Use of biodegradable PLGA microspheres as a slow release delivery system for the *Boophilus microplus* synthetic vaccine SBm7462

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Abstract

The synthetic anti-*Boophilus microplus* vaccine SBm7462 derived from the tick intestinal protein, Bm86, induced a protective immune response when emulsified in saponin and used in cattle. Using a mice model, and with the objective of improving the vaccine by continual peptide release, it was encapsulated in PLGA 50:50 microspheres and inoculated in BALB/c mice to assess the immunological response by detection of anti-peptide IgGs. Comparative studies were made with the peptide emulsified in saponin and with another synthetic vaccine, and the microsphere/peptide was characterized for efficiency of encapsulation, in vitro release profile, morphology, size, peptide integrity after encapsulation and stability in different pHs. The findings showed that saponin enhances a better immune response from SBm7462 and that the PLGA 50:50 microspheres are suitable for use with this peptide.

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1. Introduction

The tick, *Boophilus microplus*, is one of the most important arthropods in veterinary medicine because of the economic losses caused in cattle raising in Central and South America and Australia. It affects more than 75% of the cattle population worldwide.

The losses caused are classified as direct, such as blood sucking, and indirect, which are related to the transmission of pathogenic agents; the global economic losses caused by *B. microplus* have been estimated at US$ 7 per animal per year (Mc Cosker, 1979). Brazil has the fifth largest cattle herd in the world and economic losses estimated at US$ 2 billion (annually) due to the direct and indirect effects of *B. microplus* infestations (Grissi et al., 2002).
The most common control method is the use of chemical products. However, this form of control presents several inconveniences, and perhaps the principal is the crossed resistance to different chemical bases such as organophosphates (Patarroyo and Costa, 1980) formamidines, cicloamidines, piretroides (Lodos et al., 1999) and ivermectines (Martins and Furlong, 2001), besides contamination of the environment and food product of cattle origin. On the other hand, there is growing evidence that the present strategies based on the use of acaricides are not cost-effective (Pegram et al., 1991).

Currently, one of the alternative ways to control B. microplus that has been gaining importance is the use of vaccines. Protective immunity against this tick was induced after inoculating antigens obtained from the intestine of partially ingurgitated females (Johnston et al., 1986; Opdebeeck et al., 1988). With the objective of isolating and purifying these protective antigens, at the end of the 1980s a glycoprotein was isolated from the parasite intestine called Bm86 (Willadsen et al., 1989). This molecule was cloned and expressed in Escherichia coli for cattle immunization, and conferred 77% protection (Rand et al., 1989). Later, it was expressed in fungi and presented different efficiency levels in Cuba (De La Fuente et al., 1995) and Brazil, where it showed 51% efficiency when tested in cattle under field conditions (Massard et al., 1995). However, the use of chemicals associated to the immunization with rBm86 is essential to promote efficient tick control; therefore it is still necessary to research other protective antigens to control. This fact has triggered research for different immunogens, such as synthetic peptides, whose advantages include not needing fermentation in cultures and non-contamination with biological substances. The synthetic vaccine SBm7462 containing antigenic determinants of the Bm86 protein and added to saponin was tested in cattle of several breeds, and an efficacy of 81.05% was conferred (Patarroyo et al., 2002), when parameters such as reduction in the number and weight of adult females, reduction of mean weight of eggs, and decrease in fertility were assessed, according to previously established formulas (De La Fuente et al., 1995).

An effective form of increasing the efficacy of a vaccine is by the use of adjuvants that are defined as substances that act non-specifically to increase the response of an antigen (Alexander and Brewer, 1995) and the specificity is conferred by the antigen. Tick antigens have shown good results when saponin was used as adjuvant and Australian researchers working with rBm86 emulsified in saponin observed satisfactory protection levels and high antibody titles that recommended saponin as a suitable adjuvant for use of tick antigens (Heller-Haupt et al., 1988; Jackson and Opdebeeck, 1994). A promising alternative to the conventional adjuvants is the microencapsulation in biodegradable polymers that release their content continuously over time (Audran et al., 1998). Among the biodegradable polymers available commercially, the polyesters derived from lactic and glycolic acids (PLGA) present advantages that include enhancement of the immune response, reduction in the total quantity of immunogen necessary to generate a good immune response, protection of the antigen from degradation, release to a specific location and more efficient antigenic dose (Lima and Rodrigues, 1999). Several experiments have shown the efficacy of this release system when used to encapsulate some synthetic peptides (Rosas et al., 2001, 2002; Carcaboso et al., 2003). This is especially applicable to cattle raising, where vaccine administration for a large number of animals is logistically challenging, troublesome, stressing, and therefore counter-productive for the animals. The different studies on the use of slow delivery in veterinary vaccinology applied for control of some infections of ruminants often use mice as experimental model. This was the case of the bovine parainfluenza type 3 (Shephard et al., 2003) and Brucella ovis which was tested as a vaccine against B. ovis and B. abortus infections (Murillo et al., 2002). The exception is the research of Stanley et al. (2004) who used a crude extract of Toxoplasma gondii encapsulated into poly(d,l-lactide-co-glycolide) (PLG) for intranasal immunization.

The present paper describes the induction of the immune response in mice immunized with the SBm7462 oligopeptide encapsulated in PLGA microspheres compared with its emulsification in saponin and with that induced by the synthetic peptide SPf66 encapsulated in PLGA. The influence of the peptides and adjuvants in the hepatic function, the efficiency of the microencapsulation, the release profile and SBm7462 integrity after microencapsulation were also assessed.
2. Material and methods

2.1. Material

The following materials were used: PLGA (poly-D,L-lactide-co-glycolide) Resomer® RG 506 PLGA (Boehringer Ingelheim K.G., Germany) with a 50:50 copolymer ratio (lactic/glycolic acid percentage) and a 0.8 dl/g intrinsic viscosity. Polyvinylalcohol (PVA; average MW 30,000–70,000) and affinity-purified antibody peroxidase-conjugated rabbit anti-mouse IgG were supplied by Sigma Chemical Co. Kits for enzyme dosage were supplied by bioBérieux® (France). The protein assay kit (micro BCA) was purchased from Pierce, Teknovas (Bilbao, Spain). The saponin, Saponine®, derived from Quillaja saponaria was supplied by UCB Leuven, Belgium. All other chemicals were analytical grade.

2.2. SBm7462 peptide

The anti-B. microplus SBm7462 vaccine (Patent pending) consists of 43 amino acid residues, is derived from the Bm86 protein structure and was previously described by (Patarroyo et al., 2002).

2.3. SPf66 peptide

The encapsulated synthetic SPf66 peptide, specific against malaria was used to compare to the tested SBm7462 and was ceded by “Fundación Instituto de Inmunología de Colombia” (FIDIC). Its production and encapsulation in the PLGA 50:50 microspheres followed the methodology used in previous experiment (Rosas et al., 2001).

2.4. Animals

Were used 120 female BALB/c mice between 8 and 10-week-old, weighing around 20 grams, obtained from the animals’ house at the Federal University of Minas Gerais. The mice were randomly assigned into six groups. They received water and normal diet ad libitum throughout the experiment and were kept in an acclimatized environment (20–24 °C, 45–75% humidity) with a daily artificial light period of 12 h. The maintenance and handling of the animals followed the current international regulations.

2.5. Microsphere preparation

The microspheres containing the SBm7462 peptide were prepared using the biodegradable PLGA copolymer 50:50 using the extraction technique by solvents(double emulsion (w/o/w) with PVA as emulsion stabilizing agent. Briefly, 250 mg PLGA 50:50 were dissolved in 5 ml dichloro-methane and emulsified with 250 ml of 10% aqueous solution of the peptide by sonication for 30 s at 50 W (Branson® sonifier 250). The resulting emulsion (w/o) was poured into 25 ml of a 8% PVA solution in deionized water pH 6.8 and emulsified for 5 min at 9500 rpm using a turbine homogeniser (Ultraturrax® T-25) to form a double emulsion (w/o/w). Finally, 50 ml of a 2% isopropanol solution was added and agitated for 1 h. The microspheres were collected by centrifuge at 10,000 × g and resuspended in distilled water and the last procedure was repeated three times. The washed microspheres were freeze-dried.

The mean size and morphology of the microspheres were determined by laser diffractometry using a Coulter® Counter LS130 and scanning electronic microscope, respectively.

2.6. Measurement of the efficacy of SBm7462 and SPf66 microencapsulation

The total of encapsulated peptide was estimated by the bicineconic acid test (BCA) after disrupting the microspheres in a NaOH 0.2 M solution. The efficiency of the encapsulation (%) was calculated by the peptide charge obtained (w/w) and the theoretical loading of peptides (10%) in the microspheres, using the formula quoted by Castellanos et al. (2001).

The peptide associated with the surface was calculated by suspending the microparticles in 20 mM phosphate buffered saline pH 7.4 and incubation at 37 °C under continuous orbital rotation for 30 min. The microspheres were centrifuged and the supernatant analyzed to research SBm7462 and SPf66 by the bicineconic acid test. The peptide associated with the surface represented a percentage of total encapsulated peptide that was present on the microsphere surface and consequently available for initial release.
2.7. In vitro release studies

Twenty milligrams of microspheres were placed in test tubes containing 2 ml 20 mM phosphate buffered saline, pH 7.4 and incubated at 37 °C under continuous orbital rotation. Every 7 days and up to 59 days for SBm7462 and 63 days for SPf66, the samples were centrifuged at 10,000 × g for 10 min. The supernatant was collected and quantified using BCA. The peptide release study was continued after substitution with the same volume of new solution. The peptide release patterns from the microspheres were created in terms of accumulative release versus time.

2.8. Analysis of the SBm7462 peptide integrity and stability

To confirm the integrity after the microencapsulation, the freeze-dried microspheres were submitted to complete digestion with NaOH 0.2N and compared with the native peptide. The analyses were carried out by reverse phase chromatography (HPLC-RP) using a column RP-18 LiCrospher 100LiChrocart 125-4 with a gradient 0–70% B in 30 min with detection at 210 nm, solvent A: H2O:TFA(95:005) B:ACN:TFA (95:005) flow 1 ml/min.

The stability and the structural conformation of the native peptide at different pHs and the same parameters after encapsulation were assessed by circular dichroism using a JASCO J-810 spectropolarimeter. The spectra were measured in cylindrical quartz cuvettes (Hellma) with 0.01 cm light passage; calibrated with d-10-camforsulfonic acid, with correction of the base line and analysis of the results by the Jasco program. In order to analyze the peptide conformation a 2 mM solution was prepared of the peptide in acetonitrile–water (1:1), a solvent in which this peptide is completely soluble. Dilutions were prepared from this solution of the peptide in trifluoroethanol/water (TFE/water at 30%, v/v) and for stability at different pHs (pH 0.5, 12.0 and 7.0) the peptide was dissolved in distilled deionized water adjusting the pH with NaOH and HCl.

2.9. Immunization protocol

The animals of each group were inoculated subcutaneously on the back every 3 weeks, totaling three doses, as described in Table 1. They were bled before the first immunization and at 7-day periods for 21 weeks, by the retro-orbital plexus under anesthesia with ether. The serum obtained was stored at −80 °C until different tests were performed.

2.10. Detection of anti-SBm7462 and SPf66 IgGs

The ELISA plates (Nunc-Immuno™ Plate FP 96 MaxiSorp™ NUNC) were sensitized with 1 μg of each peptide per well in 0.13 M carbonate buffer solution and allowed to adsorb at room temperature for 12 h. The serum samples were diluted 1:100 in incubation buffer containing Tween 20 and tested in triplicate. Anti-peptide antibodies were detected with rabbit IgG anti-mouse IgG (heavy and light chains) conjugated with peroxidase (diluted 1:40,000). A sample was considered positive when the reaction showed an optical density (OD) higher than the mean plus two standard deviations of the OD obtained for negative controls.

2.11. Hepatic function tests

Sera from six animals selected randomly per treatment on day 0 and then at every 3 weeks up to the 15th experimental week were used for dosing the total proteins, albumin and the enzymes AST (aspartate aminotransferase) and ALT (alanin aminotransferase). An enzyme multiparametric autalyzer was used for
biological assays (Alize\textsuperscript{H}, Lisabio, France). The results were expressed from the arithmetic mean of the values found.

2.12. Statistical analysis

The Kruskal–Wallis test was used at 5% probability using the SAEG statistical program 8.X. Version (Federal University of Viçosa) and the data with a \( p < 0.05 \) were considered statistically significant.

3. Results

3.1. Microsphere characterization

In the scanning electron micrography the microspheres, formed by w/o/w, were shown to be smooth and spherical and no pores were observed on their surface (data not shown). The efficacy of encapsulation for SBm7462 was 60% and the mean size of the microspheres was 1.63 \( \mu \text{m} \); for SPf66 the encapsulation efficacy was 85% and the mean size 1.3 \( \mu \text{m} \).

3.2. In vitro release studies

As can be seen in Fig. 1, SBm7462 release showed a triphasic profile with 40.6% initial burst release followed by a second phase of slow release up to the 37th day of 33% more of the peptide; completing a total release of 98% at 51 days. A triphasic release profile was also observed for SPf66, after an initial 25% burst, there was a slow release phase reaching 30% of the total release at 29 days. The third release phase was growing and after 63 days approximately 80% had been released.

3.3. SBm7462 peptide integrity and stability

Fig. 2 shows the chromatograms obtained from different SBm7462 samples: (A) native peptide and (B) peptide extracted from the microspheres. A main signal was observed in both with retention time of approximately 21 min, corresponding to the peptide.

Fig. 3 shows the analyses by dichroism obtained from: (A) native peptide, (B) peptide after desencapsulation and (C) the native peptide at different pHs. The spectrum obtained by these analyses shows that both the native peptide and peptide after extraction presented a helicoid conformation. Equally, the native peptide conserved its helicoid conformation at the different pHs studied.

3.4. In vivo IgGs response to SBm7462 and SPf66 peptides

The immune response was measured over 21 weeks by the presence of antibodies. Fig. 4A shows that starting in the 4th and 5th weeks for the SBm7462-saponine (III) and SBm7462-PLGA 50:50 (V), respectively, there were statistically significant difference relative to the control groups (groups I, II and

![Fig. 1. In vitro cumulative release of peptides SBm7462 and SPf66 loaded PLGA 50:50 microspheres in 20 mM phosphate buffered saline pH 7.4.](image)

![Fig. 2. Peptide SBm7462 analyzed by HPLC-RP. Chromatographic profile prior of microencapsulation (A); chromatographic profile after extraction from PLGA microspheres (B).](image)
IV) the difference persisting until the end of the experiment for group III and until the 13th week for group V. When groups III and V were compared, the humoral response was superior and statistically significant in group III from the 13th week until the end of the experiment. The IgGs peak was earlier in the peptide emulsiﬁed in saponin (in the ninth week) than in the peptide encapsulated in PLGA (10th week). As shown in Fig. 4B, the anti-SPf66 peptide IgGs levels were signiﬁcantly superior to those obtained with the other two groups that used PLGA (IV and V) from the second week until the end of the experiment. The anti-SPf66 IgGs peak occurred in the 10th week and then declined, but a high IgGs proﬁle was observed until the end of the experiment.

No statistically signiﬁcant differences were observed among the controls (I, II and IV) when they were compared.

3.5. Hepatic function study

No statistical difference was observed among the different groups, when different parameters were analyzed (total protein, albumin, AST and ALT) evidencing no hepatotoxic effects (data not shown).

4. Discussion

The synthetic peptide SPf66 was used in this study to demonstrate that the microspheres were efficacious in elevation of IgG antibody when used to encapsulate synthetic peptides. It was chosen because of its similarity with SBm7462 regarding size, synthesis methodology and because it had already shown satisfactory results when encapsulated in PLGA microspheres (Rosas et al., 2001, 2002).
Controlled release systems such as PLGA microspheres permit a continuous or pulsable release of encapsulated antigens, simulating a continuous exposure such as that which occurs naturally in the parasite–host relationship, eliciting a strong cell and/or humoral response depending on the antigen. Based on these facts, this study, using the model described, investigated the possibility that the SBm7462 vaccine capsulated in 50:50 PLGA microspheres promoted an immune response superior to that promoted by this peptide emulsified in saponin. The findings showed that, although viable after encapsulation, the level of antibodies elicited by the encapsulated immunogen was significantly lower than that detected with the peptide emulsified in saponin (Fig. 4).

There are several reasons that could explain this fact, such as loss of integrity, low encapsulation efficacy and the early release profile of the peptide from the microsphere.

During the microsphere formulation antigens are exposed to extreme conditions such as evaporation solvent process, ultrasound homogenization, direct exposure to organic solvents (Igartua et al., 1998) and shear stress (Lima and Rodrigues, 1999) that make it necessary to confirm the peptide integrity. Some methodologies as HPLC-RP and circular dichroism are used to analyze the integrity of peptide conformation. Figs. 2 and 3 show that in the microencapsulation process, there was not alteration in the SBm7462 because no unfoldings were observed in the sequence nor the appearance of additional species that might suggest aggregated formation or denaturation; further, the peptide extracted from the microspheres continued to present a helicoid conformation. Another factor

Fig. 4. Serum IgG antibody response detected by indirect ELISA in serum samples from BALB/c mice. Groups I–V (A) and group V compared with IV and VI (B). Arrows indicate the immunization and the bars indicate the standard deviation (±).
related to the failure of immunogens when PLGA is
used, is the break of the in vivo polymer that induces
an acid micro environment inside the microspheres
and in the release environment that would cause
alteration in antigenic epitopes, because the fall in pH
is a factor of unfolding and irreversible inactivation of
encapsulated proteins (Rosas et al., 2002; Perez et al.,
2002) but SBm7462 was shown to be resistant to acid
pHs as shown by circular dichroism (Fig. 3C). All
evidence presented previously indicated that the low
level of antibodies was not due to alterations during
the encapsulation process nor to changes in the pH
occurred after inoculation in the animals, since the
possible alterations in the peptide that could alter the
determining antigens did not take place.

If we agree with the research of Shi et al., (2003)
where it was demonstrated that the efficiency of protein
encapsulation depends on its solubility in aqueous
medium, affinity with polymers, stability of the first
emulsion, and preparation conditions, we could explain
the low rate of encapsulation of SBm7462 because it
was diluted in an aqueous solution in the first phase of
the double emulsion process and the peptide is
hydrophobic, that does not permit the formation of a
complete solution of the peptide in water, so that it was
capsulated as a suspension that did not favor its
capsulation in the PLGA microspheres; probably a
proportion of the peptide in the suspension was not
covered by PLGA; otherwise it would have remained
adsorbed or associated to the surface of the formed
microspheres.

The peptide associated to the surface is an indicator
of the efficiency of the encapsulation and the smaller
the quantity of associated peptide the greater the
efficiency (Alonso et al., 1994). The SBm7462,
associated to the surface, was 40.6% of the total
capsulated peptide, a quantity that is considered as
very high. A clear consequence of this adsorption is
the high initial burst observed and the constant and
greater release of the peptide, in contrast to SPI66,
where the initial burst was low and the antigen was
released in small quantities, presenting a release
plateau after the initial release (Fig. 1). Probably, the
SBm7462 release speed could have affected the
immune response, because the quick processing of the
antigen did not permit the formation of a clone with
high affinity that would lead to an IgGs production
comparable with that produced by SPI66 encapsula-
tion. The IgGs kinetics of the latter was similar to that
obtained in previous studies (Rosas et al., 2002).

When the immune responses of encapsulated
SBm7462 and the emulsified in saponin were
compared, it was observed that the IgGs elicited by
encapsulation decreased from day 50 after the last
inoculation, but the antibodies elicited by the
emulsification from day 35 after the last inoculation
remained constant and greater until the last week of
the study. The former fact may be explained because
in vitro kinetics of SBm7462 release at this time all the
peptide had already been released and the quick
release in large quantity of the vaccine did not permit
the processing of a good immune response (Figs. 1 and
4).

On the other hand, the synthetic peptide SBm7462
has already been tested in cattle using saponin as
adjuvant, with highly satisfactory results when its
efficacy was assessed in the control of B. microplus.
This fact could be attributed to the production of
specific IgGs of the subclass IgG1 according to
Gonzales (2003). In addition, other components of the
immune system would be involved as complement or
different profiles of Th cells. The studies of Kimaro
and Opdebeeck (1994), García-García et al. (2000),
and Patarroyo et al. (2002) showed that the IgG
response, associated to one challenge with larvae for
assessment of biological parameters, could be
considered as indicative of protective capacity, when
immunogens derived from the so-called “concealed
antigens” for the control of B. microplus are being
tested. The challenge is absolutely necessary, because
as shown by Patarroyo et al. (2002) a high level of IgG
was elicited when the SBm19733 antigen was used,
however, its efficiency, after challenge, was extremely
low.

It was also demonstrated that the antibodies
produced against SBm7462 showed a strong reaction
on the surface of the tick intestine epithelial cells with
intense reactivity inside the digestive vacuoles,
confirming that the construction and structure of this
peptide stimulated the immune system of the
immunized animals, permitting the recognition of
the integral protein in situ by the elicited antibodies
(Patarroyo et al., 2002).

The hepatic function study showed that there was
no indication of hepatotoxicity caused by SBm7462
whether emulsified in saponin or encapsulated in
PLGA confirming previous studies carried out on cattle using saponin as adjuvant.

Based on the findings obtained in this study and with the data cited by (Patarroyo et al., 2002), it was concluded that saponin is the adjuvant that gave best response to SBm7462 peptide under the current conditions. The use of the PLGA microspheres would represent an advance in Veterinary Medical vaccinology; they are viable for use with SBm7462 but additional studies are needed to improve the encapsulation process. These studies could follow the following direction: increase in the solubility of this peptide; substitution of water in the first phase of the double emulsion by another diluents where this peptide is completely soluble.

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