Vet Res Commun
DOI 10.1007/s11259-010-9423-0

ORIGINAL ARTICLE

The effect of a single intramammary infusion of a biological response modifier in cows at drying off

Bibiana Elisabet Dallard · H. H. Ortega · I. A. Iguzquiza · N. R. Salvetti · O. A. Quaino · L. F. Calvinho

Accepted: 11 June 2010

Abstract Biological response modifiers (BRM) are compounds that interact with the immune system to regulate specific aspects of host response. The objective of this study was to describe clinical and morphological changes during involution of bovine mammary gland following a single-dose infusion of a BRM containing lipopolysaccharide and cellular fractions of *Escherichia coli* incorporated into liposomes. A massive leukocyte response and increased subepithelial stroma infiltration of mononuclear cells, eosinophils and mast cells was observed in BRM-treated quarters compared with untreated controls; however, morphologic parameters assessed at 11 days post infusion were indicative of only slightly accelerated involution compared with untreated controls. In addition, BRM infusion at the end of lactation did not interfere with mammary epithelial cell proliferation and caused only mild systemic effects.

Keywords Biological response modifiers · Mammary gland · Involution · Bovine

Introduction

The bovine mammary gland is highly susceptible to new intramammary infections (IMI) during physiological transition from lactation to involution (Oliver and Sordillo 1988; Nickerson 1989). Although not fully understood, increased susceptibility to IMI has been related to changes during the involution process that may facilitate bacterial penetration of

B. E. Dallard (✉) · H. H. Ortega · N. R. Salvetti · O. A. Quaino · L. F. Calvinho
Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Rvdo. Padre Kreder 2805, (3080), Esperanza Santa Fe, Argentina
e-mail: bdallard@fcv.unl.edu.ar

B. E. Dallard · H. H. Ortega · N. R. Salvetti
Argentine National Research Council (CONICET), Buenos Aires, Argentina

I. A. Iguzquiza · O. A. Quaino · L. F. Calvinho
Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria, C.C. 22, (2300), Rafaela, Santa Fe, Argentina

Published online: 02 July 2010

the streak canal, interfere with natural defense mechanisms and enhance bacterial growth. Conversely, fully involuted bovine mammary glands are markedly resistant to IMI (Oliver and Sordillo 1989). These observations led to the hypothesis that hastening mammary gland involution while increasing natural protective mechanisms at early nonlactating period could favor prevention of IMI during the involution process (Oliver and Smith 1982b).

Biological response modifiers (BRM) or immunomodulators are compounds capable of interacting with the immune system to regulate specific aspects of host response (Tzianabos 2000). The type of activity of these compounds depends on their mechanism and site of action, dose, route and timing of administration (Tzianabos 2000). Several BRM have been used in an attempt to enhance non-specific immune mechanisms against bovine mastitis pathogens using different administration routes (Zecconi 2000; Hu et al. 2001; Takahashi et al. 2005). However, the exact mechanisms of action and effects on the bovine mammary tissue of compounds employed are not fully understood. Early studies using a mouse mastitis model showed that intramammary infusion of *Escherichia coli* lipopolysaccharide (LPS) accelerated mammary gland involution, which was associated with increased resistance to subsequent challenge with *Staphylococcus aureus* (Anderson 1976). In addition, effect of intramammary LPS administration on subsequent development of *S. aureus* IMI was assessed in Guinea pigs. Lipopolysaccharide-pretreated Guinea pigs showed less severe disease manifestations than nontreated animals, which was associated with higher neutrophil migration into the mammary gland (Vandeputte-Van Messom et al. 1995). Consequently, compounds that could accelerate mammary involution enhancing the cow's natural defense systems may play a role in mastitis control at drying off either alone or in conjunction with dry cow antibiotic therapy.

Studies performed in cows showed that intramammary inoculation of *E. coli* LPS at drying off resulted in an increase of phagocytic cells number, lactoferrin, serum albumin, immunoglobulins concentration and pH, compared with uninfused controls. In addition, secretion volume in LPS inoculated quarters was 40% less than noninoculated quarters 7 days after cessation of lactation indicating that LPS inoculation accelerated mammary involution (Oliver and Smith 1982a). Intramammary LPS infusion at drying off was also associated with transient reduction of mastitis pathogens isolation from mammary glands during the nonlactating period (Oliver and Smith 1982b; Dallard et al. 2007). Recent studies performed in lactating cows showed that LPS infusion in mammary quarters experimentally infected with *S. aureus* elicited a heightened inflammatory response compared to uninfused quarters as demonstrated by TNF-α induction, higher milk somatic cell counts and albumin levels in milk (Kauf et al. 2007). However, there is only little information available describing morphological changes occurring in the nonlactating bovine mammary tissue as a result of intramammary injection of LPS, and this information was obtained following chronic stimulation of the gland (Nickerson et al. 1992).

The objective of this study was to describe clinical changes, morphological changes and effect on mammary epithelial cell proliferation during early involution of bovine mammary gland following a single infusion of a LPS-based BRM.

Materials and methods

Biological response modifier

The product contained lipopolysaccharide of an *Escherichia coli* strain (LN02) at two different concentrations, 0.45 and 0.73 μmol, 4.5 mg of membranous and ribosomal

fractions of the same strain incorporated into liposomes contained in 10 ml of an aqueous based vehicle (Laboratorio Neomar, Buenos Aires, Argentina). Liposomes (The Liposystem Complex®, I.R.A., Milan, Italy) composition was 40% phospholipids and 60% hydrophilic medium and active principle. Concentration of LPS was determined by quantification of 2-keto-3-deoxyoctonate (KDO) by a colorimetric method (Osborn 1963). Prior to the present study, a dose-response trial was carried out to select LPS doses. Four cows at the end of lactation were intramammarily infused with BRM containing LPS concentrations of 0.18, 0.45, 0.73 and 0.91 μmol, while the remaining BRM components were kept at the same concentration. Doses yielding the highest somatic cell count (SCC) response without gross mammary swelling or systemic adverse effects (e.g, elevated rectal temperature, increased respiratory frequency and heart rate) were selected.

Animals and experimental design

Six Holstein nonpregnant cows in late lactation (weeks 31–36) from the herd of the Rafaela Experiment Station producing approximately 8 kg of milk per day prior to experimentation were used. Cows used in this study were from parity 3 to 5, and were milked twice daily before interruption of lactation. All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS 1999). The animals were selected based on previous bacteriological studies and SCC performed 20 days before BRM infusion. All the quarters used in this study were free of infection at the time of BRM infusion and mean geometric SCC mammary quarter was 124.725 cells/ml. Cows were randomly assigned to each of three treatment groups. Two cows were infused intramammarily in all quarters with BRM containing 0.45 μmol of LPS (Group 1), two cows with BRM containing 0.73 μmol of LPS (Group 2) whereas two cows were infused with a placebo containing only vehicle (Control group). This approach was used to evaluate changes in mammary gland tissue since inflammatory responses to LPS are known to be limited to infuse glands (Lengemann and Pitzrick 1986; Shuster et al. 1991). In addition, considering that in putative field conditions this compound is to be infused in all mammary quarters simultaneously, systemic clinical changes following BRM infusion were evaluated. Milking was interrupted after infusion. Cows were fed only alfalfa hay and had free access to water for the remainder of the experiment. For bacteriological analysis and SCC mammary secretion samples were aseptically collected using standard procedures (Hogan et al. 1999) 3 days before BRM administration, immediately before inoculation and 1, 2, 3 and 7 days post-infusion. The first two streams of milk from each teat were discarded, the next 5 ml were collected in sterile plastic vials for bacteriological analysis and then 30 ml were collected into plastic vials for performing SCC. The latter samples were preserved with azidiol (0.3%) at 4°C and analyzed within 24 h. Teats were dipped in a 0.5% iodophor solution after samples were taken. Animals were slaughtered 11 days post-inoculation at a local abattoir and samples for histological analysis were taken. Mammary tissue samples were taken from two zones in each quarter based on previous descriptions (Nickerson et al. 1992); zone 1: tissue from dorsal to the gland cistern (supra cisternal) and zone 2: tissue from the dorso-lateral portion of the gland (deep parenchyma) at a depth of 4 cm. Samples were kept in 4% buffered formaldehyde before laboratory processing.

Clinical examination

Rectal temperature was recorded immediately before BRM administration and at 0.5, 1, 2 and 7 days post-infusion. Mammary quarters were clinically examined by palpation and

assessment of macroscopic alteration of mammary secretion was conducted. Mammary gland clinical status was classified based on degree of swelling as normal mammary tissue, moderate swelling and marked swelling.

Bacteriological examination

Ten μl of mammary secretion samples were streaked onto blood agar plates supplemented with 5% ovine blood and incubated aerobically for 48 h. Plates were examined for bacterial growth at 24 and 48 h. Bacterial identification was conducted following standard procedures (Hogan et al. 1999). Presence of two similar colonies of any pathogen or one colony of *S. aureus* on blood agar was considered as a positive identification; therefore detection limit was 200 colony forming units/ml (cfu/ml) for the former and 100 cfu/ml for the latter. Intramammary infection was defined as isolation of the same organism from two consecutive samples.

Somatic cell counts

Somatic cell counts per ml were determined using a Fossomatic 90 (Foss Electric, Hillerød, Denmark). A completely randomized design with data collected in a sequence of unequally spaced points in time, measured in days, was used. Time 0 corresponded to pre inoculation sample. The experimental unit was a quarter and the dependent variable was the $\log_{10}$ of SCC. Two factors were considered, treatment and time. A Proc Mixed (SAS 1999) was applied using the repeated statement with an unstructured covariance matrix. The decision process related to the covariance structure was assisted by using the log likelihood value. Since the factor "day" is quantitative the log was modeled as a polynomial function of "day". Effects included in the model were treatment, day, day*treatment, day*day, day*day*treatment and the significance level was 0.05. Individual quadratic curves were obtained for each treatment. For data analysis only quarters free from infection were considered.

Tissue samples preparation

Immediately after cows were sacrificed samples of mammary parenchyma from each mammary quarter were taken and processed for light microscopy. Tissue samples of approximate 1.0 cm^3 were fixed in 4% neutral buffered formaldehyde, washed in phosphate buffered saline (PBS, pH 7.4) and processed for inclusion in paraffin (Woods and Ellis 1994). Serial 5 μm thick sections were mounted on glass-slides previously treated with VECTABOND (Vector Labs, Burlingame, USA) and stained with Haematoxylin–Eosin for a previous observation.

Morphometric studies

For data analysis only quarters free from infection were considered. Image analysis was performed using Image Pro-Plus 3.0.1 ® system (Media Cybernetics, Silver Spring, MA, USA). Images were digitised by a CCD colour video camera (Sony, Montvale, NJ, USA) mounted on top of a conventional light microscope (Olympus BH-2) using ×4, ×10 and ×40 objective lenses. Microscopic fields covering the entire area were digitised and stored in a 24 bits true colour TIFF format. Images resolution was set to 640×480 pixels. At the used magnifications, each pixel of the image corresponded to 2.68, 1.04 and 0.26 μm^2,

respectively, and each field in the monitor represented a tissue area of 2.2, 0.33 and 0.02 mm^2, respectively. Quantitative analysis was used to determine percentage mammary tissue area composed of stroma and size of alveoli in μm^2. Mammary tissue was also examined for infiltration of mononuclear cells, neutrophils, eosinophils, mast cells and presence of *corpora amylacea*. For this purpose tissue samples were further stained with Giemsa. This method allowed for differential characterisation of eosinophils, neutrophils, mononuclear cells and mast cells. Volume density of different cell types on each microscopic field was the morphometric variable used for measuring tissue infiltration (Gundersen et al. 1988) using the relationship proposed by Weibel (1969). Prevalence of these cell populations was quantitated in tissue stroma in 25 randomly selected microscopic fields/tissue specimen at 4×. Presence of *corpora amylacea* was examined in the alveolar lumina and interalveolar stromal areas. Differences between treatments were evaluated by ANOVA and Duncan test and significance was set at $p<0.05$.

Cellular proliferation

This was evaluated through expression of proliferation cell nuclear antigen (PCNA), a protein associated to delta polymerase that is expressed in multiplying cells from final stage of phase G1 to phase M. Expression of PCNA was revealed by immumohistochemistry using specific monoclonal antibodies. A streptavidin-biotin immunoperoxidase method was performed as described (Dallard et al. 2007). Briefly, sections were deparaffinized, hydrated and microwave pre-treated (antigen retrieval). The endogen peroxidase activity was inhibited with 1% H_2O_2 and nonspecific binding was blocked with 10 % normal goat serum. Sections were incubated overnight at 4°C with PCNA monoclonal antibody (clone PC-10; Novocastra Laboratories, Newcastle, UK). Slides were washed with PBS and incubated for 30 min at room temperature with rat-preabsorbed biotinylated secondary antibody. The visualization of antigens was achieved by the streptavidin-peroxidase method (BioGenex, San Ramon, CA) and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit - Zymed, San Francisco, CA) was used as chromogen. To verify specificity, adjacent control sections were subjected to the same immunohistochemical method replacing primary antibodies by rabbit and mouse non-immune serum. To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB reagent alone.

Immunostained mammary epithelial cells showing a characteristic nuclear staining pattern were considered positive. Cells were classified in three categories according to variations in staining intensity: light (1+), moderate (2+) and intense (3+). A minimum of 1,000 cells in at least 20 microscopic fields was counted in each specimen. Only cells showing an intense nuclear staining (PCNA 3+) were quantified and results were expressed as percentage of mammary epithelial cells counted (Dallard et al. 2007).

Results

Clinical examination

Rectal temperature peaked 40.7°C 12 h post-inoculation in one cow of Group 1 and 40.4°C in one cow of Group 2. Rectal temperature of cows in the three groups ranged between 38 and 39.6°C during the remainder of the experimental period. Macroscopic changes on mammary secretions and moderate swellings were observed in three mammary quarters

(RF, LF and LH) of the cow that presented 40.7°C (Group 1) and in three mammary quarters (RF, RH and LH) of the cow that presented 40.4°C (Group 2) at 24 h post-inoculation. Two mammary quarters from a cow in control Group (RF and LH) presented an infection caused by *Streptococcus dysgalactiae* and were therefore not considered for data analysis. No side effects other than those mentioned were observed during the experimental period.

Somatic cell counts

A pronounced increase in SCC in mammary secretions of cows in Groups 1 and 2 was observed at 24 h post-inoculation (PI) which was followed by a slow and slight decrease over the remaining sampling interval. Peak log SCC at 24 h for Groups 1 and 2 were $\log_{10}$ 6.94 and 6.74, respectively. Conversely, control Group SCC showed a slow increase over the sampling period, reaching the highest value ($\log_{10}$ 6.91) by day 7 PI (Fig. 1). The effects of treatment, day, day*treatment and day*day*treatment were significant ($p<0.05$). The quadratic equation was found to be adequate for regression of $\log_{10}$ on day. Although models for Groups 1 and 2 were similar, they were found to be neither parallel, nor coincident ($p<0.05$).

Morphometric analysis

Analysis of mammary tissue components eleven days after infusion of BRM showed no differences in stromal area and alveolar size among treatment groups at zone 1. However, percentage of connective tissue stroma and alveolar size at zone 2 increased in quarters from both treatment groups; being those from Group 1 significantly different ($p<0.05$) from untreated controls (Table 1). Interlobular connective tissue was abundant at this zone reflecting higher stromal percentage. In both BRM-treated and control quarters small and large alveoli showing cuboidal epithelial cells were observed. Secretory epithelial cells contained large fat droplets at the apical area and a small number of cells presented irregular nuclei with chromatin condensation and a highly vacuolated cytoplasm. There was no evidence of extensive sloughing of alveolar epithelial cells. Mammary tissue was composed

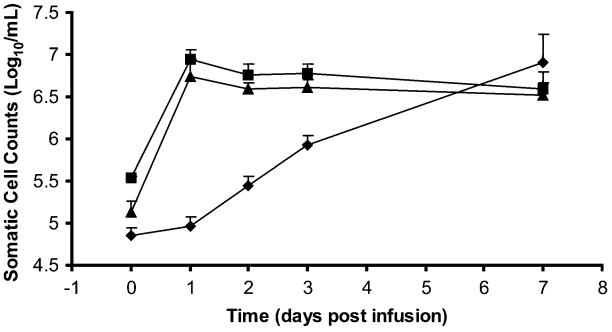

Fig. 1 Means of somatic cell counts of mammary quarters infused with a lipopolysaccharide-based biological response modifier at the end of lactation in Holstein cows. Control (◆): quarters infused with placebo; Group 1 (■): quarters infused with biological response modifier (BRM) containing 0.45 μmol of *Escherichia coli* lipopolysaccharide (LPS); Group 2 (▲): quarters infused with BRM containing 0.73 μmol of *E. coli* LPS. Error bars represent standard deviation

Table 1 Effect of treatment with a biological response modifier on mammary gland morphometric parameters eleven days post intramammary infusion. Group 1: mammary quarters treated with BRM containing 0.45 μmol of LPS. Group 2: mammary quarters treated with BRM containing 0.73 μmol of LPS. Zone 1: supra cisternal area. Zone 2: dorsal area. Data are expressed as means±Standard deviations (SD)

Tissue	Treatment	Stromal area[1] Mean±SD	Alveolar size[1] Mean±SD
Zone 1	Control	49±16.6[a]	3401.96±1335.54[a]
	Group 1	53.32±14.22[a]	3641.15±1816.44[a]
	Group 2	50.28±15.57[a]	3275.89±418.12[a]
Zone 2	Control	39.19±14.54[a]	3346.59±1233.91[a]
	Group 1	58.78±14.42[b]	5381.88±2474.49[b]
	Group 2	54.26±15.57[ab]	4148.99±1237.71[ab]

[a–b] Means with different superscripts within each zone (supra cisternal and dorsal) and morphometric parameter differ ($p<0.05$)

[1] Values are expressed in percentage

[2] Values are expressed in μm^2

mainly of alveolar lumen and little interalveolar tissue was present. Most cells infiltrating interalveolar stroma were mononuclear cells, neutrophils and eosinophils. The latter were also observed in most cases within the alveolar lumen (Fig. 2). Eosinophils, mononuclear cells and mast cells infiltration found into the stroma at both zones were greater for BRM-treated quarters compared with untreated controls ($p<0.05$) (Fig. 3). However, neutrophils infiltration was similar for control and BRM-treated quarters (Table 2). Presence of *corpora amylacea* was similar for control and BRM-treated quarters.

Cellular proliferation

No differences in expression of PCNA among control and treated groups at either zone were detected. The percentages of proliferation of mammary epithelial cells are summarized in Fig. 4.

Discussion

Escherichia coli LPS has been used extensively to study events that take place during mammary gland inflammation, and less frequently to stimulate bovine mammary gland non-specific defence mechanisms after cessation of milking (Oliver and Smith 1982a,b; Nickerson et al. 1992). In the present study the effect of a BRM containing *E. coli* LPS and cellular fractions incorporated into liposomes administered in a single dose at the end of lactation in Holstein cows was determined. Liposomes function is mainly to deliver associated antigens into macrophages, which are target cells for LPS (Alving 1993; Erridge et al. 2002). In addition, LPS incorporation into liposomes reduces part of its biological activity and therefore diminishes potential toxic effects (Erridge et al. 2002). BRM doses used in the present study proved to be safe for cows, causing only mild inflammation. In addition, presence of a single temperature peak in BRM-infused cows coincided with previous observations following endotoxin intramammary infusion (Burvenich and Peeters 1983; Lengemann and Pitzrick 1986). Infusion of a BRM at the end of lactation under field conditions would imply its use in pregnant cows. Non-pregnant cows were used in this

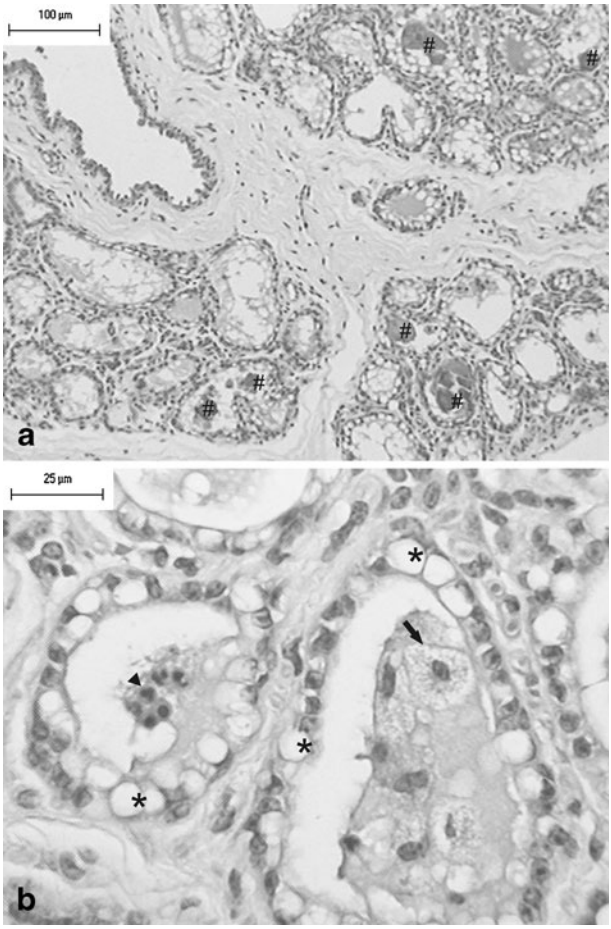

Fig. 2 Light micrographs of mammary tissue obtained 11 days post infusion of a lipopolysaccharide (LPS)-based BRM administered at drying off. (a) Alveoli at supracisternal area showing irregular lumen and containing *corpora amylacea* (#) (10x). Quarter infused with BRM containing 0.45 μmol of LPS. (b) Alveoli at deep parenchyma area showing cuboidal epithelial cells containing large vacuoles (*). Desquamated epithelial cells (arrows) and eosinophils (arrow heads) were observed in the lumen (x 40). Quarter infused with BRM containing 0.73 μmol of LPS

study to allow for morphometric evaluation without sacrificing the fetuses. Previous studies showed that infusion of intramammary IL-2 at drying off was associated with a higher rate of abortion in the treated group (Erskine et al. 1998), rising concerns about use of formulations that can affect pregnancy. Although determining the effect of the BRM on gestation was beyond the scope of this study, a similar BRM formulation containing 0.35 μmol of LPS was infused intramammarily into each quarter of 35 cows at drying off while a similar group received antibiotic treatment only. No differences on abortion rate was observed between groups (Calvinho et al., unpublished data).

A similar SCC pattern in both groups of treated quarters was observed in this study; however, a dose-dependent effect was not evident. This could have been due to small differences in LPS concentration between selected doses, which agrees with a previous study where no significant differences in SCC response following intramammary

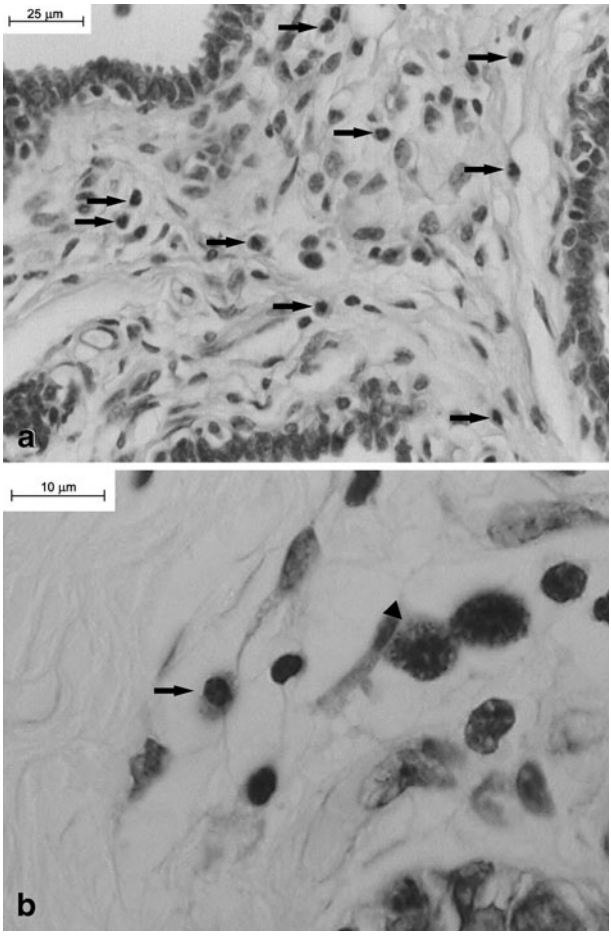

Fig. 3 Light micrographs of mammary tissue obtained 11 days post infusion of a lipopolysaccharide (LPS)-based BRM administered at drying off. (a) Stroma interalveolar showing eosinophils (arrows) (x40) Quarter infused with BRM containing 0.45 µmol of LPS. (b) Stroma interalveolar showing a macrophage (arrow) and mast cell (head arrow) (x100). Quarter infused with BRM containing 0.73 µmol of LPS

inoculation of either 1 or 10 µg of *E. coli* LPS in mammary glands of goats were detected (Lengemann and Pitzrick 1986). Differences in SCC at inoculation time between both experimental groups could have also contributed to minimize a dose-response effect. The pattern of SCC over time in untreated quarters was similar to that previously described in cows after interruption of lactation (Jensen and Eberhart 1981; Oliver and Smith 1982a); showing a steady increase along the sampling period and a peak 7 days PI. Somatic cell counts in BRM-treated quarters peaked at 24 h and differed from control quarters which did not reach these levels until 7 days PI. A similar chronological pattern was observed following a single intramammary administration of 100 µg of *E. coli* LPS or 100 µg of LPS plus 20 mg of colchicine at the end of lactation (Oliver and Smith 1982a) and of 100 µg of *E. coli* LPS at late lactation (Lehtolainen et al. 2003). In addition, although using a different sampling time, a similar SCC pattern was observed in a recent study following intramammary infusion of the same BRM at 0.35 µmol LPS in uninfected mammary

Table 2 Effect of treatment with a biological response modifier on the leukocyte infiltration into mammary gland subepithelial stroma eleven days post intramammary infusion. Group 1: mammary quarters treated with BRM containing 0.45 μmol of LPS. Group 2: mammary quarters treated with BRM containing 0.73 μmol of LPS. Zone 1: supra cisternal area. Zone 2: dorsal area. Values are expressed in volume density × 100. Data are expressed as means±Standard deviations (SD)

Tissue	Treatment	Eosinophils	Neutrophils	Monocytes	Mast cells
Zone 1	Control	0.5 ± 0.34^{a}	1.02 ± 0.17^{a}	1.56 ± 0.59^{a}	0.31 ± 0.13^{a}
	Group 1	1.71 ± 0.27^{b}	1.25 ± 0.39^{a}	4.09 ± 0.59^{b}	1.43 ± 0.51^{b}
	Group 2	2.02 ± 0.25^{b}	1.3 ± 0.3^{a}	6.05 ± 1.88^{c}	1.7 ± 0.34^{b}
Zone 2	Control	0.42 ± 0.24^{a}	0.76 ± 0.44^{a}	1.52 ± 0.35^{a}	0.29 ± 0.22^{a}
	Group 1	1.62 ± 0.31^{b}	1.13 ± 0.74^{a}	4.87 ± 1.01^{b}	1.31 ± 0.4^{b}
	Group 2	1.89 ± 0.22^{b}	0.86 ± 0.41^{a}	5.53 ± 1.31^{b}	1.65 ± 0.62^{b}

[a–b] Means with different superscripts within each zone (supra cisternal and dorsal) and cellular type differ ($p<0.05$)

quarters at drying off (Dallard et al. 2009). The different nature of the preparations used in the previous study by Oliver and Smith (1982a) does not allow for direct comparisons; however, massive influx of cells was achieved at the same time PI and followed a similar pattern remained elevated for the first 7 days PI of LPS. Conversely, Nickerson et al. (1992) administering daily intramammary infusions of 100 μg of *E. coli* LPS during 21 days of the nonlactating period did not find significantly elevated SCC performed at weekly intervals. Differences between studies can rely both on the nature of compound used and repeated stimulation of the involuting gland.

Percentage of bovine mammary tissue area composed of epithelium and lumen decreases during gland involution while percentage of stromal area increases, reaching a peak between 14 and 25 days after milking is interrupted (Sordillo and Nickerson 1988; Capuco et al. 1997). Changes in mammary epithelial cells observed in this study in both BRM-treated and untreated quarters were in accord with those described in naturally involuting mammary glands 7 to 14 days after drying off (Holst et al. 1987). Alveolar size was not affected by BRM treatment at supracisternal zone at eleven days post-infusion; however,

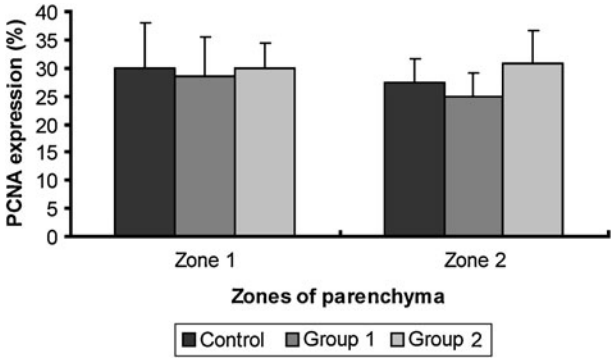

Fig. 4 Effect of treatment with a biological response modifier on expression of proliferation cell nuclear antigen (PCNA) at eleven days post intramammary infusion. Group 1: mammary quarters treated with BRM containing 0.45 μmol of LPS. Group 2: mammary quarters treated with BRM containing 0.73 μmol of LPS. Zone 1: supra cisternal area. Zone 2: dorsal area. Values represent means expressed as percentages of PCNA±Standard deviations (SD)

alveolar size of Group 1 BRM-treated quarters showed a significant increase at dorsal zone. Percentages of area occupied by stroma showed a significant increase at dorsal area in Group 2, indicating an intense tissue remodeling which was mainly due to presence of abundant interlobular connective tissue. Although the size of alveoli was larger in the same group than in untreated controls, the number of alveoli per lobule and interalveolar connective tissue were reduced. These results, together with lack of differences in number of *corpora amylacea* between treatment and control groups, indicated only a slight acceleration of involution. Previous studies (Oliver and Smith 1982a,b) reported acceleration of mammary involution following intramammary administration of 100 μg or *E. coli* LPS at the end of lactation. Although differences in products infused do not allow making direct comparisons, it has to be considered that acceleration of mammary involution in those studies was defined on the basis of biochemical measurements of mammary secretions rather than morphological changes. In addition, Nickerson et al. (1992) following daily intramammary administration of 100 μg of LPS in nonlactating cows for 21 days found that percentage of alveolar epithelial area was not affected; however, percentage of alveolar lumina was reduced and percentage of stroma increased.

Previous studies have shown that subepithelial stromal areas are the most common sites of leukocyte infiltration during mammary gland involution (Sordillo and Nickerson 1988). Mononuclear cells, eosinophils and mast cells were significantly increased in BRM-treated quarters; however neutrophil infiltration was not affected by BRM treatment. An increment in the number of infiltrating leukocytes in nonlactating bovine mammary glands has been observed after repeated administration of LPS, interleukin-2 (IL-2) and pokeweed mitogen (PWM) from *Phytolacca americana* (Nickerson et al. 1992) as well as in naturally infected quarters (Sordillo and Nickerson 1988). Macrophages are the predominant cell type both in mammary secretions (Jensen and Eberhart 1981) and subepithelial stromal areas (Sordillo and Nickerson 1988) in noninfected involuting mammary glands. Following repeated administration of LPS into bovine mammary quarters during the nonlactating period, a predominant leukocyte type infiltrating subepithelial stroma was not observed (Nickerson et al. 1992). In the present study, although the relative percent of infiltrating cells in subepithelial stroma was not determined, a significantly higher number of mononuclear cells in BRM-treated quarters indicated an increased recruitment of this cell type. This finding agrees with a recent study in which a significant increment of CD14 monocytes/macrophages was observed at 7 days of involution in mammary quarters treated with the same BRM formulation at 0.35 μmol compared with untreated controls (Dallard et al. 2009). Monocytes and macrophages recognize LPS and react releasing a range of proinflammatory mediators which in local infection sites and in moderate levels benefit the host promoting inflammation and priming the immune system (Tzianabos 2000).

Eosinophil infiltration is not common in udder tissues (Nickerson et al. 1992). Previous studies reported increased eosinophil infiltration at subepithelial stromal areas following administration of IL-2 or PWM in nonlactating (Nickerson et al. 1992) and lactating (Sordillo et al. 1991) bovine mammary glands. However, no increase in the number of eosinophils was observed following repeated administration of LPS in nonlactating quarters (Nickerson et al. 1992). Significance of eosinophils infiltration in the udder is not known. Sordillo et al. (1991) suggested that increased presence of eosinophils and neutrophils might have been associated with deleterious effects to mammary tissue structure and function caused by exogenously administered IL-2. In the present study a significantly increased number of eosinophils were detected in BRM-treated quarters. However,

maintenance of alveolar structure and lack of differences in expression of PCNA in mammary epithelial cells in BRM-treated and control quarters suggested absence of adverse effects on mammary gland function and structure.

Mast cells infiltrating bovine mammary gland subepithelial stroma decrease gradually from interruption of lactation reaching low concentrations at parturition (Sordillo et al. 1988). However, the role of these cells in involuting glands is not understood and there is only little information about significance of these cells in bovine mammary glands. Early studies directed to determine the possible role of mast cells in the pathological changes observed during *Streptococcus agalactiae* IMI were inconclusive (Zarkower and Norcross 1966). In the present study, although mast cells were found in BRM-treated quarters in numbers exceeding those normally associated with involution, no deleterious effects on mammary structures were observed.

Neutrophils infiltrating subepithelial stroma maintain similar numbers in uninfected quarters during the nonlactating period; however, this cell type increases in infected quarters (Sordillo and Nickerson 1988). In the present study, similar numbers of neutrophils in BRM-treated and control quarters were observed infiltrating subepithelial stroma. Conversely, Nickerson et al. (1992) found that neutrophil infiltration in stroma was higher in mammary quarters after repeated administration of LPS and PWM compared with those receiving IL-2 or PBS. Albeit the major component of BRM used in this study was LPS and therefore a similar infiltration pattern could have been expected in both studies, continuous LPS administration and slaughter soon after the last LPS infusion in the previous study (Nickerson et al. 1992) may have accounted for the observed differences.

Mammary involution in the dairy cow takes place with minor loss of epithelial cells as determined by morphological studies (Holst et al. 1987; Sordillo and Nickerson 1988). In addition, mammary epithelial cells showed increased incorporation of [^{3}H]Tdr during the nonlactating period indicating augmented cell division and therefore replacement of mammary cells (Capuco et al. 1997). Since previous studies showed that *E. coli* LPS inhibited proliferation of bovine mammary epithelial cells in vitro (Matthews et al. 1994; Calvinho et al. 2001), PCNA was determined to assess if the BRM used in this study had effect on mammary epithelial cell proliferation. Lack of differences in expression of PCNA between BRM-treated and control quarters indicated that this compound did not interfere with cell proliferation as determined at 11 d post infusion. In a recent study, infusing the same BRM intramammarily at drying off we found increased PCNA expression in treated quarters compared with untreated controls at 7 days post inoculation. However, no differences in PCNA expression between treated and control quarters were observed cell at 14 and 21 d post infusion (Dallard et al. 2007).

In conclusion, the BRM administered at a single intramammary dose in cows at the end of lactation yielded a massive leukocyte response with only slight clinical signs. Increased number of mononuclear cells, eosinophils and mast cells infiltrated subepithelial stromal areas. While mammary epithelial cell proliferation was not altered, morphometric parameters were not indicative of accelerated mammary gland involution which can limit the applicability to potentially reduce the risk of new IMI. Further research is granted to determine the efficacy of this BRM, in single administration or as an adjunct to antimicrobial therapy, to prevent or eliminate IMI during the dry period.

Acknowledgements This work was partially supported by the Agencia Nacional de Promoción Científica y Tecnológica (Argentinean Agency for Science and Technology) and Fundación ArgenINTA.

References

Alving CR (1993) Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants. Immunobiology 187:430–446.

Anderson JC (1976) The increased resistance of mice to experimental staphylococcal mastitis following inoculation of endotoxin. Res Vet Sci 21:64–68.

Burvenich C, Peeters G (1983) Effect of experimentally induced mastitis on blood flow in the heterolateral gland in lactating goats. Zentralbl Veterinarmed Reihe A 30:459–469.

Calvinho LF, Almeida RA, Oliver SP (2001) Influence of bacterial factors on proliferation of bovine mammary epithelial cells. Rev Argent Microbiol 33:28–35.

Capuco AV, Akers RM, Smith JJ (1997) Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. J Dairy Sci 80:477–487.

Dallard BE, Ruffino V, Heffel S, Calvinho, LF (2007) Effect of a biological response modifier on expression of growth factors and cellular proliferation at drying off. J Dairy Sci 90:2229–2240.

Dallard BE, Baravalle C, Ortega HH, Tumini M, Canavesio VR, Neder VE, Calvinho LF (2009) Effect of a biological response modifier on expression of CD14 receptor and tumor necrosis factor-alpha in *Staphylococcus aureus* infected mammary glands at drying off. Vet Immunol Immunopathol 132:237–242.

Erridge C, Stewart J, Bennet-Guerrero E, Mcintosh TJ, Poxton IR (2002) The biological activity of a liposomal complete core lipopolysaccharide vaccine. J Endotoxin Res 8:39–46.

Erskine RJ, Bartlett PC, Tavernier SR, Fowler LH, Walker RD, Seguin JH, Shuster D (1998) Recombinant bovine interleukin-2 and dry cow therapy: efficacy to cure and prevent intramammary infections, safety, and effect on gestation. J Dairy Sci 81:107–115.

Federation of Animal Sciences Societies (FASS) (1999) Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379–394.

Hogan JS, Harmon RJ, González RN, Nickerson SC, Oliver SP, Pankey JW, Smith KL (1999) Laboratory handbook on bovine mastitis. National Mastitis Council, Inc., Madison, WI. 222 pp.

Holst BD, Hurley WL, Nelson DR (1987) Involution of the bovine mammary gland: histological and ultrastructural changes. J Dairy Sci 70:935–944.

Hu S, Concha C, Johannisson A, Meglia G, Waller KP (2001) Effect of subcutaneous injection of Ginseng on cows with subclinical *Staphylococcus aureus* mastitis. J Vet Med B 48:519–528.

Jensen DL, Eberhart RJ (1981) Total and differential cell counts in secretions of the nonlactating bovine mammary gland. Am J Vet Res 42:743–747.

Kauf ACW, Vinyard BT, Bannerman DD (2007) Effect of infusion of bacterial lipopolysaccharide on experimentally induced *Staphylococcus aureus* intramammary infection. Res Vet Sci 82:39–46.

Lehtolainen T, Suominen S, Kutila T, Pyörälä S (2003) Effect of intramammary *Escherichia coli* endotoxin in early- vs late-lactating dairy cows. J Dairy Sci 86:2327–2333.

Lengemann FW, Pitzrick M (1986) Effects of endotoxin on mammary secretions of lactating goats. J Dairy Sci 69:1250–1258.

Matthews KR, Rejman JJ, Turner JD, Oliver SP (1994) Proliferation of a bovine mammary epithelial cell line in the presence of bacterial virulence factors. J Dairy Sci 77:2959–2964.

Nickerson SC (1989) Immunological aspects of mammary involution. J Dairy Sci 72:1665–1678.

Nickerson SC, Owens WE, Boddie RL, Boddie NT (1992) The effect of chronic immunostimulation of the nonlactating bovine mammary gland with interleukin-2, pokeweed mitogen, and lipopolysaccharide. J Dairy Sci 75:3339–3351.

Oliver SP, Smith KL (1982a) Bovine mammary involution following intramammary infusion of colchicine and endotoxin at drying off. J Dairy Sci 65:801–813.

Oliver SP, Smith KL (1982b) Nonantibiotic approach in control of bovine mastitis during dry period. J Dairy Sci 65:2119–2124.

Oliver SP, Sordillo LM (1988) Udder health in the periparturient period. J Dairy Sci 71:2584–2606.

Oliver SP, Sordillo LM (1989) Approaches to the manipulation of mammary involution. J Dairy Sci 72:1647–1664.

Osborn MJ (1963) Studies on the Gram-negative cell wall. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc Natl Acad Sci USA 50:499–506.

SAS Institute (1999) SAS OnlineDoc®, Version 8, Cary, NC: SAS Institute Inc.

Shuster DE, Harmon RJ, Jackso JA, Hemken RW (1991) Suppression of milk production during endotoxin-induced mastitis. J Dairy Sci 74:3763–3769.

Sordillo LM, Nickerson SC (1988) Morphologic changes in the bovine mammary gland during involution and lactogenesis. Am J Vet Res 49:112–1120.

Sordillo LM, Oliver SP, Guidry AJ, Dermody JT (1988) Humoral immune response of bovine mammary glands colonized with *Corynebacterium bovis*: enumeration of plasma cell populations in tissue and immunoglobulin concentrations in milk. Zentralbl Veterinarmed B 35:617–627.

Sordillo LM, Snider M, Hughes G, Afseth M, Campos M, Babiuk LA (1991) Pathological changes in bovine mammary glands following intramammary infusion of recombinant interleukin-2. J Dairy Sci 74:4164–4174.

Takahashi H, Komatsu T, Hodate K, Horino R, Yokomizo Y (2005) Effect of intramammary injection of RbIL-8 on milk levels of somatic cell count, chemiluminiscence activity and shedding patterns of total bacteria and *Staphylococcus aureus* in Holstein cows with naturally infected-subclinical mastitis. J Vet Med B 52:32–37.

Tzianabos AO (2000) Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. Clin Microbiol Rev 13:523–533.

Vandeputte-Van Messom G, Burvenich C, Roets E, Devriese L, Haesebrouck F (1995) Effects of *Staphylococcus aureus* mastitis after endotoxin application on milk yield and composition during subsequent lactation of Guinea pigs. J Vet Med B 42:118–126.

Weibel E (1969) Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 26:235–302.

Woods AE, Ellis CR (1994) Laboratory Histopathology. A Complete Reference. Ed. Longman Group Limited. London. 4.1–1–4.6–6.

Zarkower A, Norcross NL (1966) Histological changes in the bovine mammary gland after infusion with *Streptococcus agalactiae* extract. Cornell Vet 56:555–566.

Zecconi A (2000) Present and future of modulation of mammary gland immunity. International Dairy Federation symposium on Immunology of Ruminant Mammary Gland. Stresa, Italy. (Ed. A. Zecconi). Pp. 397–402.