A Method for Measuring Specific Antibodies in Bovine Lacteal Secretions During the Nonlactating Period[1]

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ABSTRACT

A large portion of new IMI in dairy cattle occurs during the nonlactating period. Because antibiotic infusions at the beginning of the nonlactating period are only partially effective, attempts have been made to stimulate the production of protective antibodies in lacteal secretions during this period. However, measurement of antibodies in mammary secretions during the nonlactating period has been hampered by the complex, viscous nature of these secretions. This report describes the use of caprylic acid to clarify secretions from the bovine mammary gland during the nonlactating period to provide a more accurate measurement of specific antibody. Six healthy Jersey cows were injected in the area of the supramammary lymph node with an encapsulated strain of *Staphylococcus aureus* in dextran sulfate at the beginning of the nonlactating period and 15 and 30 d later. Seven healthy unimmunized Jersey cows served as controls. Lacteal secretions taken at the beginning of the nonlactating period; at 15, 30, and 45 d into the nonlactating period; and at calving were treated with caprylic acid prior to assay for specific antibodies using ELISA. Purified *S. aureus* capsule was used as the antigen in the ELISA. Caprylic acid lowered nonspecific binding of IgG_1 and IgM in secretions during the dry period from unimmunized control cows and lowered IgM from immunized cows. The most pronounced effect of caprylic acid was an increase in IgG_2 binding in secretions from immunized cows. Treatment with caprylic acid more accurately measured specific activity of Ig in mammary secretions during the nonlactating period.

(**Key words**: dry period, isotype, antibody, lacteal secretions)

Abbreviation key: **Df** = *Staphylococcus aureus* Smith diffuse, **PMN** = polymorphonuclear neutrophilic leukocytes.

INTRODUCTION

Susceptibility of the bovine mammary gland to IMI during the nonlactating period has been recognized for decades (13). Intramammary infections occur primarily during involution and just prior to calving (6, 12, 16, 22, 26, 27). Many of these IMI persist into the next lactation, adversely affecting udder health, which in turn decreases milk production and alters milk composition (17, 18). Antibiotic therapy at the beginning of the nonlactating period is effective during early involution but does not prevent new IMI prior to or immediately following calving (15, 17).

These new IMI occur in the presence of high SCC. Jensen and Eberhart (10) reported mean SCC in uninfected quarters of 1.8×10^7 at 7 d postpartum compared with 3.6×10^5 SCC on the last day of lactation. The SCC remained high throughout the nonlactating period and decreased to 2.8×10^5 by d 6 after parturition. The relative counts of polymorphonuclear neutrophilic leukocytes (**PMN**) and macrophages were 35.4 and 35.2%, respectively, on the last day of lactation but gradually shifted as involution progressed to a 1:3 ratio of PMN to macrophages by d 21 of the nonlactating period and remained at this ratio until 1 wk prior to calving. In the involuted gland, the PMN concentration was 25-fold that of normal milk at end of the lactating period. During the week prior to calving, when the incidence of IMI is most prevalent, the ratio of PMN to macrophage increases to 1:2. Other researchers (7, 24) have found similarly high total SCC and high ratios of PMN to macrophages during the nonlactating period. This level of PMN exceeds the concentration of PMN required to protect the mammary gland against IMI in the lactating gland (20). However, Paape et al. (19) also found that, as involution progressed, secretions in the nonlactating gland were less able to support phagocytosis by donor blood PMN, despite the fact that the concentrations of primary opsonins for PMN

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(IgG_2 and IgM) (8) approach serum concentrations in the nonlactating gland (11). In-depth studies of the opsonic function of IgG_2 and IgM in secretions of the nonlactating gland have been hindered by the complex and viscous nature and limited quantity of nonlactating gland secretions.

Most attempts to quantitate Ig in secretions from the nonlactating gland have utilized isoelectrofocusing (1), electroimmunodiffusion (14, 24), electrophoresis (4, 11), or radial immunodiffusion (3). Chang et al. (5) measured specific Ig isotypes in secretions from the nonlactating gland using the ELISA, but ELISA values for unimmunized controls were not reported. Previous attempts in this laboratory have shown increases in specific antibody activity in uninfected, unimmunized controls similar to those reported by Chang et al. (5). Because the cows were neither infected nor immunized, the increase was attributed to nonspecific binding of Ig (1, 11, 23, 24). The current study attempted to measure specific isotypes more accurately by removing nonspecific binding and inhibitory factors that are present in secretions from the nonlactating gland.

MATERIALS AND METHODS

Immunization and Sampling of Cows

Staphylococcus aureus Smith diffuse (**Df**) was grown in vivo according to the methods of Watson and Prideaux (28). The organisms were killed by adding formalin (1% final concentration) and then incubated for 48 h at 37°C. Samples were washed once in PBS (0.01 *M* phosphate-buffered 0.85% NaCl; pH 7.2) and stored at 4°C in sterile PBS with 0.1%NaN_3. The vaccine consisted of 5×10^9 cfu/ml of Df in 500 mg/ml of dextran sulfate (molecular mass of 500,000; Sigma Chemical Co., St. Louis, MO) in saline.

At the end of lactation, six healthy, uninfected Jersey cows were treated with antibiotics and were immunized by injecting the vaccine in the area of the supramammary lymph node. Two booster injections were given in a similar manner at 15 and 30 d into the nonlactating period. Lacteal secretions were taken at drying off; at 15, 30, and 45 d into the nonlactating period; and at calving (colostrum).

Caprylic Acid Treatment of Lacteal Secretions

Secretions from the nonlactating glands and colostrum were treated with caprylic acid according to the method of Perosa et al. (21). Briefly, the sample was diluted (1:4, vol/vol) with acetate buffer (60 m*M*; pH 4.0), and the pH was adjusted to 4.5 with 1 *M* Tris base. Caprylic acid (30 μl/ml of diluted sample) was then added by drop with mixing. Samples treated with caprylic acid were mixed on a vortex shaker (Eppendorf, Hamburg, Germany) for 30 min at room temperature (22°C). After centrifugation for 30 min at 10,000 × *g*, the pH of the supernatant was adjusted to 7.4 with 1 *M* Tris base. The samples were refrigerated at 4°C until assayed.

ELISA for Specific Anticapsular Antibody

The Df large clearing, a mutant of Df that has a large, easily removable capsule and that has been shown to crossreact with Df, was used to prepare capsule for the ELISA (9). Approximately 10^{10} cfu of the Df mutant were mixed with PBS in a commercial Waring blender (Waring Products Corp., New York, NY) at 21,500 rpm for 15 min; the mixture was cooled with ice every 3 min. The organisms and cellular debris were removed via centrifugation at 44,000 × *g* for 30 min at 4°C. The supernatant, containing the capsule, was assayed for contamination by soluble cell-wall components using antisera to *S. aureus* cell wall in the ELISA.

The purified capsule was used as the coating antigen in a solid-phase ELISA. Two hundred microliters of polylysine (Sigma Chemical Co.) (0.1 mg/ml of PBS) were added to Immulon 2 round-bottom microtiter wells (Dynatech, Chantilly, VA) and incubated for 30 min at room temperature (22°C) in 100% humidity. Wells were washed four times with 0.85% NaCl and 0.05% Tween 20 (Sigma Chemical Co.) between each step of the ELISA using a plate washer (Skatron, Sterling, VA). The capsule preparation was diluted (1:500, vol/vol) in PBS, and 200 μl were added to each well. After incubation overnight, the wells were washed and blocked by the addition of 200 μl of gamma globulin-free horse serum diluted in PBS containing 0.05% Tween 20 and then incubated for 30 min. After washing, untreated lacteal samples and lacteal samples treated with caprylic acid and diluted in PBS and Tween 20 (IgG_1 and IgM, 1:1000; IgG_2, 1:100; and IgA, 1:500) were added and incubated for 2 h. After washing, rabbit anti-bovine IgG_1, IgG_2, IgA, or IgM was added and incubated for 2 h. After washing, alkaline phosphatase labeled sheep anti-rabbit Ig was added and incubated for 2 h. Substrate (*p*-nitrophenyl phosphate disodium; Sigma Chemical Co.) and 0.05 *M* carbonate-carbonate buffer (0.001 *M* $MgCl_2$; pH 9.8) was added. Plates were read at an optical density of 405 nm using a reference optical density of 620 nm on a Bio-Tek ELISA reader (Bio-Tek, Winooski, VT). Titers were expressed as absorbance values.

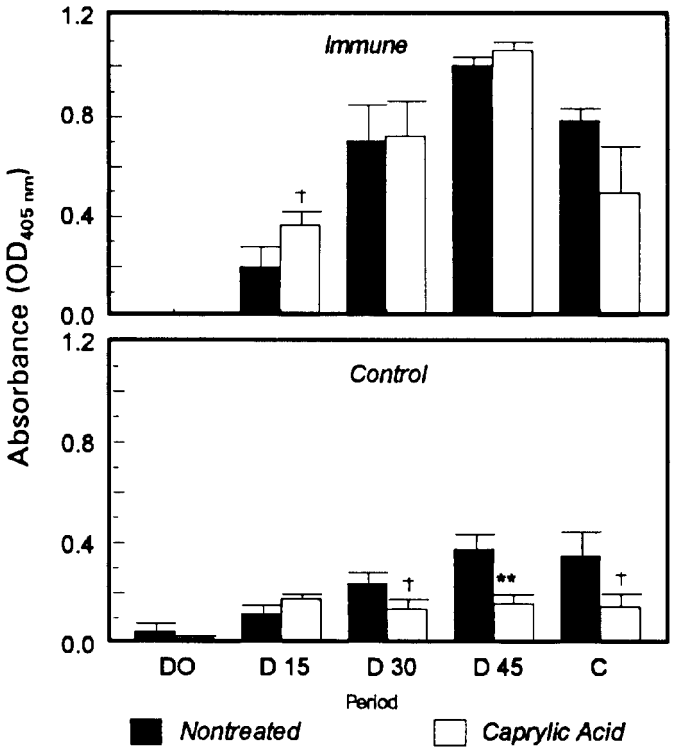

Figure 1. The mean (± SE) ELISA titers for IgG_1 in lacteal secretions taken at the end of lactation [drying off (D)]; at d 15, 30, and 45 into the nonlactating period; and at calving [colostrum (C)]. Control (n = 7); immunized (n = 6). $^{\dagger}P < 0.10$. $^{**}P < 0.01$. Absorbance values were measured at an optical density of 405 nm ($OD_{405\ nm}$).

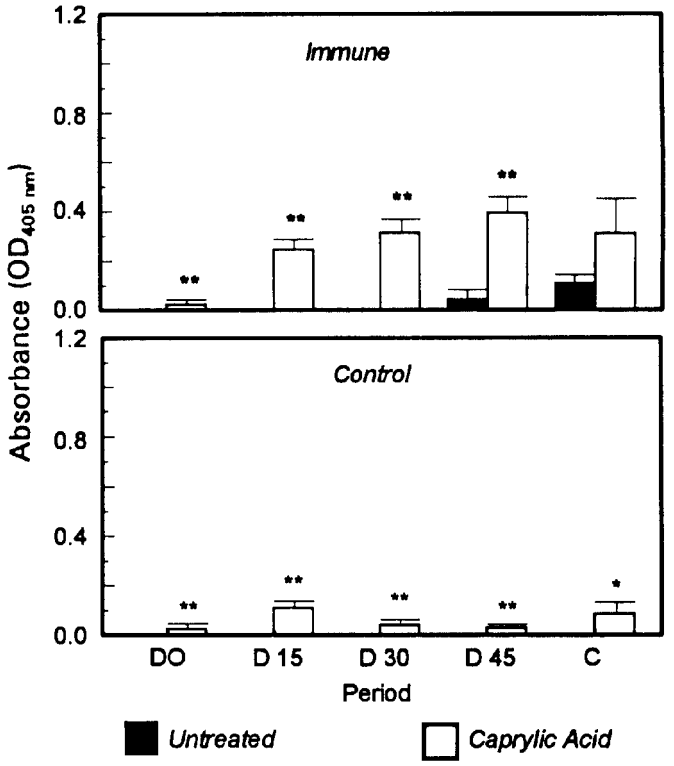

Figure 2. The mean (± SE) ELISA titers for IgG_2 in lacteal secretions taken at the end of lactation [drying off (D)]; at d 15, 30, and 45 into the nonlactating period; and at calving [colostrum (C)]. Control (n = 7); immunized (n = 6). $^{*}P < 0.05$. $^{**}P < 0.01$. Absorbance values were measured at an optical density of 405 nm ($OD_{405\ nm}$).

Statistics

Standard errors reported in the graphs were a measure of cow and assay variance for each sampling period. Student's *t* test was used to test the effect of treatment within a period.

RESULTS AND DISCUSSION

The caprylic acid clarification procedure of Perosa et al. (21) was adapted to nonlactating secretions from the mammary gland during the nonlactating period and to colostrum in an attempt to remove substances that interfered with specific antibody binding. The effect of acetate buffer was tested first (data not shown) and was found to have no effect on specific binding. Various concentrations of caprylic acid were tested to determine the most effective concentration in maximizing specific Ig titers in immune lacteal secretions. Figures 1 to 4 show ELISA values for IgG_1, IgG_2, IgA, and IgM in lacteal secretions before and after treatment with caprylic acid.

The increase in ELISA values for IgG_1, IgA, and IgM (Figures 1, 3, and 4) from cessation of lactation to d 45 into the nonlactating period in samples from unimmunized control cows was attributed to nonspecific binding because of increased concentration of these Ig during the nonlactating period (1, 11, 23, 24). Caprylic acid treatment of the control samples decreased the binding of all three isotypes to a uniform level. The resulting values were considered background for this assay. The increased binding of IgA at d 15 of the nonlactating period might have been due to the removal of inhibiting factors. No measurable IgG_2 binding occurred in unimmunized control secretions prior to treatment with caprylic acid, and only slight activity occurred after caprylic acid treatment (Figure 2).

A specific IgG_1, IgA, and IgM immune response was observed in samples from immunized cows before and after treatment of the samples with caprylic acid (Figures 1, 3, and 4). Following caprylic acid treatment, IgA values increased similar to IgA in un-

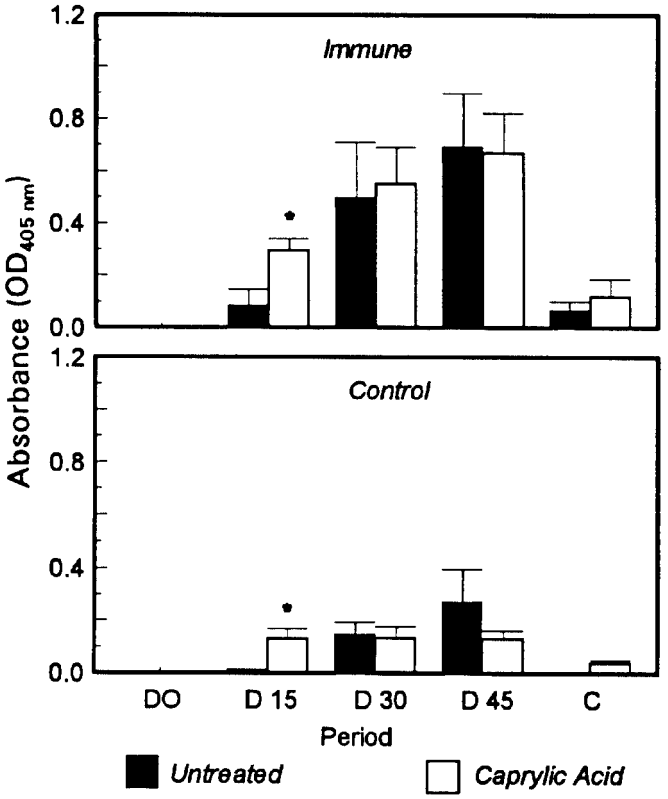

Figure 3. The mean (± SE) ELISA titers for IgA in lacteal secretions taken at the end of lactation [drying off (D)]; at d 15, 30, and 45 into the nonlactating period; and at calving [colostrum (C)]. Control (n = 7); immunized (n = 6). *$P < 0.05$. Absorbance values were measured at an optimal density of 405 nm ($OD_{405\ nm}$).

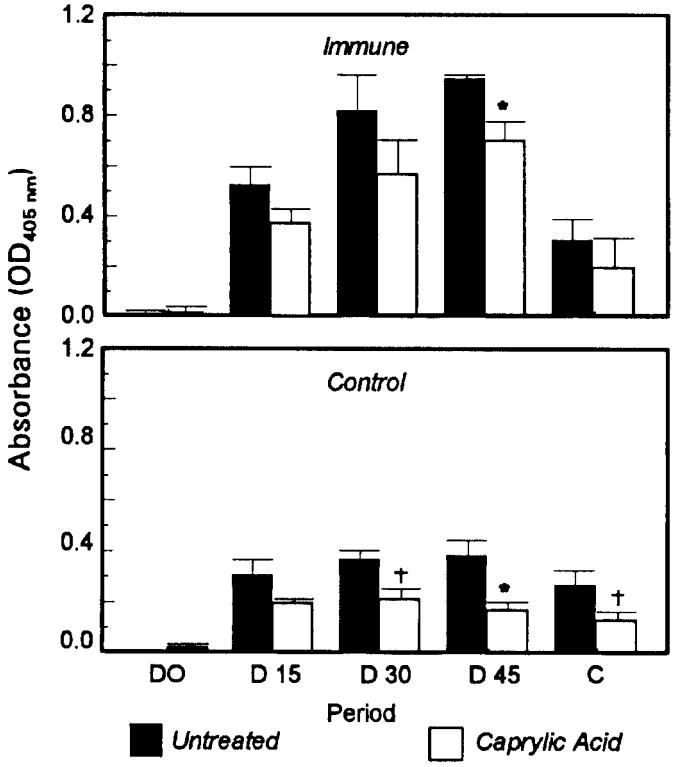

Figure 4. The mean (± SE) ELISA titers for IgM in lacteal secretions taken at end of lactation [drying off (D)]; at d 15, 30, and 45 into the nonlactating period; and at calving [colostrum (C)]. Control (n = 7); immunized (n = 6). †$P < 0.10$. *$P < 0.05$. Absorbance values were measured at an optical density of 405 nm ($OD_{405\ nm}$).

treated immunized samples. Caprylic acid tended to decrease binding of IgM, although significant only at d 45 of the nonlactating period. Prior to treatment with caprylic acid, an IgG_2 immune response was not evident until d 45 of the dry period. However, after treatment with caprylic acid, IgG_2 showed a significant response to immunization (Figure 2).

The factors affecting the increase in specific binding of IgG_2 could not be determined from this study, but a report by Butler (2) suggests that complexing IgG_2 with lactoferrin might be a contributing factor because of the high concentration of lactoferrin in secretions of the nonlactating gland (29). Others (25) found that complexing Ig with proteins in lacteal secretions interfered with antibody binding.

This study demonstrated that caprylic acid treatment gave a truer picture of specific antibodies that were present in nonlactating secretions. This procedure should help clarify the dynamics of the humoral immune system of the mammary gland during the nonlactating period.

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