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Comparison of Montanide™ GEL 01 and oily MontanideTM ISA 50 in presenting a peptide to the immune system of dogs

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Traditional vaccines based on killed or attenuated microorganisms or inactivated toxins have disadvantages associated with the risks of pathogenicity reversion, contamination with infectious material, variations between vaccine batches, storage problems, among others. Peptide identification containing important epitopes within antigens could be an attractive possibility to avoid those risks. However, in general, peptides are poorly immunogenic. Adjuvants are a part of the solution to improve the immunogenicity of these peptide based vaccines. Recently, a peptide from the tick P0 acidic ribosomal protein chemically conjugated to the Bm86 protein has been assayed as vaccine candidate against ticks. This antigen formulated in oily preparations containing Montanide™ ISA50 (SEPPIC, France) generated a specific antibody response against the P0 peptide which showed an efficacy around 85% against *R. sanguineus* ticks when used for dog immunization. This efficacy was positively correlated with the anti-P0 antibody titers. In this paper, the immunogenicity of this chemical conjugate was assayed in dogs when Montanide™ GEL 01 (SEPPIC, France) was used as adjuvant instead of oily Montanide™ ISA 50. The antibody titers against the P0 peptide did not show statistically significant differences between the experimental groups and the evaluation of changes in the inoculation site showed significantly lesser adverse side effects in the group immunized with the antigen in Montanide™ GEL. These results validate the use of this gel as a safer adjuvant for dogs than oily Montanide, encouraging the use of this adjuvant for the technological development of the pP0-Bm86 conjugate as an anti-tick vaccine for dogs.

Key words: Peptide vaccine, ticks, adjuvant, Montanide, dogs, P0 peptide.

INTRODUCTION

Vaccines are considered as one of the most successful medical inventions (Hilleman, 2000). The possibility of using antigens defined either by the recombinant DNA technology or by the use of peptide chemistry has

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encouraged many groups around the world to identify important epitopes within their antigens of interest and find ways to incorporate them into an immunogenic structure that could be used for vaccination purposes. Despite a very strong movement in this direction and demand for new and better vaccines, there is little availability in the market of synthetic peptide based vaccines. This is because peptides are bad immunogens and behave like haptens (Jackson et al., 2000). To overcome this drawback, peptides are covalently coupled to a highly immunogenic carrier protein. Among the most widely used proteins as carriers are bovine serum albumin (BSA) (Jahouh et al., 2012), ovalbumin (Simerska et al., 2013) and hemocyanin from *Megathura crenulata* (KLH, Keyhole Limpet Hemocyanin) due to their numerous epitopes, their high molecular masses and their proven efficacy in different vaccine preparations (Gil et al., 2001; Langeveld et al., 1994a, b). Delivery system to achieve ideal bio-distributions and kinetics of the antigen is also an important issue to consider in the vaccine designs and especially in peptide-based vaccines (Kurella et al., 2000). The main role associated with the adjuvant is to improve the antigen presentation to the immune system, to preserve its conformational integrity and to prolong its useful life. The inclusion of mineral oil in the vaccine preparation is a way to achieve a slow antigen release increasing the exposure time of the antigen to the immune system (Leroux-Roels, 2010). The most common side effects for this kind of vaccine formulations are local tissue reactions at the injection site such as induration, swelling and in the most critical cases, access development. Consequently, the benefits of oil adjuvant incorporation into a vaccine need to be balanced against the risk of adverse side effects taking into consideration target species (Heegaard et al., 2011).

Over the last 25 years, numerous studies have been conducted in an attempt to develop effective vaccines against ticks (Guerrero et al., 2012; Willadsen, 2008). Currently, there is no vaccine with protective antigens against infestations of *Rhipicephalus sanguineus* (Latreille, 1806) in dogs. It has been previously shown that immunization using a 20 amino acid synthetic peptide from the tick ribosomal protein P0 (pP0) chemically conjugated to Keyhole Limpet Hemocyanin of *Megathura crenulata* (KLH) or Bm86 as carrier protein in an oily formulation containing Montanide™ ISA 50 (SEPPIC, France) was effective to generate specific antibody titers against the peptide which produced a remarkable diminution in the viability and fertility of *R. sanguineus* ticks fed on vaccinated animals (Rodríguez-Mallon et al., 2012; Rodríguez-Mallon et al., 2020). These anti-tick effects were demonstrated to be positively correlated with the specific immune response against the pP0. However, the oily formulation of this effective antigen produced some side undesired effects on the inoculation site of immunized dogs (Rodríguez-Mallon et al., 2013, 2020). The objective of this study was to assess the immunogenicity of the pP0-Bm86 chemical conjugate formulated with the polymeric adjuvant Montanide™ GEL 01 (SEPPIC, France) in dogs and compare the specific antibody response obtained with that of the same antigen in oily formulation using Montanide™ ISA 50 (SEPPIC, France). General health parameters and *in situ* changes at the injection site were also evaluated in vaccinated dogs using quantitative and qualitative data.

MATERIALS AND METHODS

Immunization schedule

Twenty-two dogs of different breeds and sex ranging between 1 to 5 years old from the Cuban police service were randomly assigned to five experimental groups as shown in Table 1. Dogs were fed with a commercial pellet diet (produced by ALICAN S.A, Argentina) and water *ad libitum*. The trial was conducted in the Altahabana Veterinary Clinic of the Canine Unit in the Ministry of Interior (MININT, Cuba). Dogs were maintained in separated boxes until the end of the experiment. The sampling exercise and all procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (2011) and were approved by the Ethics Committee at the CIGB.

The P0 synthetic peptide (NH2-AAGGGAAAAKPEESKKEEAK-CONH2) (pP0) was chemically conjugated to the Bm86 protein produced by the fermentation of a recombinant *Pichia pastoris* yeast clone (Canales et al., 1997) at the CIGB of Camagüey (Gavac™ active ingredient, Lot: 14.1302-4) (pP0-Bm86). Considering the adjuvant properties described for the Bm86 nanoparticles produced by this yeast (Garcia-Garcia et al., 1998), this protein could be the most suitable and feasible candidate of carrier for the efficient presentation of pP0 to the host immune system, because this protein is itself an immunogen against ticks. The pP0-Bm86 conjugate was obtained by using the maleimide method (Carter 1994). Briefly, the carrier protein was activated using 3-maleimidopropionic acid N-hydroxysuccinimide ester (MPS) as the crosslinking reagent and one Cysteine residue was added to N-terminal end of the P0 peptide in order to be linked to the carrier protein. The immunogen diluted in PBS (NaCl 8 g/L, Na_2HPO_4 1.15 g/L, KCl 0.2 g/L, KH_2PO_4 0.2 g/L, pH 7-7.2) was formulated with two different adjuvants: Water-in-Oil (W/O) emulsion (Montanide™ ISA 50; SEPPIC, France) in a 60/40 proportion of immunogen/adjuvant and a new adjuvant based on the dispersion of a high molecular weight polyacrylic polymer in water (Montanide™ GEL 01) kindly provided by SEPPIC (France) using 3 and 8% of gel in the final preparation. Dogs were subcutaneously immunized with 1mL of pP0-Bm86 in doses of 500 μg/ animal on days 0, 21 and 36. Dogs in negative control groups were injected with PBS prepared in Montanide™ ISA50 or Montanide™ GEL 01. The general behavior of the animals was observed daily and body temperature was measured each two days throughout the test. Body weights were recorded and hematological tests were performed the same days in which dogs were immunized and fifteen days after the last immunization. Inoculation sites were evaluated during 1 week after each immunization and a score was assigned to each dog according to the observed local injuries. Body weights were compared using repeated measures such as one way ANOVA followed by Bonferroni multiple comparisons test; scores of local injuries were compared by ANOVA and Bonferroni multiple comparisons test on the statistical program Prism (version 6.0 for Windows; GraphPad Software, USA).

Characterization of the immune response by indirect ELISA

Dog serum samples were taken on days 0, 21, 36 and 51 in order

Table 1. Dogs on the experimental groups.

G1: PBS-Montanide™ ISA 50		G2: PBS-Montanide™ Gel 01 8%		G3:pP0-Bm86 Montanide™ ISA 50		G4: pP0-Bm86 Montanide™ Gel 01 3%		G5:pP0-Bm86 Montanide™ Gel 01 8%	
Breed	**Age / Sex**	**Breed**	**Age / Sex**	**Breed**	**Age / Sex**	**Breed**	**Age / Sex**	**Breed**	**Age / Sex**
CS	1.5 / F	CS	2 / F	CS	2 / F	CS	2 / F	CS	1 / F
GS	4.5 / M	GS	3 / M	GS	1.5 / F	GS	3 / F	GS	5 / M
SS	2 / M	SS	2 / F	SS	3 / M	SS	1 / M	SS	3 / M
		BM	2 / M	BM	2.5 / F	BM	3 / F	BM	5 / F
				LR	4 / M	SS	1.2 / M	SS	1 / M

Breeds: CS: Cocker Spaniel; SS: Springer Spaniel; GS: German Shepherd; BM: Belgian Malinois; LR: Labrador Retriever; Age is expressed in years. F. Female; M: male.

to evaluate the specific antibody response against pP0 by an indirect ELISA as previously described (Rodríguez-Mallon et al., 2012). Briefly, 100 ng per well of pP0 chemically conjugated to Keyhole Limpet Hemocyanin of *Megathura crenulata* (KLH) was used to coat ELISA plates overnight at 4°C because the plate binding of the peptide alone is not efficient and the use of a different chemical conjugate of this peptide will guarantee that only the immune response against pP0 is measured. Sera were serially diluted in base 1:2 in PBS 1X. The plates were incubated with the diluted sera for 1h at 37°C and then incubated with 1:10,000 anti-dog IgG–HRP conjugate (Sigma) for 1h at 37°C. The color reaction was developed with a substrate solution containing o-phenylenediamine, 0.4 mg/ml in 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 5.0 and 0.015% of hydrogen peroxide. The reaction was stopped with 2.5 M H_2SO_4 and the OD490 nm was determined. The antibody titer was established as the reciprocal of the highest dilution, at which the mean OD of the serum in question was three times the mean OD of the negative control serum. The geometric mean of antibody titers in each group was determined from individual values and the data were log base 10 transformed and compared by using ANOVA and Bonferroni multiple comparisons test on the statistical program Prism (version 6.0 for Windows; GraphPad Software, USA).

RESULTS

Specific antibody responses against pP0 were obtained in all animals immunized with the pP0-Bm86 chemical conjugate (Figure 1A). Fifteen days after booster on experimental day 36, the antibody titers against pP0 in vaccinated groups become statistically significant ($P<0.01$) compared to negative control groups (G1 and G2) reaching an average value above 1000 for all groups vaccinated with the conjugate. There were no statistically significant differences between anti-pP0 antibody titers among groups 3, 4 and 5 vaccinated with the pP0-Bm86 conjugate with different adjuvants. According to previous experiments, this specific antibody response against pP0 will produce an important reduction in the tick reproductive potential with a calculated efficacy around 85% (Rodriguez Mallon et al., 2020).

There were no statistical significant differences among the body weight averages at different times during the experiment in each group (Figure 1B). There was no change in normal behavior, or any fever in any of the animals during immunization experiment days according to the body temperature reference values reported for dogs (Kahn et al., 2010) (Figure 1C). The averages of all hematological parameters remained within the normal ranges reported for the species during the experiment (Kahn et al. 2010) as shown in Table 2. The assigned scores to local injuries at the inoculation sites showed statistical significant differences ($P<0.001$) between the groups that received Montanide™ ISA50 and those that were injected with Montanide™ GEL 01 as adjuvant (Figure 2A). Some dogs in groups 1 and 3 presented large and hard lesions with an evident volume increase. One of them in group 3 developed an access as shown in Figure 2B. This access disappeared after three days of local treatment.

DISCUSSION

These results demonstrated that the pP0-Bm86 chemical conjugate formulated with both adjuvants, Montanide™ ISA50 and Montanide™ GEL 01, was able to generate a similar specific immune response against the pP0 in dogs which is effective against ticks as previously demonstrated (Rodríguez-Mallon et al., 2020). However, the evaluation of the local reactions after subcutaneous administration of the vaccine formulations showed significant diminutions in adverse effects when Montanide™ GEL 01 is employed which could suggest the convenience to use this adjuvant when sensitive animals as pets are vaccinated.

As it is very well known, the adjuvant selection during the vaccine design is crucial for the vaccine properties. There is no universal adjuvant, and there is always a tradeoff between safety and efficacy. Therefore, the selected adjuvant will depend on the target species, the antigen, the inoculation route and the desired immune response and its duration. Alum (aluminum salts) is still the most frequently used adjuvant for human vaccines and is also used in many veterinary vaccines, especially for pets. Nevertheless, histological evidence of adverse

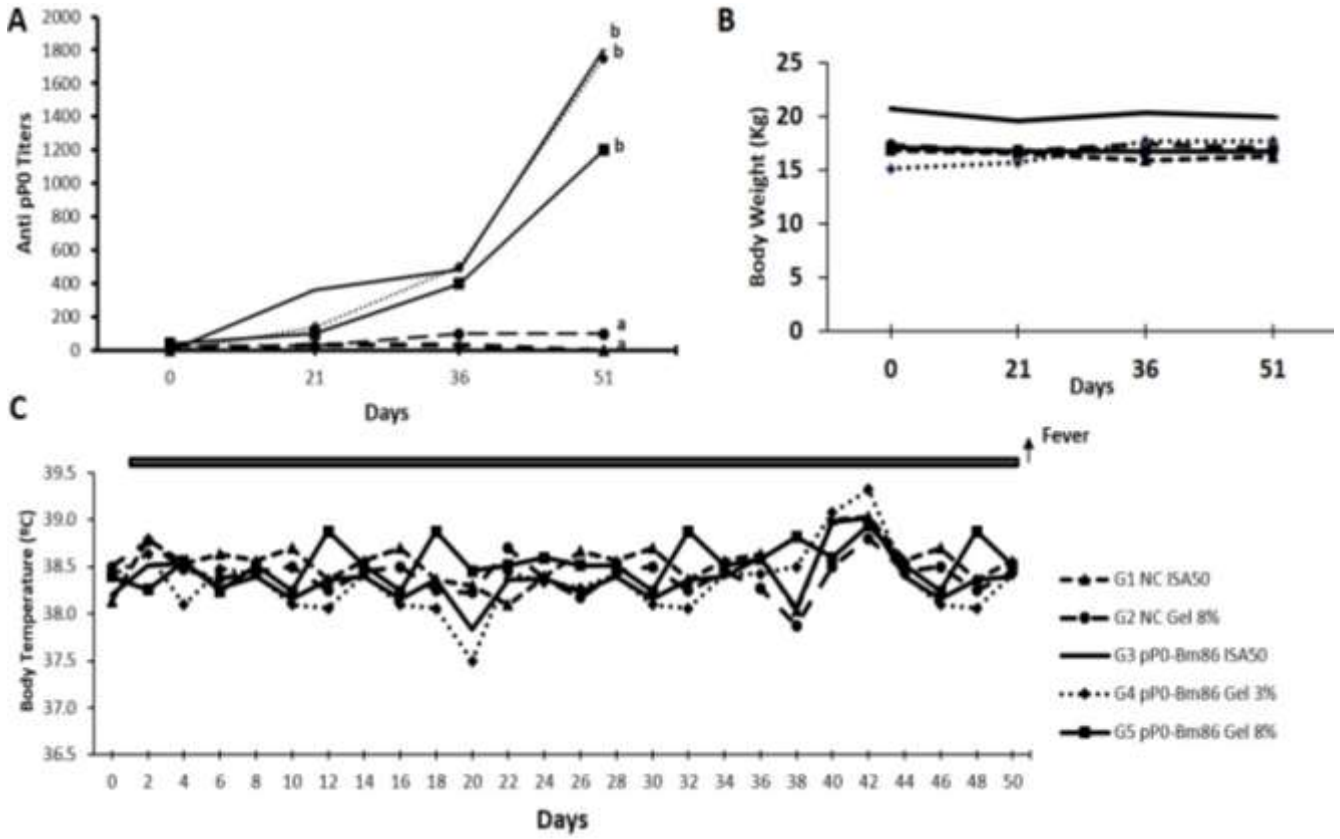

Figure 1. A-Specific antibody response against pP0 from immunized dogs. Antibody titers were determined as the reciprocal of the last serum dilution with an average OD greater than three times the average OD of the negative control groups. Each point represents the geometric mean of antibody titers in the group. Different letters mean statistically different groups (ANOVA followed by Bonferroni multiple comparison test, P<0.01). B- Body weight averages in each group at different times during the experiment. There were no statistical significant differences among this parameter at different times in each group (Repeated measures one way ANOVA followed by Bonferroni multiple comparison test, P>0.05). C- Averages of dog body temperature in the experimental groups measured each two days throughout the test. Line at 39.5ºC represents the reference value above which fever is considered in dogs (Kahn et al. 2010).

effect related to alum in pets has been described (Hendrick et al., 1992; Vascellari et al., 2003; Verdier et al., 2005). On the other hand, oil-based adjuvants as emulsions of water-in-oil, or vice versa are among the most efficient adjuvants known and they are in wide use for veterinary vaccines (Aucouturier et al., 2001). In our experience, water-in-oil (W/O) emulsions induce strong, long-term immunity but can sometimes induce local reactions which could be severe in some cases (Rodríguez-Mallon et al., 2013, 2012, 2020). Generally, water-in-oil emulsions (W/O) are recommended for bovines, small ruminants, poultry and fish when long-term immunity is required. Conversely, vaccines for pets and horses must not induce any local reactions at the injection site.

This study provides direct evidence that Montanide™ GEL 01 can act as a powerful adjuvant in a synthetic peptide based vaccine and constitute an easy-to-use and low-cost veterinary vaccine with a more safety profile in pets than oily Montanide. In summary, the results obtained in this work show the convenience of using Montanide™ GEL 01 for dogs instead of Montanide™ ISA50 because the specific antibody titers against the P0 peptide were similar with both adjuvants but the adverse events were significantly lower.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 2. Results of hematological tests performed on dogs during the immunization experiment.

	Day	HGB g/dL (12.0-18.0)	HCT % (35-55)	PLT 10^9/L (120-500)	WBC 10^9/L (6-17)	SEGS % (60-77)	LYM % (20-35)	EOS % (1-8)
G 1	0	15.2±2	44±7	210±110	10.3±2.1	71±6	26±2	4±4
	21	12±0.6	35±3	240±16	17±5	60±3	22±5	3±1
	36	14±0.8	42±3	203±90	10±5	60±14	35±11	5±3
	51	13.3±2.9	38±8	152±51	7±1	71±9	25±11	3±2
G 2	0	13.3±1.5	38±5	178±53	9.2±1	60±1	30±13	6±5
	21	13.5±2	40±6	247±22	16±1	71±7	27±6	3±1
	36	13.5±3	40±8	235±82	13±2	73±1	24±10	3±2
	51	14.6±0.6	43±2	165±26	9±2	70±7	27±8	2±2
G 3	0	12.5±1	36±5	155±50	10±1	66.4±12	31±12	8±1.2
	21	14.3±2	42±5	213±42	11±2	64±6	33±4	3±3
	36	14.2±1.8	42±6	176±88	12±3	65±12	32±14	4±2
	51	15.3±2.5	44±7	198±107	10±2	68±7	25±6	4±2
G 4	0	12±1.6	35±5	206±35	14±5	66±12	30±12	4±4
	21	12.9±2.4	38±8	287±94	9±2	67±7	35±5	3±2
	36	13.9±2	41±6	300±137	10±3	62±10	36±8	6±2
	51	13.9±1.4	40±5	275±124	13±1	73±8	24±7	8±6
G 5	0	12±2	35±13	165±65	11±4	60.6±2	26±18	3.2±1
	21	12.9±2	38±6	252±87	13±6	67±7	30±8	2±1
	36	14±2.8	41±8	280±130	12±4	65±14	30±13	3±2
	51	14.2±2	42±6	259±167	12±4	69±10	29±10	2±1

HGB – Hemoglobin; HCT – Hematocrit; PLT - Platelet count; WBC - white blood cell; SEGS - segmented neutrophils; LYM - Lymphocytes; EOS – Eosinophils, Normal reference values for each parameter appear in parenthesis (Kahn, Line, Merck, & Co, 2010). G1: PBS- Montanide™ ISA 50; G2: PBS-Montanide™ Gel 01 8%; G3: pP0-Bm86 Montanide™ ISA 50; G4: pP0-Bm86 Montanide™ Gel 01 3%; G5: pP0-Bm86 Montanide™ Gel 01 8%.

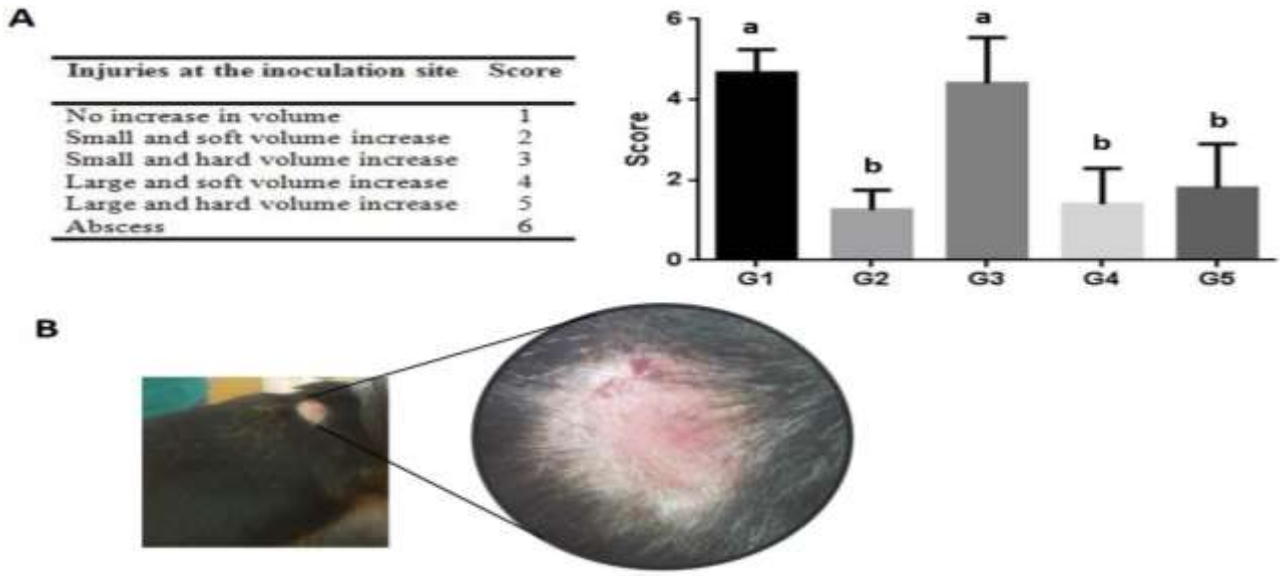

Figure 2. A-Scores to local injuries at the inoculation sites in the experimental groups. Standard deviations are represented by error bars in the positive direction. Different letters mean statistically different groups (ANOVA followed by Bonferroni multiple comparison test, P<0.001). B-Access developed by a dog in the group 3 immunized with pP0-Bm86 in oily formulation with Montanide™ ISA50. G1: PBS- Montanide™ ISA 50; G2: PBS-Montanide™ Gel 01 8%; G3: pP0-Bm86 Montanide™ ISA 50; G4: pP0-Bm86 Montanide™ Gel 01 3%; G5: pP0-Bm86 Montanide™ Gel 01 8%.

immunological control. Permission from SEPPIC has been obtained to use and mention their products in this manuscript.

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