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Concentrations of Ganglioside Type M1 and Immunoglobulin G in Colostrum Are Inversely Related to Bacterial Infection at Early Lactation in Cows

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ABSTRACT

The levels of IgG and ganglioside type M1 in the colostrum of cows and heifers were analyzed to examine their utility in predicting acquisition of intramammary infection (IMI) during the first weeks postpartum. In general, high levels of IgG and ganglioside type M1 in cows were associated with lower new incidence of IMI, and linear discriminate analysis based on these 2 variables yielded 69.4% successful classification into cows that did or did not acquire new IMI. This analysis was less successful in heifers because a high proportion of them joined the herd when already infected with bacteria in their udders. It is suggested that application of a wider range of measures that reflect the immune status would enable the identification of most cows prone to new IMI.
Key words: immunoglobulin, ganglioside, mastitis, dairy cow

INTRODUCTION

During the last weeks of pregnancy, colostrum is accumulated and stored in the lumen of the mammary gland. Colostrum is a mixture of mammary epithelial-cell secretions and organic components derived from the systemic fluid. The occurrence of blood components in colostrum relates to the leakiness of the tight junctions (**TJ**) of the epithelial cells of the alveolus during this period, and transepithelial transport of particular components, most notably Ig. Thus, the nutritional and immunity-boosting qualities of colostrum for the neonate are affected by the health and nutritional status of the dam (Quigley and Drewry, 1998; Goff, 2006). It is well established that colostrum Ig is essential for endowing the neonate with passive immunity against opportunistic infections until its own immune system matures (Miller et al., 1988; Watson, 1989; Leitner et al., 2000). Colostrum Ig is particularly rich in class G (IgG) and also contains IgM and IgA. Thus, a direct positive correlation between Ig concentration in colostrum and resistance of the young to infections has been shown in many cases (Morin et al., 1997; Jaster, 2005). Gangliosides (**GS**) are complex glycosphingolipids that occur in the neural membrane, plasma membrane, and body fluids (especially milk) of all vertebrates. The GS elicit immune responses in various cell types (Hakomori, 1993; Takamiya et al., 1998) by modulating specific receptors that bind bacteria, viruses, fungi, and bacterial toxins (Noda et al., 1980; Lee et al., 1996; Minke et al., 1999). In turn, activation of these receptors enhances phagocytosis (Yamaguchi et al., 1997) and prevents the attachment of pathogens (or their toxins) to the affected tissues. Thus, GS are additional colostrum components that provide the neonate with a nonspecific defense against infection. In milk and colostrum, GS are part of the milk fat globule membrane that surrounds milk fat droplets, which indicates that they are derived from the apical membranes of the mammary secretory cells (Keenan et al., 1972; Keenan, 1974). The concentration of GS in milk and colostrum varies with breed, climate, stage of lactation, and nutrition. Its concentration is greatest in colostrum, decreases during the first week postpartum, and increases in late-lactation milk (Puente et al., 1992; Colarow et al., 2003; Nakamura et al., 2003). The GS concentration in colostrum ranges from 7 to 10 mg/L (Puente et al., 1992; Bode et al., 2004) to 35 mg/kg (Martin et al., 2001). The main type of GS in cow's milk is GD3 (60 to 70%) followed by GM3 (20 to 25%); about 5% of GS comprises a mixture of GM1 and mono- and polysialogangliosides (Puente et al., 1992; Pan and Izumi, 2000; Martin et al., 2001). Ganglioside M1 is a natural ligand for many bacterial toxins such as the enterotoxins of *Vibrio cholerae* and *Escherichia coli* (Laegreid and Otnaess, 1987; Minke et al., 1999); it inhibits leucocidin, an endotoxin released by *Staphylococcus aureus* (Noda et al., 1980; Ozawa et al., 1994), and it serves as a binding site for the attach-

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ment of *E. coli* and *Pseudomonas aeruginosa* to cells (Lanne et al., 1995; de Bentzmann et al., 1996; Feldman et al., 1998).

In addition to conferring crucial immune protection on the neonate, the immune substances in the gland lumen also may be important for the protection of the gland itself against bacterial invasion, during the period in which it is particularly susceptible to such infection (Sordillo, 2005; Sordillo et al., 2007). Thus, although GM1 is a minor component of colostrum and milk, its ability to interact with bacteria and toxins that are widespread in mammary infections may be of specific importance for gland immunity. A similar consideration applies to the importance of IgG.

In the present study, we explored the hypothesis that the concentrations of IgG and GM1 in the colostrum of Holstein cows are important in preventing IMI during the first weeks postpartum. We determined IgG and GM1 concentrations during colostral secretion and associated them with acquisition of IMI during the first weeks postpartum, and examined whether these concentrations can be used as criteria for predicting the acquisition of IMI during this period.

MATERIALS AND METHODS

Animals

The study included 110 Israeli-Holstein cows from 1 herd: 38 dairy heifers and 72 pluriparous cows yielding, on average, approximately 12,000 L/yr; the study lasted a year. The cows were dried about 60 d before the next expected parturition and held separately from the heifers throughout parturition and lactation. During the 45 d before the end of lactation, the cows were tested 3 times at the quarter level for bacteriology and SCC. The colostrum of all cows and heifers were tested on the day of parturition, and the milk was tested at 10, 30, and 45 d postpartum for bacteriology and SCC. Infection was assessed by isolation of a single-strain bacterium accompanied by an increase in SCC. Aseptic measures were applied, and all colostrum samplings were taken from all quarters, and subsequent samplings from single quarters (Younis et al., 2000). Occurrences of clinical mastitis were recorded by the owner (edema, pain, lowered milk production, increased conductivity), and subclinical bacterial infection was identified by milk sample analysis. Bacteriological analysis was performed according to accepted standards of the National Mastitis Council (Oliver et al., 2004). A 0.01-mL aliquot was spread onto blood-agar plates (Bacto-Agar; Difco Laboratory, Becton, Dickinson and Co., Le Pont de Claix, France) containing 5% washed sheep red blood cells and onto MacConkey plates. Plates were incubated aerobically at 37°C and examined for growth after 18 and 42 h. Somatic cell count performed using a Fossomatic 360 (Foss Electric, Hillerød, Denmark) at the Israel Cattle Breeders' Association Laboratory (Caesarea, Israel). Total IgG and GM1 were determined in the mixed colostrum; that is, from all 4 quarters.

Antibody Levels (IgG)

The IgG levels were determined with ELISA using Bovine IgG, bovine reference serum standards, and sheep anti-bovine IgG-horseradish peroxidase (HRP) conjugate (all from Bethyl Laboratories, Montgomery, TX). Ninety-six-well immunoplates (Nunc, Kamstrup, Denmark) were loaded with sheep anti-bovine IgG-affinity purified antibody in carbonate-bicarbonate buffer (pH 9.6) at 100 µL/well (10 µg/well) and incubated for 1 h at 37°C. The plates were washed 3 times for 5 min each with PBS (pH 7.6) containing 0.5% Tween 20, and blocked with 3% casein, at 200 µL/well, for 20 min at room temperature. After washing the plates 3 times, 100-µL samples of colostrum at $1:10^6$ dilution were added in duplicate and the plates were incubated for 1 h at 37°C. To each plate, bovine reference serum standards at dilutions of 500 to 7.8 ng/mL were added in duplicate. The plates were washed 3 times, sheep anti-bovine IgG-HRP conjugate (at $1:10^4$ dilution) was added to each well, and the plates were incubated for 1 h at 37°C. After the plates had been washed, the bound antibodies were detected by adding 100 µL of 2.2′-azino-di-(3-ethyl-benzthiazolin sulfonate) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The respective plates were read in a model MR5000 microplate autoreader (Dynatech, Guernsey, UK) at 410 nm. The IgG levels were determined by comparison with the reference standards linear plot.

Ganglioside Type GM1

The GM1 levels in colostrum and milk were determined based on the concept of Laegreid et al. (1986), which was adapted for ELISA. Relative values of GM1 (**GM1-RV**) were determined as the reduction of binding of cholera toxin (**CT**) to PolySorb plates (Nunc) coated with GM1 by competition with the ß-chain of cholera toxin (**β-CT**, Sigma-Aldrich, St. Louis, MO). Ninety-six-well plates were loaded at 100 µL/well with GM1 (Sigma) in PBS at 100 ng/mL (pH 7.6), and incubated overnight at room temperature. The plates were washed 3 times for 5 min each with PBS that contained 0.5% Tween 20, and blocked with 1% BSA at 200 µL/well for 320 min at 37°C. The colostrum samples were diluted 1:10 with PBS and mixed in the tubes with per-

oxidase-conjugated ß-chain of ß-CT (Sigma), to reach a final concentration of 100 ng/mL, and incubated for 1 h at room temperature. Plates were washed 3 times for 5 min each, and 100 µL of the incubated colostrum samples was added to each plate in duplicate and incubated for 1 h at room temperature. Peroxidase-conjugated ß-CT at a final concentration of 100 ng/mL was added to each well. Peroxidase-conjugated ß-CT with no colostrum, which was considered as 100% bound, was added to 2 wells of each plate. After the plates had been washed 3 times for 5 min each, the bound antibodies were detected by adding 100 µL of ABTS to each well, and the optical intensity at 405 nm was determined in a microplate reader (Tecan GENios Plus, Männedorf, Switzerland). The GM1 relative values (CT neutralization activity) were calculated with the following formula:

$$\text{GM1} - \text{RV} = [1 - (\text{OD sample}/\text{OD control})] \times 100.$$

Statistical Analysis

The main analyzed parameters were total IgG (mg/mL) and GM1-RV. The differences between uninfected and infected udders in heifers and cows (second lactation and upward) were subjected to Student's *t*-test. The effects of udder infection and lactation (first compared with second and upward) on total IgG (mg/mL), GM1-RV, and SCC were determined by a 2-way ANOVA model in a random design mode.

The statistical model was:

$$Y_{ijk} = \mu + \alpha_i + ß_j + \alpha ß_{ij} + e_{ijk},$$

where μ = mean of all data, α_i = difference between the mean of udder infection i and the trial mean, $ß_j$ = difference between the mean of lactation j and the trial mean, $\alpha ß_{ij}$ = interaction between udder infection and lactation, and e_{ijk} = residual variance between measurements (random error).

Linear discrimination analysis was used to distinguish between the classification variable (uninfected or infected group) based on total IgG and GM1-RV levels and records of infection. Canonical functions are the linear combinations of the IgG and GM1 levels that maximize the separation between the uninfected and infected groups. The analysis was carried out with the JMP statistical software (SAS Institute, 2002), and it was found that:

$$\text{canonical 1} = (0.02 \times \text{IgG}) + (0.03 \times \text{GM1}),$$

whereas

$$\text{canonical 2} = (-0.02 \times \text{IgG}) + (0.04 \times \text{GM1}).$$

The Mahalanobis value, which is the distance from each point to the multivariate mean of each group, was calculated. This factor was used to classify the points within their group (uninfected or infected group) and was derived with the JMP statistical software (SAS Institute, 2002) from the correlation between the responses, taking into account their individual variances and the covariance between them.

RESULTS

Pre- and postpartum bacteriological tests of cows' udders revealed that most of the infections were new and occurred during dry-off, during the early phase of the new (current) lactation, or both. Milk yield and SCC at dry-off did not affect new infection rates (data not shown). Of the 72 tested cows, 40 (55.55%) were found free of bacteria, and their SCC during the first 45 d of the new lactation were <50,000 cells/mL. In 8 of the 35 cows identified as infected with bacteria in the current lactation, the infection was in the same udder quarter and with the same bacterium—mostly *Staphylococcus chromogenes*—as in the previous lactation; these cows were designated as chronically infected. Of the 27 newly infected cows, in which infection was detected in the current lactation, 13 were infected with *E. coli*, 5 with *Streptococcus dysgalactiae*, and 9 with *Staph. chromogenes*. In heifers, 9 (23.68% of the group) were found to be infected with *Staph. chromogenes* at early lactation and a single animal was found to be infected with *Streptococcus pyogenes*.

No associations were found between the species of the bacterial infection, the type of infection (chronic or new), and the levels of IgG or GM1 in colostrum. Therefore, further analyses focused on the interaction between bacterial infections per se and the levels of total IgG or GM1 in colostrum.

Antibody Levels (IgG)

The distribution of IgG concentration in the colostrum, with respect to the infection status (uninfected or infected udders) in heifers and cows is depicted in Table 1. The mean IgG level in the colostrum of heifers was not significantly ($P = 0.0904$) lower than that of the cows (Table 1). Infected quarters of heifers or cows had significantly ($P = 0.0011$) lower IgG levels than uninfected quarters.

Gangliosides

The distribution of GM1 concentration in the colostrum with respect to the infection status (uninfected or infected udders) in heifers and cows is depicted in

Table 1. The mean IgG levels of all combinations of udder infection and lactation, and the significance level (*P*-value) of the ANOVA main effects[1]

Group	Infected	Uninfected	*P*-value
Heifers	61.7 ± 3.6	85.2 ± 15.3	0.0011
Pluriparous cows	74.3 ± 9.7	103.7 ± 6.4	
P-value		0.0904	

[1]The interaction between these main effects was not significant ($P = 0.7400$).

Table 2. The mean GM1 in the colostrum of infected heifers was significantly ($P = 0.0035$) greater than that of the cows. This difference was not found in uninfected animals ($P = 0.4778$). Moreover, in the heifers, GM1 in infected udders was not significantly different than in uninfected ones (45.0 and 38.5, respectively), whereas in the cows, GM1 levels were significantly lower in the infected than in the uninfected quarters ($P = 0.0091$; Table 2).

Prediction of IMI Based on Linear Discriminated Analysis

The canonical plot (Figure 1) presents the discrimination between bacteria-free and infected cows based on their colostral IgG and GM1 contents. The ability to distinguish between the 2 groups of cows was successful in 69.4% of the cases; that is, only 22 out of 72 cows were misclassified.

DISCUSSION

General and Pluriparous vs. Heifer Responses

It is well established that accumulation of IgG in colostrum is an active process that occurs during late pregnancy, providing the neonate with essential passive immune protection. However, IgG is a major component of the immune response that protects the mammary gland itself against bacterial infection. Thus, it was shown that IgG levels in mammary secretions increased dramatically when cows were induced into drying-off and reached their peak levels as involution was established; that is, about 3 wk after the start of the involution, when mammary secretions became bactericidal against a range of pathogenic bacteria typically involved in mammary gland infections (Nickerson, 1989; Oliver and Sordillo, 1989). Furthermore, Shamay et al. (2003) showed that it was possible to accelerate the rate of IgG accumulation in mammary secretion by intramammary treatment with casein hydrolyzate, a substance containing powerful immunostimulants. This treatment induced the secretion of soluble components of the innate immune system as well as the influx of leukocytes from the systemic fluid.

In addition, it should be remembered that the TJ between the mammary gland lumen and the systemic fluid close only after the termination of colostrum evacuation and the start of lactogenesis. Because of the opening of the TJ during colostral secretion, the concentration distribution of components in samples of colostrum should reflect that in the systemic fluids at that time. Thus, sampling of colostral secretion represents an opportunity for noninvasive evaluation of the cows' immune status during a time window in which their immune system tends to be stressed by the pregnancy burden; a problem that might be exacerbated by inappropriate nutrition (Sordillo, 2005; Sordillo et al., 2007). However, in the present study all cows received similar high-quality food and management conditions including dry-off treatment of pluriparous cows. Therefore, it seems that these were not the major factors that could influence the levels of IgG and GM1 in the colostrum.

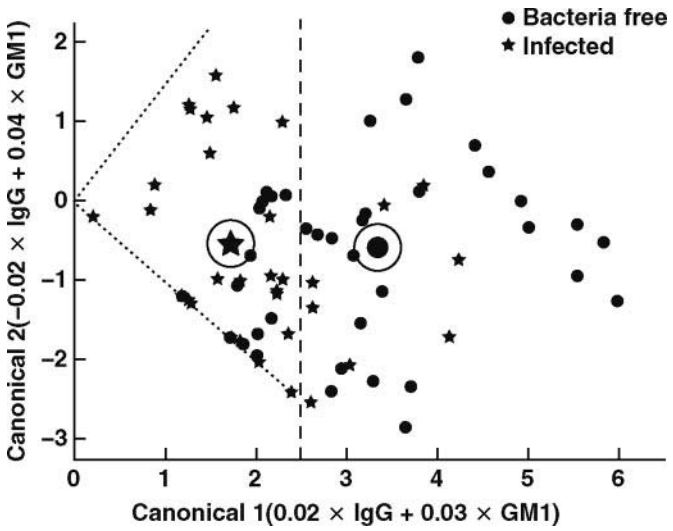

Figure 1. Canonical plot of 2-dimensional multivariate least squares means (dotted lines), with best separation between uninfected or infected group, indicated by a vertical dashed line. The multivariate means of the 2 groups are marked with circles corresponding to the 95% confidence region.

Table 2. The mean ganglioside M1 levels of all combinations of udder infection and lactation and the significance level (*P*-value) of the *t*-tests within each main effect, determined due to significant interaction between the ANOVA main effects

Group	Infected	Uninfected	*P*-value
Heifers	45.0 ± 7.4	38.5 ± 4.9	0.4691
Pluriparous cows	19.7 ± 4.3	34.2 ± 4.1	0.0091
P-value	0.0035	0.4778	

In this study, primiparous cows were confirmed to have greater levels of GM1 than pluriparous cows, in agreement with the results of Piccinini et al. (2007), which showed the same pattern for other components of the soluble innate components in milk. This made the prediction of susceptibility to mastitis based on measurements of immune components in colostrum less reliable in the case of heifers. In primiparous cows and goats, a high prevalence of IMI was reported, with indications that it occurred before the first lactation (Fox et al., 1995; Nickerson et al., 1995; Leitner et al., 2007). Thus, new infections in heifers might not be related to immune status during the transition period; that is, the period between immediate prepartum and immediate postpartum, which would probably account for the differences noted above between cows and heifers.

The difference found between heifers and cows in the absolute levels of IgG is well documented and explained by the maturation of the udder tissue and longer exposure of mature cows to pathogens. However, the lack of similar differences (in infection) in heifers can be explained by the above-described differences at the moment when udder infection occurred. Thus, in heifers, the infection probably occurred more than a month before parturition and therefore is not related to normal accumulation of IgG in a healthy animal during that period. In contrast, the pluriparous cows entered the dry-off period with or without infected udders and all were treated with antibiotic at that time. Thus, all the new IMI in pluriparous cows must have occurred during the dry-off period or close to parturition, suggesting that cows with potent immune systems resisted new IMI, whereas those with physiological or health conditions, which result in lower IgG, were susceptible to new IMI. Analogical, but with more dramatic differences between heifers and pluriparous cows, responses were found in respect to GM1: the absolute levels of GM1 in heifers were greater than in the cows, but were not associated with infection; thus, all heifers were at parturition in good health and irrespective to IMI status (i.e., infected or uninfected). In contrast, in pluriparous cows, most of the infected cows had a significantly lower GM1 compared with the uninfected ones, reinforcing the above-stated conclusion.

IgG and GM1 as Predictors of IMI in Pluriparous Cows

Tables 1 and 2 show wide differences in the levels of IgG and GM1 between heifers and pluriparous cows and, within each age group, between infected and uninfected animals. Moreover, high variability was found within each group, which reflects effects of additional factors that might affect the level of immune components in colostrum, such as age, days in milk, season, temperature, and length of day. Thus, the present results suggest that when SCC is low (i.e., as with most of the cows in the present study that acquired new infections), the general innate immune status of the cows (GM1-RV) and the levels of soluble components of the gland acquired immune system (IgG) are very important elements of gland defense. Several other studies support the concept that soluble components of the immune system are, at some stages, equally or more important than the cellular innate components (i.e., polymorphonuclear cells) in preventing new infections (Cross and Gill, 2000; Piccinini et al., 2007; Sordillo et al., 2007). Posttranscriptional regulation of milk xanthine oxidase by transepithelial secretion of its substrates (xanthine or hypoxanthine) provides passive protection to the alveolar lumen of cows through hydrogen peroxide formation coupled to conversion of purine to urate (Silanikove et al., 2007), which is a further example of a noncellular mode of immune defense.

Here we show that, by combining 2 criteria (GM1-RV and total IgG) we attained approximately 70% accuracy in predicting subsequent infection of cows. This result raises the hope that examining a broader selection of indicators of immunity that reflects the systemic status would enable reliable identification of most of the cows in a given herd that are prone to mastitis in early lactation. Such indicators may include additional GS, acute phase proteins, proinflammatory cytokines such as tumor necrosis factor-α, and soluble components of the innate immune system such as lactoferrin (Nickerson, 1989; Oliver and Sordillo, 1989) and albumin (Shamay et al., 2005). This seems to be a feasible goal given the wide range of appropriate high-throughput analytical techniques available today. In turn, such an approach might provide dairy cow breeders with a valuable management tool to diminish the economic burden posed by new infections in early lactation by applying preventive treatments to cows that are prone to new infections and by selecting for more-resistant cows.

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