

Research article

The evaluation of yeast derivatives as adjuvants for the immune response to the Bm86 antigen in cattle

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Abstract

Background: The Gavac™ vaccine against the cattle tick *Boophilus microplus* has proven its efficacy in a large number of controlled and field experiments. However, this vaccine could be further improved by searching for new alternative adjuvants that would induce a stronger long-lasting immune response. We conducted several experiments to assay the adjuvant effect of fractions of the recombinant yeast *Pichia pastoris* in mouse and cattle models. In previous experiments, the combination of the yeast membrane with saponin was the most effective formulation in stimulating the humoral immune response in mice, eliciting a response higher than Montanide 888. The response was predominantly of the IgG1 isotype. Here, we evaluated the response in cattle and compared the results with that obtained in mice.

Results: Bm86 on the membrane of *P. pastoris* plus saponin produced high antibody titers in cattle, though the protection level against tick infestations was lower when compared to Gavac™, probably due to a decrease in the IgG1/IgG2 ratio. The predictive value of the mouse model was studied through correlation analysis between the isotype levels in mice and the efficacy of formulations in cattle. Good correlation was established between the level of antibodies in mice and cattle, and between the amount of anti-Bm86 IgG1 in mice and the degree of protection in cattle.

Conclusion: Mouse model have the potential to predict immunogenicity and efficacy of formulations in cattle. These results also support the use of the yeast expression system for recombinant vaccine formulations, enabling the prediction of more cost-effective formulations.

Background

In the last seven decades, a great number of studies have been devoted to the use of adjuvants to potentiate the immune response to antigens. These efforts have been particularly important in recent years with the development of synthetic, purified subunit and recombinant vaccines, which are generally poor immunogens. Recent studies describe the advantages of using saponin over oil emul-

sions, although the latter still constitutes the most commercially available adjuvant for veterinary vaccines.

Recently, the *B. microplus* Bm86 antigen was isolated and expressed in the yeast *P. pastoris* to prepare the recombinant vaccine Gavac™ (Heber Biotec S.A., Havana, Cuba) [1,2]. This vaccine against cattle ticks contains 100 µg Bm86 per dose in 2 ml of a Montanide 888 / mineral oil in water emulsion and has proven its efficacy in a

large number of controlled and field experiments [2,3,4,5]. However, this vaccine could be further improved by searching for new alternative adjuvants that would induce a stronger long-lasting immune response, and a reduction in production cost.

The immunostimulating properties of some components of certain species of yeast have been previously reported [6,7]. The recombinant Bm86 antigen expressed in *P. pastoris* remains associated to the plasma membrane [1], that surrounds the protein with a hydrophobic environment similar to that of oil emulsion or liposomes. Taking advantage of this fact, we made several experiments to test the adjuvant effect of fractions of the recombinant yeast in mice [8].

Here, we report the results obtained when we use yeast derivatives as adjuvants for the immune response in cattle, the analysis of the predictive potential of the mouse model, and the effect of the quality of the immune response on the degree of protection. The membrane of the yeast *P. pastoris* was shown to serve as an adjuvant for the humoral immune response in both animal species, adding new advantages to the yeast expression system for the production of recombinant vaccine formulations.

Results

Experiment I

Kinetics of the antibody response

Table 1 shows the results obtained in the quantification by ELISA of the level of anti-Bm86 antibodies in the serum samples from immunized mice [8]. Control groups kept a basal level of Bm86-specific reactivity equal to that of preimmune sera, indicating the specificity of the assays. Mice injected with Bm86 in the membrane plus saponin produced the highest serological response, reaching an immunological peak on day 30. Animals from the Bm86 / cell group showed practically no immunological response against the Bm86 antigen (Table 1).

Quality of the immune response

Sera extracted on day 40 were chromatographed through a column of Protein A Sepharose and the resulting peaks were collected for analysis, which was restricted only to subclasses IgG1, IgG2, and IgG2b, since the level of IgG3 was low and hardly varied among the different groups [8].

The total amount of protein corresponding to each subtype was determined and thus expressed as the relative level of antibodies (Table 2). The quantification level of the different IgG isotypes from Bm86 / Montanide 888 or Bm86 / cell groups were similar, with a predominance of IgG1 within the IgG subclasses. Groups immunized with membrane plus saponin showed a higher propor-

tion of the IgG2 isotype, which was of over 30% of the total IgG content.

Forty days after the immunization, pooled sera were titrated by ELISA to compare the relative amounts of anti-Bm86 IgG subclasses in the vaccinated groups (Table 3). The levels of Bm86-specific immunoglobulins showed a pattern similar to that of total IgG. Cells produced low levels of anti-Bm86 antibodies. In contrast, specific antibody levels were higher for groups injected with membrane and with Montanide 888. In the group immunized with the membrane plus saponin, the production of anti-Bm86 IgG2a was strongly stimulated. In control groups immunized with Dextranase-containing preparations, no specific anti-Bm86 antibodies were detected.

Experiment II

Serology

In calves immunized with preparations containing Bm86, high anti-Bm86 antibody titres were obtained with the formulations based on Montanide 888 and the membrane plus saponin, while the response obtained with cells was extremely poor (Table 4). Although the saponin-based formulation produced an immune response with a higher IgG2/IgG1 ratio than GavacTM, the levels of the IgG isotypes were not significantly different between both formulations (Table 4).

Response to challenge with tick larvae

Calves were challenged with 1000 larvae per animal per day for three days two weeks after the third immunization. The ability of each formulation to elicit a protective immune response was determined and expressed as overall efficacy (Table 5). The Montanide 888 adjuvant produced the most effective response, while the percentage of tick control was very low in the groups immunized with Bm86 associated to cells.

Mouse as a predictive model

The value of mice in predicting the immunogenicity of Bm86 formulations in cattle was studied through a correlation analysis of the response with the formulations tested in both animal systems. At week 9 antibody levels in cattle were correlated with mouse antibody levels at day 30 or day 40, obtaining correlation coefficients of 0.96 and 0.99, respectively.

A relationship was established between the mean anti-Bm86 IgG1 levels in mice and the protection reached in cattle with each formulation, giving a correlation coefficient of 0.99.

In contrast, anti-Bm86 IgG2a levels in mice were poorly correlated to protection in calves ($R^2 = 0.49$).

Table 1: Experiment I. Anti-Bm86 levels in mice immunized according to the experimental groups^a.

Immunogen / adjuvant	Days			
	10	20	30	40
Bm86 / Montanide 888	0.23 ± 0.24	1.34 ± 0.58	2.17 ± 0.54	1.02 ± 0.12
Bm86 / cells	0.17 ± 0.05	0.16 ± 0.10	0.24 ± 0.31	0.25 ± 0.07
Bm86 / membrane	0.17 ± 0.07	1.20 ± 0.67	2.09 ± 0.48	1.09 ± 0.27
Bm86 / membrane / saponin	0.23 ± 0.09	2.16 ± 0.39	2.68 ± 0.18	1.09 ± 0.12
Cells	0.15 ± 0.07	0.07 ± 0.05	0.18 ± 0.06	0.19 ± 0.05
Membrane	0.22 ± 0.08	0.19 ± 0.11	0.26 ± 0.11	0.26 ± 0.14
Membrane / saponin	0.21 ± 0.06	0.12 ± 0.03	0.23 ± 0.08	0.25 ± 0.11

^a Ten mice per group were immunized with 5 µg Bm86 adjuvated as indicated (Garcia - Garcia *et al.*, 1995). ELISA was used to determine total antibody response against Bm86. Briefly, 100 µl of antiserum diluted 1:100 in PBS were added to the wells, incubated for 2 h at 37°C, washed four times with PBST and developed with HRP-conjugated goat anti-mouse IgG as describe in material and methods. Antibody titres were expressed as the mean absorbance value (O.D._{492nm}) per group ± SD (N = 10).

Table 2: Experiment I. Immunoglobulin in mice immunized according to the experimental group^a.

Immunogen / adjuvant	IgG1	IgG2a	IgG2b	Total IgG
Bm86 / Montanide 888	0.519 (71.39) ^b	0.128 (17.61)	0.080 (11.00)	0.727
Bm86 / cells	0.271 (60.90)	0.094 (21.12)	0.080 (17.98)	0.445
Bm86 / membrane	0.566 (74.57)	0.127 (16.73)	0.066 (8.70)	0.759
Bm86 / membrane / saponin	0.637 (54.17)	0.465 (39.54)	0.074 (6.29)	1.176
Cells	0.278 (66.03)	0.083 (19.72)	0.060 (14.25)	0.421
Membrane	0.498 (70.14)	0.170 (23.94)	0.042 (5.92)	0.710
Membrane / saponin	0.717 (67.64)	0.251 (23.68)	0.92 (8.68)	1.060

^a Ten mice per group were immunized with 5 µg Bm86 (except the controls) adjuvated as indicated. Antibody levels were expressed as the absorbance at 280 nm of the pooled serum samples at day 40 (Garcia - Garcia *et al.*, 1995). ^b Within the parentheses the percent of the total IgG that corresponds to the subclass.

Table 3: Experiment I. Anti-Bm86 titer in the sera of mice immunized according to experimental groups^a.

Immunogen / adjuvant	IgG1	IgG2a	IgG2b	IgG1/IgG2a
				Ratio
Bm86 / Montanide 888	2000	200	100	10
Bm86 / cells	32	4	8	8
Bm86 / membrane	400	<50	<50	>8
Bm86 / membrane / saponin	1000	400	100	2.5

^a Experiments were conducted as described in Table 2. The IgG samples were homogenized to the same protein concentration and specific anti-Bm86 antibody levels were determined by ELISA and expressed as antibodies titres (Garcia - Garcia *et al.*, 1995).

Table 4: Experiment II. Anti-Bm86 titer in immunized cattle^a.

Immunogen / adjuvant	Total IgG	IgG1	IgG2
Bm86 / Montanide 888	23 744	11 763	1 114
Bm86 / cells	44	N/D	N/D
Bm86 / membrane / saponin	24 639	8 611	2 560

^a Three cross-bred Holstein calves per group were immunized with 100 µg Bm86 (except the controls) adjuvated as indicated. Antibody titers are expressed as the geometric mean titer determined by LISA.

Table 5: Experiment II. Protection of vaccinated calves against tick infestations^a.

Immunogen / adjuvant	Tick Number ^b	Tick Weight ^c	Percent of reduction of		Efficacy ^f (%)
			Egg laying Capacity ^d	Fertility ^e	
Bm86 / Montanide 888	35	45	61	40	85
Bm86 / cells	0	6	14	0	14
Bm86 / membrane / saponin	18	26	27	30	58

^a Experiments were conducted as described in Table 4. ^b Percent reduction of the adult female ticks. ^cPercent reduction on the mean weight of the engorged tick. ^d Percent reduction in the laying capacity of the adult female ticks. ^e Percent reduction in the mean weight of the larvae per gram of eggs. ^f Efficacy (%) = $100 \times \{1 - (\text{CRT} \times \text{CRO} \times \text{CRF})\}$; CRT: Reduction in the number of engorging ticks; CRO: Reduction in the egg laying capacity; CRF: Reduction in fertility.

Table 6: Vaccine formulations used in the experiments.

Group	Immunogen/adjuvant	Brief description
1	Bm86 / Montanide 888	Gavac TM , purified recombinant Bm86 emulsified with Montanide 888 / mineral oil.
2	Bm86 / cells	Recombinant Bm86 associated to disrupted cells of <i>P. pastoris</i> .
3	Bm86 / membrane	Recombinant Bm86 associated to the membrane of <i>P. pastoris</i> .
4	Bm86 / membrane / saponin	Recombinant Bm86 associated to the membrane of <i>P. pastoris</i> .
5	Cells	Cells of <i>P. pastoris</i> .
6	Membrane	Membrane of <i>P. pastoris</i> .
7	Membrane / saponin	Membrane of <i>P. pastoris</i> plus saponin.

Discussion

The *B. microplus* Bm86 antigen has been proven to induce a protective immune response in immunized cattle [1,2,4,5,9]. Furthermore, the control of tick populations in the field is correlated to the level of anti-Bm86 antibodies elicited by vaccination [4,10,11]. The vaccine developed by our group uses a recombinant Bm86 antigen

obtained from *P. pastoris*-expressing cells [1,2], which is expressed while associated to the plasma membrane [1,2]. Its hydrophobic microenvironment and the possible immunostimulating ability of certain components of the yeast cell wall led us to evaluate the effect of using some yeast derived preparations as the adjuvant of the humoral immune response to the Bm86 antigen.

The membrane-associated Bm86, when given to mice, quantitatively and qualitatively similar antibody responses to that of Bm86 / Montanide 888 [8]. The mechanism by which membrane-based formulations act could be similar to that of oil-based adjuvants [12], forming a depot that slowly releases the antigen prolonging its persistence and exposure time to immunocompetent cells. The addition of saponin to the membrane formulation strongly stimulated the immune response in mice, increasing the IgG2/IgG1 ratio (Table 3). Similar results have been previously reported with the use of saponin in mice [13]. This formulation was also effective in calves, producing a quantitatively similar response to that of Montanide 888 (Table 4). The response elicited by the membrane plus saponin showed an increase in the level of IgG2 and a decrease in the amount of anti-Bm86 IgG1 antibodies. The ability of the membrane / saponin formulation to potentiate the antibody response in cattle to levels similar to those of Montanide 888 was therefore demonstrated. However, the increase in the IgG2 response had a negative effect on the level of protection against tick infestation (Table 5), evidencing the role of IgG1 antibodies in the mechanism of action of anti-tick vaccines. In previous results animals immunized with midgut membrane antigens from *B. microplus* were protected against tick populations showing a collaborative action between the complement proteins and IgG1 antibodies [10,14]. IgG1 is the isotype that most efficiently fixes the complement in cattle [14, 15].

The inability of animals to respond to Bm86 associated to cells could be explained by the sub-cellular localization of the protein, which seems to be associated to the periplasmic side of the plasma membrane being unexposed to the immune system. Perhaps this formulation produces a stronger cell response, but this does not seem to play an important role in the Bm86-mediated mechanisms for the protection of cattle from *B. microplus* infestation [16].

The potential of the mouse as a predictive model of the immune response of cattle to the recombinant antigen Bm86 was also studied. Under experimental conditions, the response to Bm86 could be effectively predicted. The levels of antibodies specifically against Bm86 were proportional in mice and cattle. Furthermore, there was a high correlation between the titer of anti-Bm86 IgG1 elicited in mice and the efficacy of the formulation in cattle. These results could simplify future studies in the search of new adjuvants for the recombinant Bm86 antigen.

Although the inclusion of saponin in anti-tick vaccines should not be considered in the future because it produces a reduction in the anti-Bm86 IgG1 subtype and in-

creases the IgG2a. The use of alternative yeast derivatives as adjuvants of the immune response in cattle are of increasing interest, particularly for antigens like Bm86 that remain associated to the membrane. The dual use of the yeast expression system can make this vaccine more cost-attractive.

Materials and Methods

Preparation of *P. pastoris* fractions

The recombinant vaccine Gavac™ against the cattle ticks and the genetically engineered strain of *P. pastoris* that expresses the recombinant Bm86 antigen were obtained as previously reported [1,2]. A control strain of *P. pastoris* expressing a fungal dextranase [17] was used in the study to prepare control formulations.

Yeast cells were harvested from methanol induced cultures of both strains. Cells were mechanically disrupted with glass beads [2] or processed to purify the plasma membrane [18]. Briefly, cells were re-suspended in disruption buffer (50 mM sodium phosphate, 5 mM EDTA, 10% sucrose, 300 mM NaCl, 1 mM β -mercaptoethanol, pH 7) and disrupted using a ball mill disruptor. The disruption pellet was obtained by centrifugation and then homogenized in washing buffer (50 mM sodium phosphate, 5 mM EDTA, 0.5% Triton X-100, 300 mM NaCl, 1 mM β -mercaptoethanol, pH 7). The pellet was finally recovered by centrifugation. The plasma membrane was obtained eliminating the cell wall by treating with the hydrolytic enzyme Zymolase 100T (Sigma, USA) and purified by differential ultra-centrifugation on sucrose gradients [18].

The fractions obtained were analyzed by SDS-PAGE and Western blot. The concentration of Bm86 associated to the cell and membrane preparations was determined to adjust the amount of Bm86 antigen in each formulation (Table 6) to 50 μ g/ml. Equivalent amounts of the preparations from the control strain were added to obtain control vaccine formulations (Table 6). Saponin (saponin white pure, Merck) was added to the final concentration of 1 mg/ml when indicated.

Animals, immunization and serum collection

Experiment I

Seven groups of 5-week old Balb/c mice were randomly distributed into four test groups and three control groups (Table 6). Three doses of 100 μ l of vaccine (test groups containing 5 μ g of Bm86) were sub-cutaneously injected at 0, 10 and 20 days. Sera from animals were collected on days 0, 10, 20, 30 and 40 and stored at -20°C for a later immune response analysis.

Experiment II

Five groups of 3 one-year-old Holstein calves each were used in the experiment (Groups 1,2,4,5 and 7 in Table 6). Animals were immunized with 100 µg of Bm86 in doses of 2 ml by the intra-muscular route (IM) at 0, 4 and 7 weeks. Jugular blood samples were collected from cattle at 9 weeks and stored at -20°C until they were assayed.

Immunoglobulin levels in sera

The anti-Bm86 antibodies were determined by ELISA [19]. Briefly, 100 µl of a solution with 1 µg/ml of purified Bm86 were dispensed into each well of Nunc Polisorp immunoplate (MaxiSorp F96 and PolySorp F96). Plates were coated overnight at 4°C, washed twice with PBS containing 0.005% Tween 20 (PBST) and then blocked with 2% skim milk (Oxoid, UK) for 1 hour at 37°C. Plates were washed four times with PBST and 100 µl serum was added in a serial two-fold dilution of serum in phosphate buffer saline (PBS). Plates were incubated 2 hour at 37°C, washed with PBST and labeled with 100 µl of HRP-conjugated goat anti-mouse IgG or sheep anti-bovine IgG. Plates were incubated for 40 min. at 37°C, washed four times with PBST and 100 µl of a substrate solution (0.4 mg/ml orthophenylene diamine diluted in 0.005 M citric acid/ 0.1 M Na₂HPO₄ containing 1 µl/ml 30% H₂O₂ (BDH, Poole, UK) were added to each well. Color was allowed to develop for approximately 10 min. Adding 50 µl per well of 2.5 M H₂SO₄ terminated the reaction. The optical density (OD) was measured using a Flow Titertek Multiskan ELISA reader equipped with a 492 nm filter.

Separation of IgG subclasses and analysis

Mouse IgG subclasses were separated based on their affinity to Protein A. Pooled sera from day 40 were loaded in a column of Protein A-Sepharose-CL4B (Pharmacia, Sweden) and the immunoglobulin subclasses were eluted with a stepwise pH gradient of 0.1 M citric acid. The subclasses obtained by affinity chromatography were concentrated, dialyzed, and then assayed by ELISA to determine the subclass-specific antibody levels to Bm86, as described above.

Bovine subclasses were determined using goat anti-bovine subclass-specific secondary antibodies (Sigma, USA) in the ELISA analysis of the sera.

Challenge with tick larvae

Two weeks after the third immunization, calves were controlled-infested with 1000 larvae of *B. microplus* (Camcord strain) per animal per day for three days. Adult engorged female ticks were collected, counted, weighed and their egg laying capacity and the fertility of the eggs were assessed [3]. The efficacy of the adjuvants

used was determined employing the following parameters [1,3,5]:

Reduction on the number of adult female ticks:

$$\% \text{RAFT} = 100 [1 - (\text{NTV} / \text{NTC})]$$

NTV: Number of adult female ticks in the vaccinated group.

NTC: Number of adult female ticks in the control group.

Reduction of the weight of the engorged ticks

$$\% \text{RWT} = 100 [1 - (\text{WTV} / \text{WTC})]$$

WTV: Mean weight of the adult female tick in the vaccinated group.

WTC: Mean weight of the adult female tick in the control group.

Reduction of the egg laying capacity of the adult female tick:

$$\% \text{RECT} = 100 [1 - (\text{EWTV} / \text{EWTC})]$$

EWTV: Mean weight of the eggs per tick surviving in the vaccinated group.

EWTC: Mean weight of the eggs per tick surviving in the control group.

Reduction of fertility:

$$\% \text{RF} = 100 [1 - (\text{WLV} / \text{WLC})]$$

WLV: Mean weight of the larvae per gram of eggs in the vaccinated group.

WLC: Mean weight of the larvae per gram of eggs in the control group.

Efficacy (%) = $100 \times \{1 - (\text{CRT} \times \text{CRO} \times \text{CRF})\}$; CRT: Reduction in the number of engorging tick; CRO: Reduction in the egg laying capacity; CRF: Reduction infertility

Statistical analyses

Differences between mean anti-Bm86 antibody titers were determined by analysis of variance according to Student's t test.

Correlation analysis was carried out between the antibody levels elicited in mice and those elicited in cattle in order to determine the predictive value of the mouse

model for immunogenicity. Relationships between the specific level of anti-Bm86 IgG subclasses in mice and the protection level achieved in cattle with each formulation (expressed as efficacy) were also established by correlation analyses.

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