002).

Water-in-oil-in-water (W/O/W) multiple emulsions (ME) are three-phase systems in which oil droplets containing an internal aqueous phase are dispersed in an external aqueous phase. These emulsions have significant potential in the pharmaceutical field as they provide prolonged release of the encapsulated drugs and they have already been studied as potential candidate adjuvants for vaccines (Silva-Cunha et al. 1998; Bozkir and Hayta 2004; Oliveira et al. 2009; Leclercq et al. 2011). The adjuvancity of multiple emulsions is directly related to their structure. The antigen present in the external aqueous phase is released immediately to the immune system as in aqueous vaccines, while the antigen in the internal aqueous phase is released slowly as in water-in-oil (W/O) emulsions, promoting the increase in short and long time immune response (Aucouturier et al. 2001). For example, multiple emulsions containing rabies virus were evaluated in mice. It was able to induce humoral response and showed good *in vivo* protection (Leclercq et al. 2011).

Microemulsions (MIE) are dispersions of water and oil that require surfactant and cosurfactant agents in order to stabilize the interfacial area. They are thermodynamically stable, transparent, isotropic and low viscosity dispersions that can be sterilized by filtration due to their small droplet size of the dispersed phase (<1.0 μm) (Fialho and Silva-Cunha 2004). These systems, because of their high solubilizing capability and thermodynamic stability, are attractive vehicles for parenteral administration and consequently for adjuvant formulation. Very few studies have been made using microemulsions as adjuvant formulations although their use as drug delivery systems and absorption promoters have been widely studied (Tenjarla 1999). Leclerq et al. (2011) studied microemulsions as adjuvants for rabies virus immunization and the developed system could induce humoral response in mice and presented good protection against the virus. Furthermore this formulation apparently did not cause any local reaction in the mice.

The present study aimed to develop inactivated vaccines against BTV serotype-4, using as adjuvants aluminium salts, water-in-oil-in-water (W/O/W) multiple emulsions and microemulsions. These formulations were inoculated in the rabbits to evaluate the humoral response and local reaction and then they were compared to traditional aluminum hydroxide gel and oil-in-water emulsion adjuvants.

MATERIALS AND METHODS

Preparation of the inactivated bluetongue virus serotype 4

The bluetongue virus serotype 4 (BTV-4) was propagated in VERO cells monolayers (ATCC/CCL-81, USA) in Minimum Essential Media (MEM) supplemented with fetal bovine serum (5%) and treated with Penicillin/Streptomycin (1%) and Anfotericin B (0.5%). When the cells presented 85 to 90% of citopathic effect, they were frozen and thawed for three times and then stored at -80ºC. The cell culture suspension was collected and presented a titer of $10^{5.8}$ $TCID_{50}$/50 μL. Virus was inactivated by adding betapropiolactone (0.2% v/v) for 2 h at 37ºC (Parker et al. 1975). Dialysis was performed using 12000-14000 Da Molecular Weight Cut-off (MWCO) membranes and sterile phosphate buffered saline as dialysis medium at 4ºC for 24h. In order to confirm the inactivation, the final inactivated virus suspension was diluted 1:2 and 1:4 with sterile PBS (pH 7.2) and inoculated on VERO cells monolayers at 37ºC and 5% CO_2 for 96 h after no cytopathic effect was observed.

Preparation of the adjuvants

Multiple emulsion

W/O/W multiple emulsion was prepared by the two-step method (Silva-Cunha et al. 1998). In the first step, a water-in-oil (W/O) single emulsion was formulated using isopropyl myristate (Sigma-Aldrich, Brazil) as oily phase, α-hidroxy-ω-hidroxypropyl-oxyethylen)-poly(oxypropylen)poly(oxyethylen) (Pluronic L121®, BASF Corporation, USA) as surfactant and the inactivated virus suspension as the aqueous phase. The stirring was performed using an Ultraturrax T25 (IKA Labortechnik, Germany) set at the rate of 2000 rpm for 30 min. In the second step, the primary W/O single emulsion previously formed was dispersed in an outer water phase

containing the surfactant (polysorbate 80) under stirring at 400 rpm at room temperature using an Ultraturrax T25 (IKA Labortechnik, Germany) during 20 min. After complete introduction of the primary single emulsion, the stirring was continued for 10 min for W/O/W multiple emulsion formation. The formulation was prepared under aseptic conditions. The physical stability of the formulation was evaluated during 30 days at 4ºC, room temperature (25ºC) and at 40ºC.

The osmolarities of the internal and external aqueous phases were measured using an osmometer (Osmomat 030, Gonotec, Germany) at constant temperature of 25ºC. This analysis was necessary to predict the use of the adequate solution to disperse the formulation in later analysis. After preparation, the W/O/W multiple emulsion globules were visualized using a microscope. One drop of the formulation was placed in an optical microscope (Microscope Leica DM4000B, Germany) set at 1000 x magnification and the images were captured using a Leica digital camera (DFC 280, Leica, Germany).

Microemulsion

The microemulsion was prepared following the method introduced by Hoar and Schulman (1943), called titration with the cosurfactant. First, the inactivated virus suspension was dispersed in the oily phase (isopropyl myristate), containing the surfactant (polysorbate 80) using an Ultraturrax T25 (IKA, Labortechnik, Germany) set at the rate of 8000 rpm for 20 min to form an oil-in-water single emulsion. Next, the cosurfactant (propylene glycol) was added to the single emulsion previously prepared and rotated using the same stirrer set at the rate of 8000 rpm for 20 min, until a transparent system was formed. The formulation was prepared under aseptic conditions. The physical stability of the formulation was evaluated during 8 months at 4ºC, room temperature (25ºC) and at 40ºC.

Aluminum hydroxide

For the formulation of the aluminum hydroxide adjuvant, the concentrated gel (Rehydragel LV®, Reheis Inc., USA) and the inactivated virus suspension were added to a recipient and rotated using an Ultraturrax T25 (IKA, Labortechnik, Germany) set at the rate of 1500 rpm for 20 min. The formulation was prepared under aseptic conditions and stored at 4ºC until use.

Oil-in-water (O/W) emulsion

The O/W emulsion adjuvant was prepared using aluminum hydroxide gel (Rehydragel LV®, Reheis Inc., USA), an emulsion base (Emulsigen®, MVPLabs, USA) and the inactivated virus suspension, according to manufacter instructions. Briefly, the gel and the virus suspension were added to a recipient and rotated using an Ultraturrax T25 (IKA, Labortechnik, Germany) set at the rate of 2000 rpm for 1 h. Then, the obtained suspension was added to the recipient containing the emulsion base and they were rotated using an Ultraturrax T25 (IKA, Labortechnik, Germany) at 2000 rpm for 1 h. The formulation was prepared under aseptic conditions and stored at 4ºC until use.

Characterization of the adjuvants

Determination of pH

The pH values of the prepared formulations were measured using a pH-meter Q-400 MT (Quimis, Brazil) in triplicate at a constant temperature of 25ºC. For microemulsion and W/O/W multiple emulsion analyses, the formulations were previously dispersed in ultrafiltrated water and iso-osmotic solution, respectively, in order to obtain a convenient concentration between 8% and 12%.

Determination of particle size

The average diameter of the droplets in the microemulsion was determined by quasielastic light scattering (QELS) using a nanosizer Coulter N4 (Coulter Electronics, USA) in triplicate at 25ºC. A sufficient amount of the formulation was added to the cuvette and the analysis was performed at an angle of 90 degrees. For the determination of the mean diameter of the W/O/W multiple emulsion globules, five blades containing one drop of the formulation each and one drop of an iso-osmotic solution with the external aqueous phase of the multiple emulsion were placed individually in an optical microscope (Microscope Leica DM4000B, Germany) set at 1000 x magnification. The images were captured using a Leica digital camera (DFC 280, Leica, Germany). Thirty multiple globules for each blade were measured using the software Leica application suite version 3.3.0 (Leica, Germany). The mean diameter of the other adjuvants (O/W emulsion and aluminum hydroxide) was measured using photon correlation spectroscopy (PCS) with a Malvern 4700 photon correlation spectrometer (Malvern Instruments, Malvern, U.K.).

Determination of viscosity
The viscosity of the prepared adjuvants was measured using a rotational viscometer apparatus equipped with a LV-3 spindle model (Brookfield HADV III+, Brookfield Engineering Lab. Inc, USA, Brookfield Engineering Laboratory, USA) in triplicate at 25ºC. Twenty milliliter of each formulation was added to the apparatus set at the rate of 60 rpm.

***In vivo* studies**
Animals
New Zealand male rabbits weighing from 2.0 to 3.0 kg were used. Throughout the studies period, the animals were maintained in the animal facility of the Veterinary School of the Federal University of Minas Gerais, Belo Horizonte, Brazil. They were kept in a quiet and climatically controlled environment with free access to standard mice chow and water. The experiments were carried out in accordance with the guidelines set forth by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (Belo Horizonte, Brazil) and they met the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences. The animals were used in the Vaccination and evaluation of humoral response study and in the Safety evaluation test.

Vaccination and evaluation of humoral response
Animals were divided into six groups of ten animals as follows: 1 (Saline), 2 (inactivated virus suspension), 3 (multiple emulsion), 4 (microemulsion), 5 (aluminum hydroxide) and 6 (O/W emulsion). Rabbits received 2.0 mL of the preparation twice by subcutaneous route in intervals of 21 days. Blood samples were collected from five animals of each group by ear vein section on days 0, 21, 35 and 60 and tested for the presence of neutralizing antibodies.

Determination of antibodies titers by serum-neutralization test
The antibodies titration was performed by serum-neutralization test, according to the method previously described by Lobato (1996). Briefly, rabbit serum samples were inactivated for 30 min at 56ºC and then submitted to serial dilutions (1:2 to 1:256) in MEM. Positive hiperimune anti BTV-4 serum and negative control serum obtained before the adjuvants application were inactivated and diluted in the same way. Viral suspension containing 100 TCID50/ 50μL was then applied to each well containing the samples. The plates were incubated at 37^0C for 1 h and 5% CO_2, 50 μL of VERO cells suspension containing 500,000 cells/mL were applied to each well and the plate was incubated again for 96 h. After that, the plates were evaluated by the observation of cytopathic effect on the cells layer.

Safety evaluation
Skin thickness at the site of vaccine application was measured with a cutimeter before and at 7, 14 and 21 days after vaccination. After having collected the last blood samples, the rabbits were sacrificed and the presence of lesions at the site of injection due to adjuvants reaction was evaluated. All tissue sections collected were fixed in phosphate buffered 10% neutral formalin and processed by the routine technique of paraffin embedding. Histological sections (5 μm) underwent hematoxylin-eosin (HE) staining and were observed using an optical microscope at 25 X or 100 X magnifications. For use as positive control, Freund's adjuvant was applied to other three animals, using the same procedure as described in item 2.4.2.

RESULTS

Preparation and characterization of the adjuvants

Multiple emulsion
The multiple emulsions containing inactivated BTV suspension was prepared by the two-stage emulsification process. They were white in color, macroscopically homogeneous and the microscopic analysis confirmed the formation of the W/O/W multiple emulsion (Fig. 1).
The pH value, average diameter of the globules and viscosity of the developed multiple emulsions are shown in Table 1. The developed multiple emulsions present globule size and viscosity that allowed their utilization in the proposed route of administration.

Microemulsion
A microemulsion formulation containing inactivated BTV suspension was prepared. It was macroscopically homogeneous and transparent. The pH value, average diameter of the globules and viscosity of the developed microemulsion are

presented in Table 1. The characteristics of the developed microemulsion allowed its administration in the proposed route.

Aluminum hydroxide and O/W emulsion

The aluminum hydroxide gel and O/W emulsion adjuvants were easily prepared as indicated by the manufacturer. Aluminum hydroxide adjuvant appeared as a colloidal dispersion and the O/W emulsion was white in color with a homogeneous aspect. The d90 particle size of aluminum hydroxide gel adjuvant and the average diameter of the globules of O/W emulsion adjuvant formulations were evaluated. The pH and viscosity of both formulations were also determined. The results are presented in Table 1.

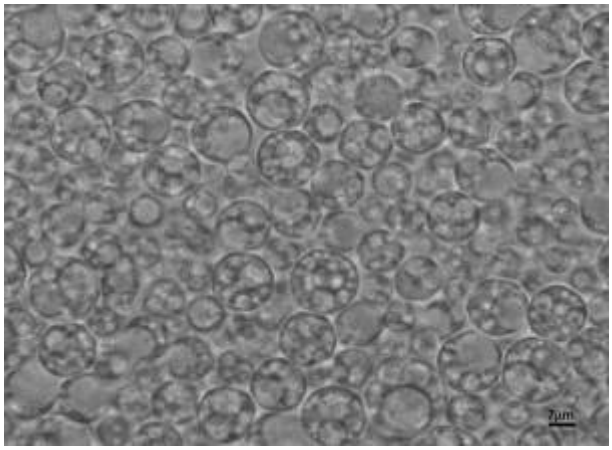

Figure 1 - Photography showing the multiple globules of the W/O/W multiple emulsion developed visualized under optical microscope at 1000x magnification.

Table 1 - Parameters determined of the prepared adjuvants.

Parameters	Adjuvant formulation			
	Microemulsion	**Multiple emulsion**	**O/W emulsion**	**Aluminum hydroxide**
pH	7.72 ± 0.21	6.27 ± 0.30	7.21± 0.27	6.59 ± 0.24
Particle size (nm)[a]	51.60 ± 3.91	12 300 ± 1 100	987 ± 140	34 290 ± 4 821
Viscosity (Pa.s)	0.24 ± 0.10	4.09 ± 0.99	2.04 ± 1.03	$3.71x10^{-3} \pm 0.85x10^{-3}$

The values are shown as mean ± standard deviation (n = 3)

[a] For microemulsion, multiple emulsion and O/W emulsion, the particle size is related to the average droplet diameter; for aluminum hydroxide, the particle size is related to the suspended particles in the gel.

In vivo studies

Vaccination and evaluation of humoral response

Sera collected from the experimental rabbits were evaluated by serum-neutralization. The results, analysed statistically by the unpaired t-test, are presented in Figure 2.

After 21 days of vaccine administration, all the formulations induced the production of antibodies against BTV. However, there was no difference in the mean antibody titers when compared the adjuvants formulations and virus alone. On day 35, rabbits injected with O/W emulsion showed higher anti-BTV antibody titers when compared to the other formulations tested. However, no statistical difference was observed between the O/W emulsion group and the control group (virus alone). At this time, all the adjuvants formulations showed higher antibody titers than on day 21. After 60 days, O/W emulsion still demonstrated better response to immunization. There was no statistical difference when comparing antibody titers of multiple emulsion, microemulsion, aluminum hydroxide groups and the control group (virus alone).

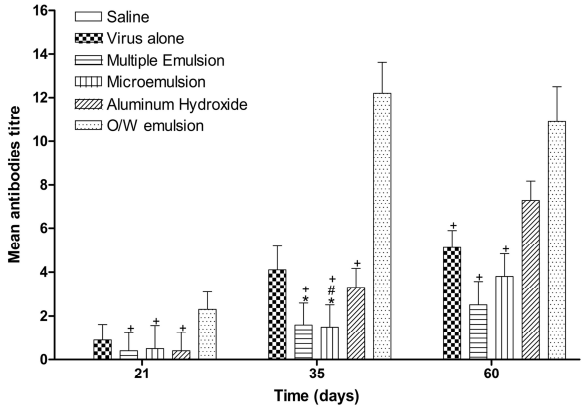

Figure 2 - Skin thickness of rabbits after subcutaneous administration of the evaluated vaccines: saline, virus alone, multiple emulsion (ME), microemulsion (MIE), aluminum hydroxide (AH), Oil-in-water (O/W) emulsion. The values are shown as media ± standard deviation.

Safety evaluation

Comparison of local injuries among the different adjuvants used was performed. The results are presented in Figure 3.

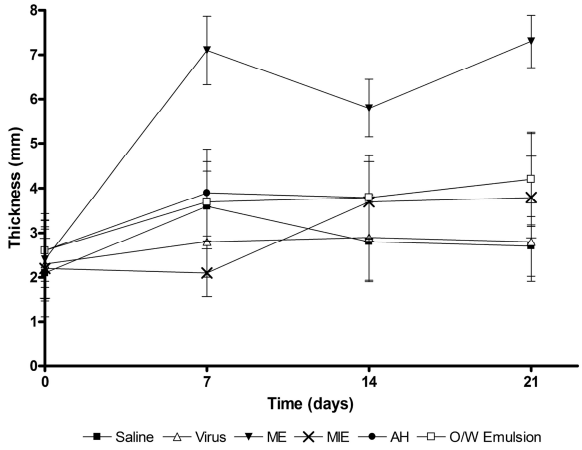

Figure 3 – Mean antibodies titers in rabbits immunized with adjuvant formulations containing BTV virus. The values are shown as mean of antibodies titer ± SD. Statistical difference was determined by the unpaired *t*-test. * $p < 0.05$ compared to virus group; # $p < 0.05$ compared to aluminum hydroxide group and + $p < 0.05$ compared to O/W emulsion group. The comparison was made at each time point separately.

A significant difference in local inflammatory response induced by the multiple emulsions was observed in comparison to the other adjuvants containing oily phase (microemulsion and O/W emulsion). After euthanasia, gross lesions at the site of inoculation were evaluated in each rabbit. No macroscopic lesions were detected in rabbits from saline, the inactivated viral suspension alone and microemulsion groups.

However, two rabbits from aluminum hydroxide, five of multiple emulsion and two from O/W emulsion group showed nodules under the skin that were collected and evaluated upon microscopy. For comparison, the lesions caused by the application of Freund's adjuvant, used as positive control, were also evaluated. Reaction caused by Freund's adjuvant formed encapsulated nodules with a significant infiltration of macrophages around haloes of negative images associated to lipids, disperse vacuolation separated by collagen, and a few polymorphonuclear cells without any area of necrosis (Figs. 4A and 4B).

Nodules formed by the administration of the O/W emulsion adjuvant had necrosis with multifocal areas of mineralization, moderate lymphocytic and histiocytic infiltration and rare eosinophils surrounded by a well organized capsule (Fig. 4C and 4D). The application of aluminum hydroxide adjuvant induced the formation of coalescent small nodules that contained central eosinophilic and macrophagic infiltrate (Figs. 4E and 4F). The reaction caused by the multiple emulsions formulation was characterized by the central collagen necrosis, intense eosinophils and lymphocytes infiltrate and, mild macrophages and neutrophils infiltration (Figs. 4G and 4H).

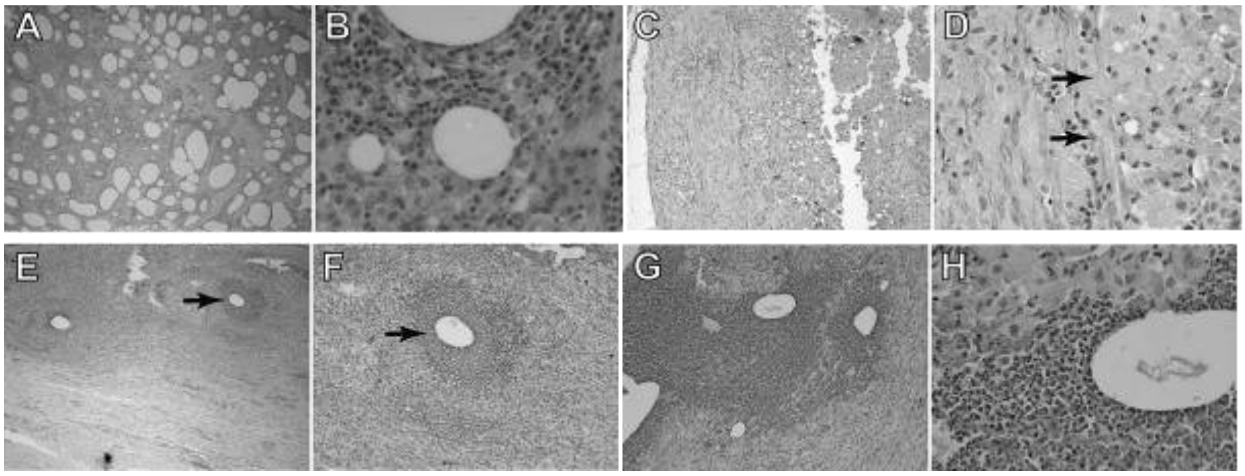

Figure 4 - Photomicrographs of the rabbits tissue reactions induced by the injection of different adjuvants: A and B – Freund's adjuvant showing disperse vacuolation separated by collagen with significant infiltration of mononuclear and a few polymorphonuclear cells, without any necrotic area; C and D - O/W emulsion showing fibrotic capsule with macrophages, and a few polymorphonuclear cells surrounding the necrosis area and foamy macrophage adjacent to the capsule (arrow) (D); E and F – AH showing halo (arrow) surrounded by polymorphonuclear cells, mainly eosinophils, and macrophages, without any necrostic area; G and H – W/O/W multiple emulsion, with collagen degeneration and halo surrounded by polymorphonuclear cells, mainly eosinophils, and macrophages. Hematoxilin-eosin A, C, E and G 100x and B, D, F and H 400x.

Necrotic areas were encapsulated and frequently interspersed by some areas containing bulky cells similar to aggregated macrophages with evident cytoplasm. In some fibrotic areas, near to the necrosis, there were areas of calcification. Eosinophilic infiltration was observed in the conjunctive tissue adjacent to the nodule, mainly around the haloes of negative images associated to lipids.

DISCUSSION

The process of vaccination, to be effective, has to stimulate the immune response against antigen and protect the organism against subsequent contacts. This goal can be reached by the use of adjuvants that are able to improve the immunogenicity. Adjuvants have been used for about 70 years even before the mechanism of their action had been understood (Choi et al. 2006). Nowadays, according to several studies, adjuvants can be classified by their mode of action that includes immunomodulation, presentation, induction of cytotoxic T lymphocytes, targeting and depot formation (Cox and Coulter 1997; Horzinek et al. 1997; Bowersock and Martin 1999; Khan et al. 2007; Gregorio et al. 2009).

The use of micro and multiple emulsions as drug delivery systems has been widely described in the literature. The difference from the present study was that these systems generally contained purified drugs and proteins (Hasse and Keipert 1997; Peltota et al. 2003). The possibility that complex aqueous phases (such as cells and virus suspensions) could be incorporated into these systems, without causing alterations in its characteristics, has still not been well evaluated. In the present study, the aqueous phase was composed of virus and residual proteins and lipids from cell and medium growth and the results of the preparation of the adjuvants showed that this complex aqueous phase could be well incorporated into W/O/W multiple emulsion and microemulsion systems without changing their characteristics and stability. When the stability of the developed adjuvants was evaluated, it was observed that the microemulsion did not present any physical or chemical alteration during a period of at least eight months, when it was stored at 4ºC, room temperature and 40ºC. The W/O/W multiple emulsion was not stable at 4ºC, showing immediate phase separation when stored at this temperature, probably due to changes on the properties of the emulsifier Pluronic L121®. At 40ºC, the multiple emulsions started to present instability after 15 days and at room temperature, it was stable for 30 days.

The pH of all adjuvants formulations evaluated was around neutral, which should not cause potential irritation reactions at the site of application. The small droplets obtained for microemulsion formulation was already expected due to cosurfactant molecules penetration into the surfactant film, lowering the fluidity and surface viscosity of the interfacial film, decreasing the radius of curvature of the microdroplets and forming transparent systems (Fialho and Silva-Cunha 2004). The multiple globules size obtained for multiple emulsion were similar to those described by Silva-Cunha et al. (1997) that evaluated a W/O/W emulsion containing insulin in the internal aqueous phase for parenteral administration. So, both formulations developed in this study could be administered via the subcutaneous route.

As the adjuvant has to be administered through syringes, the viscosity is of critical importance. According to Jain et al. (2010), it is well known that the viscosity of parenteral formulations may affect their syringeability. The low viscosity observed for microemulsion adjuvant, due to its small droplets, ensures ease syringeability. Although the viscosity of multiple emulsions prepared was higher, it was not difficult to administer this formulation through the syringes.

The presence of local reaction due to adjuvants administration is very common but the limitation for their use is dependent on the intensity of the reaction. The safety evaluation test performed showed that multiple emulsions and microemulsion adjuvants did not cause harmful reactions (data not shown), which led to conclude that, after 40 days of injection, the lesions were resolved. However, after vaccination no reduction of local inflammatory reaction due to multiple emulsion administration was observed. This could be explained by the presence of viral proteins that were absent in the safety evaluation test and that might be able to induce the immunological response.

In a previous work conducted by Toledo et al. (2001) in humans, the administration of the adjuvant alone did not induce important tissue reactions on the site of injection but the

application of the adjuvant with antigen led to the formation of granulomas and sterile abscess. This effect indicated that the activation of the specific components of the immune system was essential to trigger severe adverse reactions.

The microscopic evaluation of the nodules developed on the rabbits after adjuvants administration revealed differences among the lesions, which was dependent on the type of the adjuvant used. Comparing multiple emulsion and microemulsion formulations, it is noted that they differ mainly by an emulsifier (Pluronic L121®) that is present in the first and not in the second. The large incidence of granuloma and the formation of necrosis after administration of multiple emulsions could be related to the use of the emulsifier. In a previous study (Hunter et al. 1981), formulation with Pluronic L121®, containing mineral oil, induced high antibody titres but induced an edema at the site of injection that remained for weeks. Pluronic L121® is a POE/POP copolymer that after autoclavation can release the residues of ethylene oxide or propylene oxide that may modify its structure without changing the capacity to stabilize the emulsion. These modifications could be associated to the reactions observed in the animals that received multiple emulsions. Further studies should be conducted in order to evaluate the presence of free ethylene and propylene oxides on the formulation.

The results of mean antibody titers obtained in the present study, concerning to the production of neutralizing antibodies (NAb), did not differ from what had been published, where low titres of NAb were produced after the administration of inactivated virus (Barber and Campbell 1984). Humoral immune response against BTV, represented by NAb is detected between 7 and 14 days after infection with the live virus but the administration of the inactivated virus does not always lead to the production of this type of antibodies (Foster et al. 1991). This result correlated with some reports that showed that serum antibody titers were not consistent indicators of protection from virus infection (Luedke and Jochim 1968; Stott et al. 1979; Jeggo et al. 1984; Stott et al. 1985; Schijns 2000; Niederhäuser et al. 2008).

O/W emulsion and aluminum hydroxide were commercial adjuvants that presented a known profile of stimulation of antibodies response and, in this study, showed the highest mean antibodies titres. Nevertheless, these results were not statistically different from those obtained after administration of the virus alone, which could indicate that the induction of NAb by using inactivated virus was not efficient. Evaluating the low response observed after the administration of multiple emulsions, it could be hypothesized that the intense inflammatory reaction, with the presence of necrosis inside the nodule, could hide the presentation of the antigen, consequently reducing the humoral response. The lesion obtained after the injection of this adjuvant might have attracted phagocytic cells that promoted the reabsorption and reparation of the injured tissue so that they could not respond to BTV.

Microemulsion also did not show satisfactory antibodies titers but it might have stimulated cellular immune response. The high amount of emulsifiers in this formulation can present antigens to the cells by their adsorption in the surface and also by induction of apoptosis. This could change the presentation of VLA in the microemulsion adjuvant to class I CPH, inducing cellular response and inhibiting the humoral response. In a study by Yang et al. (2004), the cellular death by apoptosis induced by the presence of polisorbate 80 and Pluronic L121®, was evaluated, isolated or not, in cell culture. The results showed that both the emulsifiers were able to induce apoptosis and necrosis. Although the mean antibody titer of the developed adjuvants was not high, the present study reinforced the growing interest in emulsion systems, due to its depot formation that could increase the humoral response along the time. Microemulsion and multiple emulsions were able to induce the production of antibodies against BTV and could be able to induce a cellular immune response.

In addition to the strong immune response induced, one criterion in choosing an adjuvant is the lack of pathological effect. Analyzing the incidence of local reactions among the animals that received the adjuvants, it was interesting to see that microemulsion was the only formulation that did not exhibit any sign of local reaction (Fig. 4). The absence of toxicity could be related to its composition, in which the emulsifier used for the development of the formulations was nonionic. In this regard, nonionic surfactants have been found to be favorable for pharmaceutical applications since they are less toxic and less affected by changes in pH and ionic strength (Jain et al. 2010).

In this study, the rabbits were chosen because the main objective of the study was to evaluate the

potential of the developed adjuvants in the induction of immune response. After selecting the best adjuvant to bluetongue virus, further studies should be done targeting the species for this virus in order to evaluate the specific action of this virus incorporated in the developed adjuvant in the production of antibodies and also to evaluate the effects in ruminants.

CONCLUSION

The formulations developed in this work – multiple emulsions and microemulsion – were able to maintain their stability even in the presence of complex components. They were able to induce an immune response against BTV, despite of being smaller than the traditional adjuvants. Microemulsion was easier to prepare, more stable and did not induce any local reaction (formation of granuloma), which showed that it could be safer than the other adjuvants evaluated.

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