RESEARCH LETTER

Functional role of antibodies generated in heifers through immunization with *Staphylococcus aureus* vaccines in invasion and phagocytosis assays

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Abstract

A successful *Staphylococcus aureus* vaccine should elicit a long-term antibody response that prevents establishment of the infection. The aim of the present study was to evaluate the functional role of antibodies raised against different *S. aureus* CP5 vaccines in invasion to bovine mammary epithelial cells (MAC-T) and phagocytosis by bovine milk macrophages *in vitro*. Sera and whey from cows immunized with a whole-cell *S. aureus* CP5 vaccine adjuvanted with Al $(OH)_3$ or with ISCOM Matrix, significantly reduced internalization of *S. aureus* in MAC-T cells without significant differences between both groups. The effect of antibodies generated by a *S. aureus* whole-cell and a lysate vaccine formulated with ISCOM Matrix was also evaluated. Sera and whey from both immunized groups significantly reduced *S. aureus* internalization in MAC-T cells without significant differences between both groups. Whey antibodies against whole-cell and lysate vaccines were also able to inhibit internalization in MAC-T cells of a heterologous *S. aureus* strain. In addition, sera from animals vaccinated with *S. aureus* lysate or bacterin promoted milk macrophage phagocytosis. These results provide an insight into the potential mechanisms by which these vaccines can afford protection to the mammary gland against *S. aureus* intramammary infection.

Introduction

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections (IMI) worldwide (Zecconi *et al.*, 2006). Control of *S. aureus* IMI is based on milking-time hygiene, antibiotic therapy and culling of chronically infected cows. The cure rate of *S. aureus* IMI following antibiotic treatment is low and therefore in many herds the disease is not effectively controlled (Barkema *et al.*, 2006). Due to these limitations, the development of vaccines to complement current measures to control *S. aureus* mastitis is of considerable interest to the milk production industry (Middleton, 2008).

Several experimental immunogens for *S. aureus* mastitis control have been evaluated during the last two decades (reviewed by Pereira *et al.*, 2011). However, only two vaccines, composed of *S. aureus* strain lysates expressing capsular polysaccharides (CP) adjuvanted with $Al(OH)_3$ (Lysigin™, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) and inactivated *S. aureus* expressing slime-associated antigenic complex formulated with an oil-based adjuvant (Startvac®, Laboratorios Hipra, S.A.), are currently commercially available worldwide. The mechanisms by which whole-cell or lysate vaccines may protect the mammary gland against *S. aureus* have not been fully explored. Since the main goal of *S. aureus* mastitis vaccination is prevention of new IMI (Middleton, 2008), immunogens that elicit a long-term antibody response against pathogen factors involved in early host–pathogen interactions should contribute to prevent

establishment of the infection. Enhancement of blood polymorphonuclear neutrophil (PMN) phagocytosis through production of antibodies raised against *S. aureus* CP2 bacterial lysates encapsulated in microspheres has been demonstrated (O'Brien *et al.*, 2001). In addition, it has been shown that *S. aureus* CP5 whole cell and lysate vaccine formulated with ISCOM Matrix adjuvant are able to stimulate strong antibody responses in blood and milk that increase PMN opsonic capacity (Camussone *et al.*, 2014). However, phagocytosis in these studies has been performed using blood PMN, since once in the milk environment these cells undergo morphologic changes and reduced phagocytic activity (Paape *et al.*, 2003). Macrophages are the major cell type in dry mammary gland secretions, colostrum and milk (Rainard & Riollet, 2006); among several other functions, they recognize microorganisms, alert the immune system, recruit PMN and initiate an inflammatory reaction (reviewed by Mosser & Edwards, 2008). Although macrophage bactericidal capability is considered limited, these cells have receptors for IgG_1 and IgG_2 and actively phagocytose bacterial pathogens (Desiderio & Campbell, 1980). However, there is no information about the effect of antibodies generated following immunization with *S. aureus* whole-cell and lysate vaccines on phagocytosis by milk macrophages.

The ability of *S. aureus* to attach to and internalize into mammary epithelial cells (MEC) is instrumental to mammary gland colonization and development of IMI (Almeida *et al.*, 1996; Dziewanowska *et al.*, 1999; Kerro Dego *et al.*, 2002; Zecconi & Scali, 2013). Several studies have addressed the role of antibodies directed against key antigens involved in adherence to/invasion of MEC (Olmsted & Norcross, 1992; O'Brien *et al.*, 2001; Shkreta *et al.*, 2004; Nour El-Din *et al.*, 2006). However, there is little information about the inhibition of adherence to MEC by antibodies raised against a *S. aureus* cell lysate (O'Brien *et al.*, 2001). In this study, the role of antibodies raised against a *S. aureus* CP5 whole-cell vaccine formulated with $Al(OH)_3$ or ISCOM Matrix and against a whole-cell and lysate vaccines formulated with ISCOM Matrix in phagocytosis by bovine milk macrophage and invasion of MEC *in vitro* was evaluated.

Materials and methods

Immune sera and whey

Sera and whey were obtained from two previous experiments. In the first, pregnant heifers were vaccinated with a whole-cell vaccine composed of *S. aureus* CP5 strain (Reynolds) (Fournier *et al.*, 1987) formulated with 15% $Al(OH)_3$ (Alhydrogel™), the same vaccine formulated with 2 mg per dose of immune-stimulating complexes (ISCOM Matrix, kindly supplied by Isconova, Uppsala, Sweden) or a placebo consisting of sterile saline solution (Camussone *et al.*, 2013). In the second study, pregnant heifers were vaccinated with a whole-cell and a bacterial lysate vaccine of *S. aureus* CP5 strain (Reynolds) formulated with 2 mg per dose of ISCOM Matrix and a placebo consisting of sterile saline solution and adjuvant (2 mg ISCOM Matrix per dose) (Camussone *et al.*, 2014). In both experiments, heifers were injected subcutaneously with 1 mL of vaccine in the supramammary lymph node area, 45 and 15 days before the expected calving date. Heifers were bled by puncture of the coccygeal vein before each inoculation, and on day 7 after calving blood was allowed to clot and sera were collected via centrifugation. After parturition, aseptic quarter foremilk samples were collected on day 7 according to standard procedures (Oliver *et al.*, 2004). An aliquot of 500 μL of milk from each quarter was used to prepare a composite sample. Samples were centrifuged at 300 ***g*** for 15 min; supernatants were collected and stored at −20 °C until processed. Sera and whey collected from heifers immunized with *S. aureus* bacterins and lysates showed significantly higher specific antibody levels (IgG and IgG_2 subtype) compared with nonvaccinated controls (Camussone *et al.*, 2013, 2014). Sera and whey from day 7 post calving were used for adherence/internalization and phagocytosis assays since this was the period at which the highest specific antibody levels were detected (Camussone *et al.*, 2013, 2014).

Cell culture

The established bovine mammary epithelial cell line (MAC-T; Huynh *et al.*, 1991) was generously provided by Dr. C. Porporatto, Universidad Nacional de Villa María, Córdoba, Argentina. MAC-T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), insulin (5 μg mL^{-1}), hydrocortisone (1 μg mL^{-1}), penicillin (100 U mL^{-1}), and streptomycin sulfate (100 μg mL^{-1}) (Sigma Chemical Co., St. Louis, MO). Prior to each experiment, MAC-T cells were seeded at 6×10^4 cells/well in 24-well tissue culture plates and grown for 3 days at 37 °C with 5% CO_2.

Bacteria

Staphylococcus aureus Reynolds and a *S. aureus* isolate (IR61) obtained from a cow with spontaneous clinical mastitis characterized by classical (Oliver *et al.*, 2004) and molecular methods (Martineau *et al.*, 1998) were used. Both strains were *cap5* type as determined by conventional

polymerase chain reaction (PCR), produced CP5 *in vitro* (Camussone *et al.*, 2012) and showed different pulsotypes evaluated by pulsed-field gel electrophoresis (PFGE; Chung *et al.*, 2000); the similarity between these two types was < 20%.

MEC internalization assay

The bacterial invasion assay was performed as described by Almeida *et al.* (1996) with modifications. Bacteria were activated from frozen stocks (−80 °C) by culture on Columbia agar with 2.5% NaCl added, incubated overnight at 37 °C for CP expression induction, and preincubated with a 1/10 dilution of whey or a 1/100 dilution of sera obtained on day 7 postcalving from each animal included in the study, for 60 min at 37 °C with gentle shaking. After incubation, the bacterial suspension was co-cultured with a confluent monolayer of MAC-T cells in DMEM at a multiplicity of infection (MOI, ratio of *S. aureus* organisms to cells) of 100. The number of epithelial cells per well was estimated by counting in a hemocytometer to determine the bacteria : epithelial cell ratio used during invasion experiments. Monolayers were washed three times with phosphate-buffered saline (PBS; pH 7.4) and treated with gentamicin (100 μg mL^{-1}, Sigma) in DMEM at 37 °C in 5% CO_2 for 2 h to kill extracellular bacteria. Supernatants were then collected and plated on trypticase soy agar (TSA) with 5% calf blood added to verify killing by gentamicin. Monolayers were then washed three times with PBS, treated with 0.25% trypsin-0.1% EDTA (Gibco BRL), and further lysed with Triton X-100 (Amersham, Arlington Heights, IL) at a final concentration of 0.025% (v/v) in sterile distilled water to release intracellular staphylococci. MAC-T cell lysates were serially diluted 10-fold, plated on TSA with 5% calf blood added and incubated overnight at 37 °C. Colony-forming units per mL (CFU mL^{-1}) of *S. aureus* associated with MAC-T cells were determined by standard colony-counting techniques. Each assay was run in duplicate (for whey-preincubated samples) or in triplicate (for sera-preincubated samples) with three observations per assay. Data were expressed as intracellular CFU mL^{-1} or as percentage of internalization relative to control group (whey from animals that were vaccinated with a placebo consisting of sterile saline solution considered as 100% of internalization).

Milk macrophage phagocytosis

Mammary secretions were collected from nonlactating healthy cows 10–15 days after cessation of milking and macrophages were isolated as described previously (Dosogne *et al.*, 2001). Phagocytosis assay was performed by flow cytometry using fluorescein isothiocyanate (FITC)-labeled *S. aureus* Reynolds as described (Camussone *et al.*, 2013) with modifications. Briefly, 100 μL of FITC-labeled *S. aureus* Reynolds suspension (1×10^8 CFU mL^{-1}) was incubated with pooled sera for 30 min at 37 °C with gentle shaking. Then, 100 μL of a 1×10^7 cells mL^{-1} suspension of bovine mammary secretion macrophages was added and incubated for another 30 min at 37 °C with gentle shaking. Phagocytosis was stopped by the addition of NaCl 0.85%/EDTA 0.04%. Finally, the mixture was stained with ethidium bromide to quench extracellular fluorescence (Weingart *et al.*, 1999) and analyzed by flow cytometry (FACSCanto II, BD Biosciences). The macrophage population was gated based on forward and side light scatter parameters (Region 1, R1). Data were collected using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using WINMDI software. The percentage of macrophages with associated bacteria was assessed and the mean fluorescence intensity (MFI) was used to estimate the number of bacteria internalized per positive cell (Zetterlund *et al.*, 1998).

Statistical analysis

A statistical software package (SPSS version 17.0) was used to perform statistical analysis. Means of CFU mL^{-1} obtained from adherence/internalization assays in MAC-T cells and percentages of FITC-positive macrophages in phagocytosis assays were compared using analysis of variance, followed by Tukey test to detect differences between pairs. Percentages of internalization in MAC-T cells were compared by Student's *t*-test. The significant level was set at $P < 0.05$. Results were expressed as mean ± SEM.

Results

Effect of sera and whey from heifers immunized with whole cell vaccine formulated with ISCOM Matrix and $Al(OH)_3$ on internalization in MAC-T cells

Whey and sera from ISCOM Matrix and $Al(OH)_3$ vaccinated groups inhibited internalization of *S. aureus* in MAC-T cells (Fig. 1). *Staphylococcus aureus* internalization was inhibited by sera from $Al(OH)_3$ and ISCOM Matrix immunized groups ($P = 0.001$ and $P = 0.004$, respectively), and by whey from $Al(OH)_3$ and ISCOM Matrix immunized groups ($P = 0.046$). No differences were observed between the two vaccinated groups (for sera: $P = 0.495$ and for whey: $P = 0.741$).

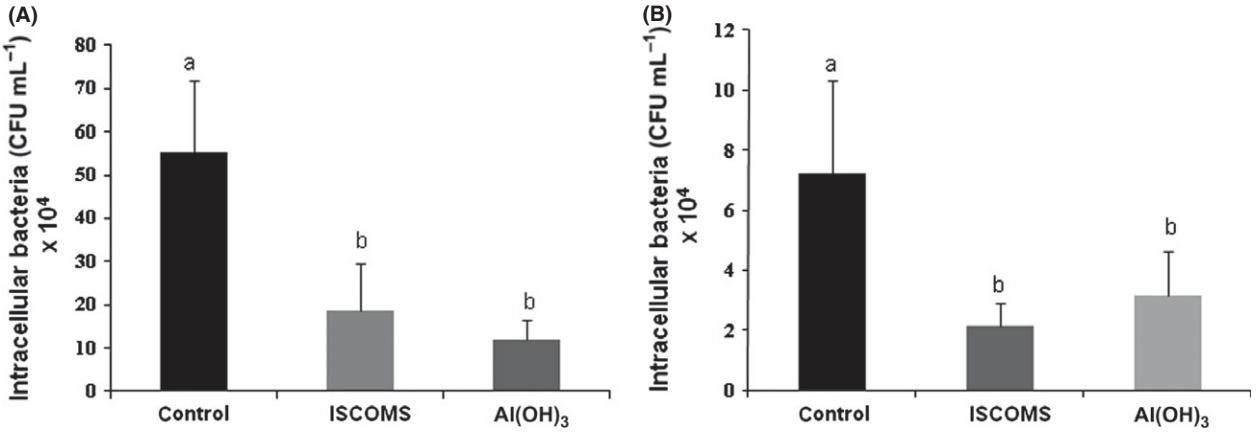

Fig. 1. Effect of antiserum against *Staphylococcus aureus* Reynolds formulated with ISCOM Matrix and $Al(OH)_3$ on internalization of homologous strain into bovine MEC. (A) *Staphylococcus aureus* Reynolds pretreated with 1/10 dilution of whey or (B) 1/100 dilution of sera from heifers immunized with *S. aureus* Reynolds in ISCOM Matrix, in $Al(OH)_3$ or sterile saline solution (as control group). Bacteria were co-cultured with bovine MEC and internalization calculated. Data are expressed as intracellular CFU mL^{-1}. Bars represent the mean of eight individual whey per group run in duplicate (A) or eight individual sera per group run in triplicate (B). Error bars represent the SEM. Different letters correspond to statistically significant differences ($P < 0.05$). 254 × 190 mm (300 × 300 DPI).

Effect of sera and whey from heifers immunized with whole-cell or lysate vaccine formulated with ISCOM Matrix on internalization in MAC-T cells

Whey and sera from both vaccinated groups formulated with ISCOM Matrix inhibited internalization of *S. aureus* in MAC-T cells (Fig. 2A and B). *Staphylococcus aureus* internalization was inhibited by sera from lysate and bacterin immunized groups ($P = 0.004$ and $P = 0.005$, respectively), and by whey from lysate and bacterin immunized groups ($P = 0.007$ and $P = 0.01$, respectively). No differences were observed between the two vaccinated groups (for sera: $P = 0.956$ and for whey: $P = 0.726$). In addition, the ability of whey from vaccinated groups to inhibit internalization of a heterologous *S. aureus* isolate (IR61) compared with *S. aureus* Reynolds was evaluated. Whey from both vaccinated groups inhibited internalization of heterologous *S. aureus* in MAC-T cells in the same way as the homologous *S. aureus* strain ($P < 0.001$) (Fig. 3). No differences in the percent internalization between the vaccinated groups were observed (homologous $P = 0.494$ and heterologous $P = 0.601$).

Phagocytosis assays

Sera were used in this assay. First, FITC+ *S. aureus* Reynolds were pretreated with pooled sera and then incubated with bovine milk macrophages (Fig. 4). Based on light scatter properties, we defined the Region 1 (R1) to further analyze macrophage phagocytosis (Fig. 4A). Sera from animals vaccinated with *S. aureus* lysate or *S. aureus* bacterin enhanced bacterial uptake by milk macrophages compared with sera from control animals ($P = 0.003$ for lysate and $P = 0.014$ for bacterin) (Fig. 4C). Furthermore, the MFI of the *S. aureus* phagocytosis by milk macrophages showed an increase when sera from both vaccinated groups were used as opsonins compared with sera from control animals (Fig. 4D) ($P = 0.047$ for lysate and $P = 0.010$ for bacterin). No differences between the two vaccinated groups were observed for percent phagocytosis ($P = 0.335$) or for MFI ($P = 0.403$). Two individual sera from groups of vaccinate were evaluated yielding similar results (data not shown).

Discussion

In this study we compared the functional effects of blood and milk antibodies generated in pregnant heifers during immunization with a *S. aureus* CP5 whole-cell and lysate vaccine on mammary epithelial cell internalization and macrophage phagocytosis. A variety of experimental vaccination studies using the new generation adjuvant ISCOMs including several organisms and animal species have been reported (Sun *et al.*, 2009). However, there is little information available about their use for vaccination against *S. aureus* bovine mastitis and this was generated through the use of defined antigens (Nelson *et al.*, 1991; Morein *et al.*, 2007). Only recently, the use of this adjuvant for the formulation of a *S. aureus* whole-cell and lysate vaccines has been reported (Camussone *et al.*, 2013, 2014).

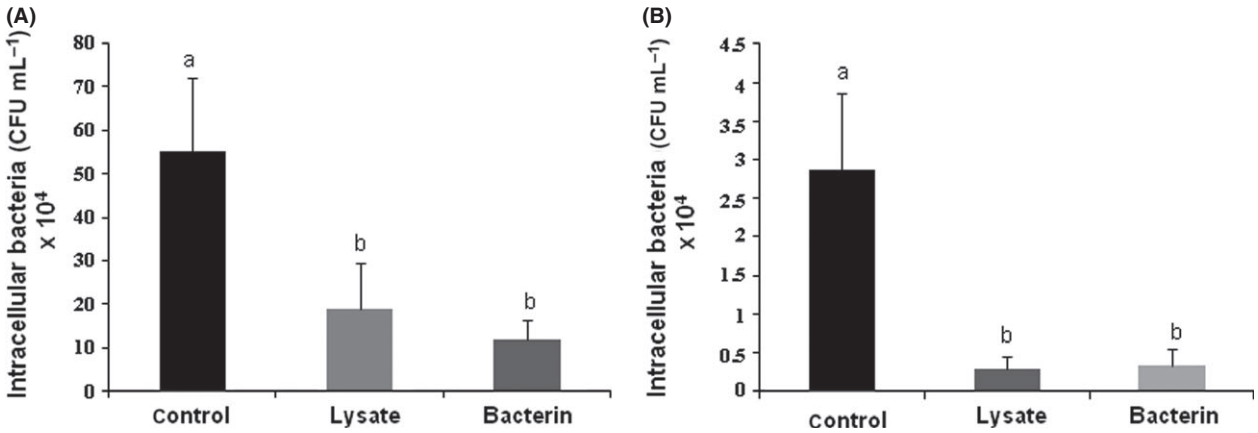

Fig. 2. Effect of antiserum against *Staphylococcus aureus* Reynolds lysate and bacterin on internalization of homologous strains in bovine MEC. (A) *Staphylococcus aureus* Reynolds pretreated with 1/10 dilution of whey or (B) 1/100 dilution of sera from heifers immunized with *S. aureus* Reynolds lysate, bacterin or sterile saline solution (as control group) in ISCOM Matrix. Bacteria were co-cultured with bovine MEC and internalization calculated. Data are expressed as intracellular CFU mL^{-1}. Bars represent the mean of eight individual whey per group run in duplicate (a) or eight individual sera per group run in triplicate (b). Error bars represent the SEM. Different letters correspond to statistically significant differences ($P < 0.05$). 254 × 190 mm (300 × 300 DPI).

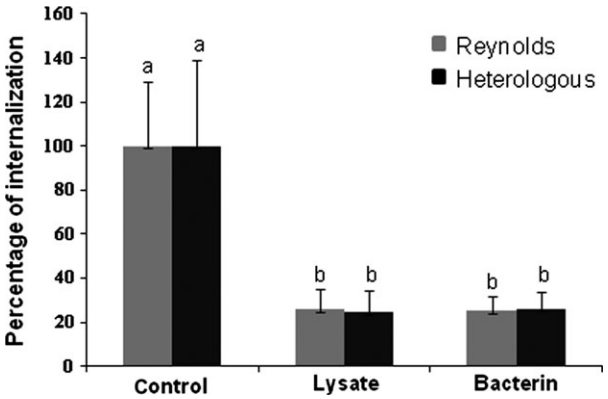

Fig. 3. Effect of antiserum against *Staphylococcus aureus* Reynolds lysate and bacterin on internalization of heterologous strains in bovine MEC. *Staphylococcus aureus* Reynolds (homologous) and *S. aureus* IR61 (heterologous) pretreated with 1/10 dilution of whey from heifers immunized with *S. aureus* Reynolds lysate, bacterin or sterile saline solution (as control group) in ISCOM Matrix. Bacteria were co-cultured with bovine MEC and internalization calculated. Data are expressed as percentage of controls (100%). Bars represent the mean of eight individual whey per group run in duplicate. Error bars represent the SEM, Different letters correspond to statistically significant differences ($P < 0.05$). 254 × 190 mm (300 × 300 DPI).

We first compared the functional capacity of antibodies generated during immunization with *S. aureus* CP5 whole-cell vaccine formulated with $Al(OH)_3$ or with ISCOM Matrix on internalization assays. Since adherence to MEC and extracellular matrix proteins is considered the first step in establishing a bacterial infection, antibodies generated through vaccination could block early host–pathogen interactions and favor macrophages and neutrophil phagocytic activity, thus helping to clear the organism from the mammary gland (Kerro Dego *et al.*, 2002; Middleton *et al.*, 2009).

The role of antibodies generated in cows in response to immunization with different *S. aureus* vaccines has been evaluated using adherence/internalization assays (Olmsted & Norcross, 1992; O'Brien *et al.*, 2001; Shkreta *et al.*, 2004). In the present study, sera and whey from cows immunized with whole cell *S. aureus* Reynolds adjuvanted with $Al(OH)_3$ and ISCOM Matrix significantly reduced internalization of *S. aureus* in bovine MEC, compared with sera and whey from control animals. Olmsted & Norcross (1992) examined the effect of serum and milk antibodies generated in one cow immunized with a *S. aureus* whole-cell vaccine on the bacterial adherence to primary bovine MEC by two different methods, demonstrating antiadherent capabilities. We decided to use a cell invasion model in the present study based on the fact that *S. aureus* can internalize in MEC (Almeida *et al.*, 1996) even in the absence of well-characterized adhesive proteins (Brouillette *et al.*, 2003) and that there is no information about the ability of antibodies generated by whole-cell vaccines formulated with classical or new adjuvants to inhibit bacteria internalization in these cells. A significant internalization inhibition by sera and whey from both immunized groups was observed. However, no significant differences in internalization inhibition between sera and whey from cows immunized with ISCOM Matrix or with $Al(OH)_3$ groups were detected, although sera and whey from ISCOM Matrix-immunized

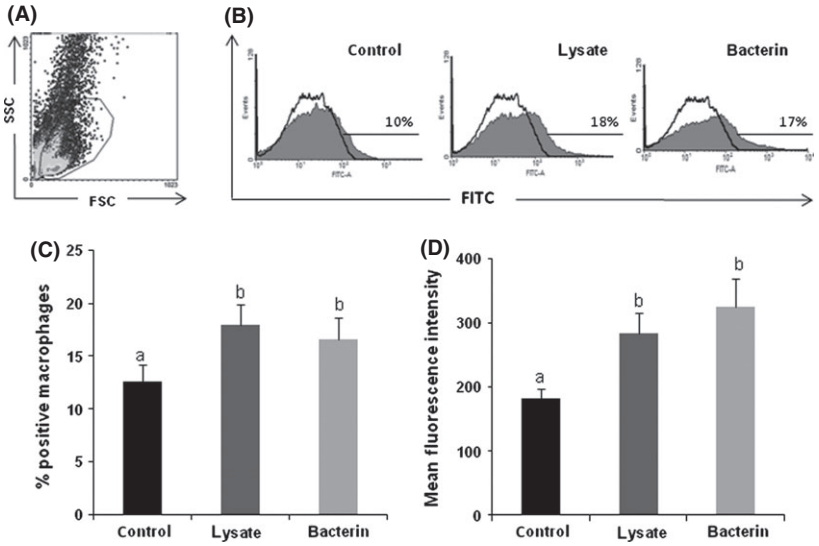

Fig. 4. Effect of antisera against *Staphylococcus aureus* Reynolds lysate and *S. aureus* Bacterin on bovine mammary secretion macrophages phagocytosis of FITC positive *S. aureus* Reynolds. FITC-positive *S. aureus* Reynolds were pretreated with 1/100 dilution of pooled sera from heifers immunized with *S. aureus* lysate, *S. aureus* bacterin or sterile saline solution (as control group) formulated with ISCOM Matrix and then co-cultured with bovine mammary secretion macrophages for phagocytosis assays by flow cytometry. (A) Representative forward scatter vs. side scatter density plots showing gate in R1 for further macrophages phagocytosis analysis. (B) Representative histograms showing fluorescence intensity for macrophages incubated with PBS (empty) or FITC-positive *S. aureus* Reynolds opsonized with sera from heifers immunized with *S. aureus* lysate, bacterin or control groups (gray-filled). Percentages of FITC-positive macrophages (C) or MFI (D) are shown. Error bars represent the SEM. Different letters correspond to statistically significant differences ($P < 0.05$). 254 × 190 mm (300 × 300 DPI).

animals was shown to have significantly increased IgG and IgG_2 levels compared with those immunized with Al $(OH)_3$ (Camussone *et al.*, 2013).

The effect of antibodies generated by a *S. aureus* whole cell and a lysate vaccines formulated with ISCOM Matrix was also evaluated in the epithelial cell internalization model. Preincubation with sera or whey from bacterin and lysate-immunized animals significantly reduced internalization of *S. aureus* in MAC-T cells compared with sera and whey from control animals; however, no significant differences were observed between the two immunized groups. Our findings are in agreement with those from O'Brien *et al.* (2001). These authors demonstrated that sera from cows immunized with a *S. aureus* CP2 lysate encapsulated in biodegradable microspheres or in Freund's incomplete adjuvant, significantly reduced bacterial adherence to primary mammary secretory epithelial cells cultured on rat tail collagen compared with preimmune sera. *Staphylococcus aureus* expressing CP5, like the strain used in the present study, has been shown to interfere with staphylococcal adherence to cells *in vitro* (Pöhlmann-Dietze *et al.*, 2000; Risley *et al.*, 2007). However, since CP are not equally expressed during the different phases of bacterial growth (Luong *et al.*, 2002), production of antibodies against antigens involved in the adherence/internalization process and against CP is desirable for vaccine development.

Whey antibodies against whole cell and lysate vaccines were able to inhibit internalization in MAC-T cells of a heterologous *S. aureus* strain. This strain presented < 20% similarity as evaluated by PFGE with the Reynolds strain and, although they shared the CP5 type, antibodies raised against CP are not considered to interfere with adherence to MEC *in vitro* (O'Brien *et al.*, 2001). Therefore, both vaccine formulations generated antibodies capable of interacting with common cell antigens present on the bacterial cell wall, suggesting that they may provide protection against unrelated isolates. Since currently available commercial vaccines are based either on multiple strains (Middleton, 2008) or on the presence of a *S. aureus* common antigen (Prenafeta *et al.*, 2010), the relative importance of staphylococcal antigens that should be included as vaccine components for achieving a protective response deserves further studies.

Macrophages are the predominant cell type in milk and secretions from nonlactating mammary glands and are the first immune cells to encounter invading bacteria (Rainard & Riollet, 2006). Bovine milk macrophages take part in early interactions with pathogens, express receptors for IgG_1 and IgG_2, are active phagocytes and are involved in antigen presentation in association with major histocompatibility complex (MHC) class I and class II molecules (Desiderio & Campbell, 1980; Craven, 1983; Sordillo & Streicher, 2002; Rainard & Riollet, 2006).

Several studies have evaluated the opsonic capacity of antibodies raised against *S. aureus* whole-cell or lysate vaccines for bovine blood PMN (Guidry *et al.*, 1991, 1994; Camussone *et al.*, 2013, 2014), since milk PMN show a markedly decreased activity compared with blood PMN (Paape *et al.*, 2003). However, there is no information about opsonic capacity of sera from vaccinated heifers for bovine milk macrophages. An increase in the percentage of milk macrophages that phagocytosed *S. aureus*, and in the number of bacteria ingested per cell, was observed when sera from animals vaccinated with *S. aureus* lysate or *S. aureus* bacterin were used as opsonins. This is in accordance with previous results from our laboratory where both neutrophil phagocytic capacity and the number of bacteria internalized per positive cell were increased upon opsonization with these sera, which was attributed to high anti-CP5 IgG levels in both vaccinated groups (Camussone *et al.*, 2014). Considering that both macrophage numbers (Hurley, 1989) and IgG levels (Sordillo *et al.*, 1987) increase as mammary involution progresses, a vaccination booster with whole cells and lysates shown to achieve high antibody levels at the end of lactation (Camussone *et al.*, 2014) could therefore favor macrophage activity during this period. However, this hypothesis should be tested under experimental or natural challenge conditions.

In conclusion, sera and whey from pregnant heifers immunized with *S. aureus* whole or lysed cells formulated with a classical adjuvant and ISCOM Matrix stimulated antibodies production that inhibited internalization in MEC and increased phagocytosis by milk macrophages, providing insight into the putative mechanism by which these vaccines can afford protection to the mammary gland against *S. aureus* IMI.

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