Efficacy of polyethylene glycol–conjugated bovine granulocyte colony-stimulating factor for reducing the incidence of naturally occurring clinical mastitis in periparturient dairy cows and heifers

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OBJECTIVE
To evaluate effects of various doses of polyethylene glycol (PEG)–conjugated bovine granulocyte colony-stimulating factor (bG-CSF) on the incidence of naturally occurring clinical mastitis in periparturient dairy cattle.

ANIMALS
211 periparturient Holstein cows and heifers.

PROCEDURES
Approximately 7 days before the anticipated date of parturition (day of parturition = day 0), healthy cattle received SC injections of sterile saline (0.9% NaCl) solution (control treatment) or PEG–bG-CSF at 5, 10, or 20 μg/kg. Cattle were commingled and housed in a pen with dirt flooring, which was kept wet to maximize the incidence of naturally occurring clinical mastitis. Within 24 hours after parturition, each animal again received the assigned treatment. Mammary glands and milk were visually scored for abnormalities twice daily for 28 days after parturition. Milk samples were aseptically collected from mammary glands with an abnormal appearance or abnormal milk and submitted for microbial culture. Daily milk production was recorded, and milk composition was assessed on days 3, 5, 7, and 10.

RESULTS
Cattle treated with PEG–bG-CSF at 10 and 20 μg/kg had significantly fewer cases of clinical mastitis (9/54 and 5/53, respectively), compared with control cattle (18/53). Administration of PEG–bG-CSF did not significantly affect daily milk production or milk composition.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that PEG–bG-CSF was effective for reducing the incidence of naturally occurring clinical mastitis in periparturient dairy cattle. Further investigations of the use of PEG–bG–CSF as a potential preventative intervention should be conducted. (*Am J Vet Res* 2015;76:231–238)

Despite decades of research and the use of multiple methods for controlling bacterial infections, including antiseptic teat dips, teat sealants, improvements in milking equipment and animal housing, vaccines against specific pathogens, and new antimicrobial preparations for use during and at the end of lactation, mastitis remains a major source of economic losses for dairy producers globally. Animal genetics and husbandry practices can substantially affect mammary gland immunity and susceptibility to intramammary infection.[1]

Beyond the anatomic barriers in the teats, protection of mammary glands from microbial invasion requires a complex and coordinated interaction between both the innate and acquired immune responses. Cattle have periods of increased susceptibility to clinical mastitis during the periparturient period beginning 1 to 2 weeks before parturition and continuing for up to 3 weeks after calving.[2] Neutrophils play a key role in defense during intramammary infections and are the primary cell type in mammary glands at the onset of an infection.[3] Impairment of immune responses during the periparturient period has been well characterized.[4] Investigators in 1 study[5] found that neutrophil functions are impaired in cows during the periparturient period. Results of that study[5] indicate bovine neutrophils have decreases in oxidative burst and myeloperoxidase-H_2O_2-halide activity, both of which play important roles in neutrophil-mediated antimicrobial activity.

Granulocyte colony-stimulating factor is an endogenous hematopoietic growth factor that stimu-

ABBREVIATIONS

bG-CSF	Bovine granulocyte colony-stimulating factor
CMT	California mastitis test
G-CSF	Granulocyte-stimulating factor
PEG	Polyethylene glycol
SCC	Somatic cell count

lates the production and differentiation of neutrophils by progenitor cells located in the bone marrow.[6,7] Recombinant human G-CSF is used to increase circulating neutrophil numbers in patients undergoing myelosuppressive chemotherapy for cancer. Recombinant bG-CSF has activities similar to those of human G-CSF, and daily administration of bG-CSF to periparturient cows induces pronounced neutrophilia and increased phagocytic and cytotoxic activity of neutrophils.[8,9]

The pharmacodynamic response to native-sequence bG-CSF as determined by changes in absolute neutrophil counts indicates that daily administration of the protein would be required for therapeutic benefit. In modern dairy operations, husbandry practices used to manage periparturient cattle during the transition period are not consistent with administering daily injections for several weeks to restore protective neutrophil function throughout the periparturient period. Modifying native proteins by covalent binding of water-soluble polymers such as PEG extends the duration of activity by increasing the hemodynamic volume of the protein, reducing first-pass renal clearance, and reducing proteolytic degradation.[10] A single dose of PEG–human G-CSF can provide efficacy equivalent to that after 11 daily doses of native human G-CSF.[11]

A variant of PEG–bG-CSF was found[12] to be capable of increasing absolute neutrophil counts for approximately 7 to 10 days following a single SC injection. Administration of PEG–bG-CSF to periparturient dairy cattle approximately 7 days prior to the anticipated date of parturition and within 24 hours after calving enhances the myeloperoxidase-H_2O_2-halide activity of neutrophils, which is depressed during the periparturient period.[12] On the basis of these results, the authors hypothesized that periparturient cattle receiving PEG–bG-CSF would have improved resistance to clinical mastitis. The objective of the study reported here was to evaluate the effects of various doses of PEG–bG-CSF on the incidence of naturally occurring clinical mastitis in periparturient dairy cattle.

Materials and Methods

ANIMALS

Pregnant Holstein cows and heifers (n = 211) from a commercial dairy herd located in Hanford, California, were enrolled in the study, which was conducted at that dairy. Approximately 25% of the cattle were heifers and 75% were multiparous (second and third lactation) cows. Each animal was enrolled as it approached the anticipated date of parturition (day of parturition = day 0). On day −14, pregnant cattle were moved to a designated pen, and a physical examination that included evaluation of all major body systems, respiratory rate, heart rate, and rectal temperature was conducted. The physical examination was repeated on day −7. Cattle that were clinically normal on days −14 and −7 were eligible for enrollment in the study.

Owners provided informed consent for participation of the cattle in the study. The study protocol was reviewed by the Elanco Animal Health Institutional Review Board to ensure compliance with applicable guidelines for conduct of research on animals. Edible products from cattle treated with PEG–bG-CSF were discarded for 7 days after the last dose of PEG–bG-CSF in compliance with a slaughter authorization issued by the US FDA.

PREPARATION OF PEG–BG-CSF

Bovine G-CSF was cloned and expressed in *Escherichia coli* by use of recombinant technology. Expressed protein was isolated from inclusion bodies and refolded. It was then chromatographically purified and covalently bound to a 20-kDa activated PEG at position T133, in accordance with the methods of Cho et al,[13] to yield mono-PEG–bG-CSF. The protein was formulated at a concentration of 7.83 mg/mL in 10mM sodium acetate buffer (pH, 4.0), sorbitol (50 mg/mL), and polysorbate 20 (0.033 mg/mL). Sterile saline (0.9% NaCl) solution was used as a negative control treatment. The PEG–bG-CSF was stored frozen at −70°C until use. Saline solution was stored under ambient conditions until use.

ANIMAL HUSBANDRY

Cattle were housed as a group in a drylot pen with partial shade and dirt floors prior to parturition. After they had calved, cattle were moved to another drylot pen with partial shade and a dirt floor, which was kept wet to facilitate exposure of the mammary glands to mastitis-inducing pathogens. On days when there was no precipitation, water was added to the dirt with an oscillating sprinkler placed on the pen floor. The surface was maintained damp but without freestanding puddles of water.

Cows were milked twice daily in a herringbone parlor with automated milking equipment. Premilking and postmilking teat dipping were performed with an iodine disinfectant. Cattle for which parturition was imminent were fed a total mixed ration composed of alfalfa, corn, straw, distiller's grains, minerals, corn gluten, and corn silage prior to parturition. After calving, cattle were fed a total mixed ration composed of alfalfa, barley, corn, canola, wet distiller's grain, and corn silage. Both rations met National Research Council nutrient requirements for dairy cattle. Cattle had ad libitum access to water through automatic waterers located in the pens.

Cows were vaccinated against *E coli* with a J5 bacterin at the end of lactation and approximately 30 days prior to the subsequent parturition. They also were treated with an antimicrobial[a] (penicillin G procaine and dihydrostreptomycin) at the end of lactation.

STUDY DESIGN

The study was conducted as a completely randomized design. No blocking factors were used. Cattle were assigned to each of 4 treatment groups. Cattle

in the 4 treatment groups received SC injections of PEG–bG-CSF at a dose of 5 µg/kg (n = 51 [38 cows and 13 heifers]), 10 µg/kg (54 [41 cows and 13 heifers]), or 20 µg/kg (53 [40 cows and 13 heifers]) or saline solution (control treatment) at a volume equal to that used for the 20 µg/kg dose of PEG–bG-CSF (53 [39 cows and 14 heifers]); treatments were administered on day –7 and within 24 hours after parturition (day 0). Separate randomization tables containing a list of treatment groups were used for heifers and multiparous cows to ensure equal proportions of heifers and multiparous cows were included in each group. Cattle were randomly assigned to groups on the basis of the sequence in the randomization tables on day –7. Animals that calved < 24 hours or > 10 days after receiving the first dose were removed from the study and replaced with another animal of the same parity assigned to the same treatment.

TREATMENT ADMINISTRATION

Cattle were weighed on day –7 to enable calculation of individual doses, which were administered on days –7 and 0. The PEG–bG-CSF was thawed immediately before administration, and the solution was mixed by gentle inversion of the vials. A syringe and 18-gauge, 1-inch needle were used to administer the appropriate dose of PEG–bG-CSF or saline solution by SC injection in the prescapular region of the neck of each animal.

ASSESSMENT OF ABSOLUTE NEUTROPHIL COUNT

In a previous study,[12] treatment of cattle with PEG–bG-CSF induced significant increases in absolute neutrophil counts. Therefore, blood samples were collected by jugular venipuncture immediately before treatment administration on day –7 and again on day –6 (approx 24 hours after treatment administration). Additional blood samples were collected from all cattle on day 7.

Blood samples were analyzed with an automated cell counter,[b] and light microscopy was used for WBC differentiation. Automated cell counts and results for light microscopy were used to calculate the absolute neutrophil count of each sample. Both segmented neutrophils and immature neutrophils (eg, band cells, myelocytes, and metamyelocytes) were included in calculations of absolute neutrophil counts.

ASSESSMENT OF MASTITIS

The clinical status of each mammary gland was recorded at the morning and evening milking on days 1 to 28 by the milker, who was unaware of the treatment assignments for each animal. Clinical score was assigned by use of a 5-point scale as follows: 1 = visually or palpably normal mammary gland and visually normal milk, 2 = visually or palpably normal mammary gland but visually questionable milk, 3 = visually or palpably normal mammary gland but visually abnormal milk, 4 = visually or palpably abnormal mammary gland and visually abnormal milk, and 5 = visually or palpably abnormal mammary gland, visually abnormal milk, and evidence of systemic inflammation. When an animal had a clinical score ≥ 2, a CMT (scores of negative, trace, 1, 2, and 3) was performed on milk from the affected mammary gland. When the CMT result was greater than trace, duplicate milk samples from the affected mammary gland were obtained and the rectal temperature of the animal was determined. Cattle with a rectal temperature ≥ 40°C were considered to have systemic inflammation (clinical score, 5).

A clinical score ≥ 2 and CMT score greater than trace at any of the evaluations were considered indicative of clinical mastitis. Each new case of clinical mastitis was recorded as a separate event. A new case of clinical mastitis was defined as 2 mastitic episodes separated by an evaluation that yielded a clinical score of 1 (ie, visually or palpably normal mammary gland and visually normal milk).

BACTERIOLOGIC EVALUATION

Milk samples were aseptically collected from the affected mammary glands of cattle with signs of clinical mastitis and used for identification of microbial pathogens. Samples were collected at the time clinical mastitis criteria were met; samples were frozen at –70°C and stored at the study site. After all cattle enrolled in the study had reached 28 days of lactation, frozen milk samples were sent to a diagnostic laboratory[c] for analysis. Each sample was cultured on blood agar and *Mycoplasma* direct plates. Biochemical tests were used to identify the genus and species of each isolate.

MILK PRODUCTION AND COMPOSITION

To assess the effect of treatment on milk yield, milk production was recorded at each milking for cattle with no morbidity events or for cattle up to the time of their clinical morbidity event (ie, healthy animals only) on days 1 to 28. Mean daily milk yield was determined for each group for the first 28 days of lactation.

To assess the effect of treatment on SCC and milk composition, composite milk samples were collected from all healthy mammary glands at the morning milking on days 3, 5, 7, and 10. Samples were sent to the Tulare County Dairy Herd Improvement Association Laboratory for analysis of SCC and concentrations of fat, lactose, protein, and milk solids.

DURATION OF GESTATION AND PERCENTAGE OF LIVE BIRTHS

To determine whether administration of PEG–bG-CSF had an effect on the duration of gestation, the number of days between the first dose (estimated day –7) and the day of parturition (day 0) was determined for each animal, and mean values were calculated. The total number of calves born alive for each treatment group was tabulated and compared to determine whether PEG–bG-CSF had detrimental effects on the viability of calves in utero.

STATISTICAL ANALYSIS

Statistical analysis was performed with the aid of commercial software.[d] For all analyses, values of $P \leq 0.05$ were considered significant.

The primary efficacy criterion was the failure rate as measured by the incidence of clinical mastitis events. Clinical mastitis incidence rates were evaluated 2 ways. First, an animal was denoted as clinically affected if 1 or more mammary glands met the criteria for clinical mastitis (clinical score ≥ 2 and CMT score greater than trace) at any evaluation time during the first 28 days of lactation. A generalized mixed model for binomial data was used to compare the failure rates for the control treatment with the failure rates for each PEG–bG-CSF treatment. Parity (heifers vs multiparous cows) and treatment were fixed effects. If the parity-by-treatment interaction was not significant, treatments were compared across both levels of parity. If the parity-by-treatment interaction was significant, treatments were compared within each of the 2 levels of parity. A generalized mixed model for binomial data was used to model the relationship among treatment failure rates. If the parity-by-treatment interaction was not significant, dose response was compared across both levels of parity. If the parity-by-treatment interaction was significant, the dose response was evaluated for each of the 2 levels of parity. Second, the total number of clinical mastitis events per group was evaluated. Clinical mastitis events that occurred at any time during the study were determined (eg, if an animal had > 1 clinical mastitis event or clinical mastitis in > 1 mammary gland). The resulting data were expressed as count data and were analyzed by the method of analysis described for the clinical mastitis incidence data.

Duration of abnormal clinical status scores was determined for each morbidity event for each animal. Depending on the distribution of the data, an ANOVA or an appropriate nonparametric method was used for analysis and to compare treatment means (or medians) with the control mean (or median). A 2-way analysis was performed to evaluate the parity-by-treatment interaction. If the parity-by-treatment interaction was not significant, treatments were compared across both levels of parity. If the parity-by-treatment interaction was significant, the treatments were compared within each of the 2 levels of parity.

Secondary efficacy variables (milk yield, milk composition, and absolute neutrophil count) that were continuous in nature were analyzed by use of an ANOVA or an appropriate nonparametric method for each time point. The treatment-by-parity interaction was compared within each time point. Secondary efficacy variables that were qualitative (eg, clinical scores) were analyzed by use of the Cochran–Mantel-Haenszel row mean test at each time point, whereby results for the control group were compared with results for each treatment group, both across and within both levels of parity.

Mean SCC and concentrations of fat, protein, lactose, and milk solids were determined on days 3, 5, 7, and 10 at the morning milking for each group. Repeated-measures ANOVA was used, whereby parity, treatment, and day were fixed effects and animal nested within treatment by parity was considered a random effect. The possible covariance structures evaluated were compound symmetry, compound symmetry heterogeneous variances, autoregressive, autoregressive heterogeneous variances, and unstructured. The minimum Akaike information criterion was used to select the covariance structure. The least squares mean was used to compare the effects of PEG–bG-CSF with those of the control treatment on the basis of unadjusted *P* values. When compound symmetry, compound symmetry heterogeneous variances, and unstructured were used as covariance structures, the random effect of cattle nested within treatment by parity was eliminated from the model specification. Repeated-measures ANOVA identical to that described for milk composition was used for analysis of total daily milk yield.

Repeated-measures ANOVA was used for analysis of absolute neutrophil counts, with particular interest in the means for treatment-by-day interactions. These means provided information on the effect of exposure to PEG–bG-CSF.

Results

ABSOLUTE NEUTROPHIL COUNTS

Saline solution–treated cattle had relatively constant absolute neutrophil counts across the time points **(Figure 1)**. In contrast, cattle treated with PEG–bG-CSF had marked increases in absolute neutrophil counts within 24 hours after administration. Absolute neutrophil counts remained elevated, relative to those in control cattle, 7 days after the second dose

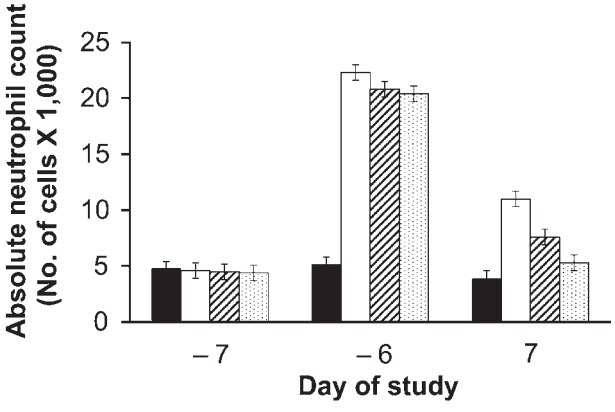

Figure 1—Mean ± SEM absolute neutrophil count for 211 periparturient Holstein cows and heifers injected SC with PEG–bG-CSF at a dose of 5 µg/kg (stippled bars; n = 51 [38 cows and 13 heifers]), 10 µg/kg (diagonal-striped bars; 54 [41 cows and 13 heifers]), or 20 µg/kg (white bars; 53 [40 cows and 13 heifers]) or saline (0.9% NaCl) solution (control treatment) at a volume equal to that used for the 20 µg/kg dose of PEG–bG-CSF (black bars; 53 [39 cows and 14 heifers]). Cattle received 2 injections (the first on day –7 and the second within 24 hours after parturition). Day of parturition was designated as day 0.

Table 1—Results of statistical analyses (*P* value) of absolute neutrophil count in 211 periparturient Holstein cows and heifers injected SC with PEG–bG-CSF at a dose of 5 µg/kg (n = 51 [38 cows and 13 heifers]), 10 µg/kg (54 [41 cows and 13 heifers]), or 20 µg/kg (53 [40 cows and 13 heifers]) or saline (0.9% NaCl) solution (control treatment) at a volume equal to that used for the 20 µg/kg dose of PEG–bG-CSF (53 [39 cows and 14 heifers]).

	Day of study		
Comparison	**–7**	**–6**	**7**
Saline solution vs 5 µg/kg	0.685	< 0.001	0.149
Saline solution vs 10 µg/kg	0.737	< 0.001	< 0.001
Saline solution vs 20 µg/kg	0.887	< 0.001	< 0.001
5 µg/kg vs 10 µg/kg	0.944	0.665	0.017
5 µg/kg vs 20 µg/kg	0.794	0.042	< 0.001
10 µg/kg vs 20 µg/kg	0.848	0.108	< 0.001

Values were considered significant at $P \leq 0.05$.

Cattle received 2 injections (the first on day –7 and the second within 24 hours after parturition). Day of parturition was designated as day 0.

Table 2—Incidence of clinical mastitis in cattle treated with saline solution or various doses of PEG–bG-CSF.

Group	**No. (%) of cattle with clinical mastitis**
Saline solution (n = 53)	18 (34)
5 µg/kg (n = 51)	10 (19.6)
10 µg/kg (n = 54)	9 (16.7)*
20 µg/kg (n = 53)	5 (9.4)†

,†Value differs significantly ($P = 0.044$; †$P = 0.004$) from the value for saline solution.

Table 3—Incidence of clinical mastitis in mammary glands of cattle treated with saline solution or various doses of PEG–bG-CSF.

Group	**No. of mammary glands with clinical mastitis**	**No. of cattle with multiple clinical mastitis events***
Saline solution	22	4
5 µg/kg	10†	0
10 µg/kg	10‡	1
20 µg/kg	7§	2

*Cattle had > 1 mammary gland with clinical mastitis or cattle had > 1 episode of clinical mastitis. †,‡,§Value differs significantly (†$P = 0.035$; ‡$P = 0.020$; §$P = 0.003$) from the value for saline solution.

of PEG–bG-CSF at 10 and 20 µg/kg but not at 5 µg/kg. Results of statistical analysis of group comparisons were summarized **(Table 1)**.

CLINICAL MASTITIS INCIDENCE

The proportion of cattle in each group with signs of clinical mastitis in at least 1 mammary gland was summarized **(Table 2)**. Approximately one-third of the cattle receiving saline solution developed clinical mastitis during the first 28 days of lactation. Cattle treated with PEG–bG-CSF at 10 or 20 µg/kg had significant reductions in the incidence of clinical mastitis, relative to the incidence for the control group. Cattle treated with PEG–bG-CSF at 5 µg/kg had a nonsignificant reduction in the incidence of clinical mastitis. There were no significant differences in clinical mastitis rates among the PEG–bG-CSF treatment groups. Similarly, the treat-

Table 4—Severity of clinical mastitis at the time of diagnosis and duration of clinical mastitis for cattle treated with saline solution or various doses of PEG–bG-CSF.

	Clinical score at diagnosis				
Group	**2**	**3**	**4**	**5**	**Mean ± SEM duration (d)**
Saline solution	1	21	0	0	10.8 ± 1.4
5 µg/kg	1	8	0	1	5.3 ± 2.1
10 µg/kg	0	8	0	2	8.4 ± 2.1
20 µg/kg	0	7	0	0	6.9 ± 2.5

Clinical score was assigned by use of a 5-point scale as follows: 1 = visually or palpably normal mammary gland and visually normal milk, 2 = visually or palpably normal mammary gland but visually questionable milk, 3 = visually or palpably normal mammary gland but visually abnormal milk, 4 = visually or palpably abnormal mammary gland and visually abnormal milk, and 5 = visually or palpably abnormal mammary gland, visually abnormal milk, and evidence of systemic inflammation.

Table 5—Frequency distribution of bacterial isolates cultured from milk samples collected from individual mammary glands with clinical signs of mastitis for cattle treated with saline solution or various doses of PEG–bG-CSF.

Bacterial isolate	**Saline solution**	**5 µg/kg**	**10 µg/kg**	**20 µg/kg**
Streptococcus uberis	5	2	5	5
Streptococcus dysgalactiae	1	0	1	1
Streptococcus acidominimus	0	0	1	0
Aerococcus urinae	1	0	0	0
Aerococcus viridans	0	1	1	0
Granulicatella adiacens	0	0	0	1
Enterococcus durans	1	1	0	0
Lactococcus lactis	0	0	1	0
Escherichia coli	2	0	1	0
Staphylococcus xylosus	2	1	0	0
Staphylococcus hyicus	2	0	1	0
Staphylococcus chromogenes	0	0	1	2
Staphylococcus lentis	0	1	0	0
No growth	8	4	2	1

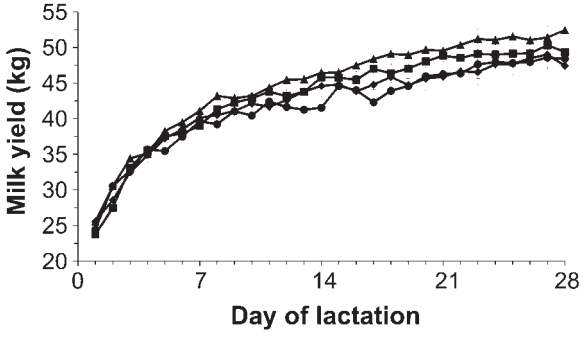

Figure 2—Effect of administration of PEG–bG-CSF at a dose of 5 µg/kg (circles; n range = 41 to 51), 10 µg/kg (triangles; n range = 45 to 54), or 20 µg/kg (squares; n range = 48 to 53) or saline solution (diamonds; n range = 35 to 53) on mean ± SEM daily milk production by healthy cattle during the first 28 days of lactation. Cattle received 2 injections (day –7 and within 24 hours after parturition). Day of parturition was designated as day 0.

Table 6—Effects of PEG–bG-CSF on SCC and milk composition for healthy cattle treated with saline solution or various doses of PEG–bovine G-CSF.

Variable	Day	Saline solution	5 μg/kg	10 μg/kg	20 μg/kg
SCC (X 10^3 cells/mL)	3	989 ± 313	668 ± 144	858 ± 183	1,212 ± 288
	5	987 ± 337	524 ± 165	394 ± 115	364 ± 108
	7	415 ± 178	317 ± 105	160 ± 44	481 ± 155
	10	415 ± 183	164 ± 38	115 ± 30	172 ± 56
Fat (%)	3	1.97 ± 0.18	2.15 ± 0.18	2.15 ± 0.28	2.73 ± 0.31
	5	1.95 ± 0.20	2.16 ± 0.29	2.16 ± 0.20	2.23 ± 0.32
	7	1.32 ± 0.11	1.64 ± 0.32	1.58 ± 0.12	1.67 ± 0.22
	10	1.06 ± 0.09	1.56 ± 0.24	1.36 ± 0.18	1.18 ± 0.09
Lactose (%)	3	4.17 ± 0.13	4.41 ± 0.06	4.39 ± 0.07	4.30 ± 0.08
	5	4.43 ± 0.09	4.59 ± 0.06	4.56 ± 0.07	4.54 ± 0.07
	7	4.65 ± 0.06	4.73 ± 0.06	4.76 ± 0.04	4.72 ± 0.05
	10	4.79 ± 0.06	4.82 ± 0.05	4.90 ± 0.04	4.87 ± 0.04
Protein (%)	3	4.51 ± 0.08	4.55 ± 0.06	4.61 ± 0.16	4.59 ± 0.08
	5	4.13 ± 0.05	4.02 ± 0.06	4.13 ± 0.07	4.12 ± 0.05
	7	3.78 ± 0.05	3.76 ± 0.05	3.81 ± 0.05	3.81 ± 0.05
	10	3.50 ± 0.04	3.58 ± 0.05	3.52 ±0.04	3.50 ± 0.04
Milk solids (%)	3	9.50 ± 0.14	9.81 ± 0.08	9.82 ± 0.15	9.71 ± 0.11
	5	9.41 ± 0.11	9.48 ± 0.10	9.55 ± 0.10	9.51 ± 0.09
	7	9.30 ± 0.09	9.36 ± 0.10	9.44 ± 0.07	9.41 ± 0.09
	10	9.16 ± 0.09	9.28 ± 0.09	9.31 ± 0.07	9.27 ± 0.07

Values reported are mean ± SEM. Day of parturition was designated as day 0.

ment-by-parity interaction was not significant, which suggested that multiparous cows and heifers had similar responses to treatment.

The number of mammary glands with clinical mastitis in the various groups was summarized **(Table 3)**. Cattle in all 3 PEG–bG-CSF treatment groups had significant decreases in the number of affected mammary glands and a nonsignificant reduction in the proportion of cattle with multiple infected mammary glands, relative to results for the control group. There were no significant differences in clinical mastitis rates among the PEG–bG-CSF treatment groups. Similarly, the treatment-by-parity interaction was not significant, which suggested that multiparous cows and heifers had similar responses to treatment.

Relative severity of each case of clinical mastitis at the time of diagnosis was assessed on the basis of clinical score **(Table 4)**. The distribution of clinical scores was similar among groups, and most affected mammary glands had a clinical score of 3 at the time of clinical mastitis diagnosis. The effect of treatments on the duration of abnormal milk or mammary glands for each clinical mastitis case was summarized. Mean duration of clinical mastitis episodes did not differ significantly among groups.

MICROBIOLOGICAL ANALYSIS

Fifty-one milk samples collected from individual mammary glands with clinical signs of mastitis were evaluated to identify bacteria associated with each infection **(Table 5)**. Results of the microbiological analyses indicated that the clinical mastitis cases in this study population were associated with both gram-positive and gram-negative bacteria. The most frequent isolates were common mastitis pathogens, including *Streptococcus uberis*, *Streptococcus dysgalactiae*, coagulase-negative *Staphylococcus* spp, and *E coli*. There were also a variety of isolates, including *Aerococcus* spp, *Enterococcus* spp, and *Lactococcus* spp, that are not usually associated with intramammary infections.

Table 7—Effect of PEG–bG-CSF on mean ± SEM duration of gestation and percentage of live births for cattle treated with saline solution or various doses of PEG–bG-CSF.

Group	Duration of gestation from first dose to parturition (d)	Live births*
Saline solution	6.5 ± 0.4	54/56 (96.4)
5 μg/kg	6.8 ± 0.4	52/53 (98.1)
10 μg/kg	7.0 ± 0.4	52/54 (96.3)
20 μg/kg	6.0 ± 0.4	50/56 (89.3)

Cattle received 2 injections, the first of which was administered on day –7 and the second within 24 hours after parturition; day of parturition was designated as day 0.

*Value reported is number of live births/number of treated cattle (percentage).

MILK PRODUCTION AND COMPOSITION

Mean daily milk production for healthy cattle in each group was summarized **(Figure 2)**. All groups had steadily increasing milk production over the first 28 days of lactation. There were no significant differences in mean daily milk production among groups in the study.

The effects of PEG–bG-CSF administration on SCC and milk composition (concentrations of fat, lactose, protein, and milk solids) in healthy animals were sum-

marized **(Table 6)**. Administration of PEG–bG-CSF at the 3 doses evaluated had no significant effect on SCC or milk composition.

DURATION OF GESTATION AND PERCENTAGE OF LIVE BIRTHS

Effects of PEG–bG-CSF administration on the duration of gestation and percentage of live births were summarized **(Table 7)**. Administration of PEG–bG-CSF had no significant effect on the duration of gestation after the initial dose ($P = 0.47$) or percentage of live births ($P = 0.14$).

Discussion

Cattle treated with PEG–bG-CSF at 10 and 20 µg/kg 7 days prior to the anticipated date of parturition and again within 24 hours after calving had sustained increases in absolute neutrophil counts. These results are consistent with the biological function of G-CSF as an inducer of proliferation and differentiation of bone marrow myeloid precursor cells.[14] Previous studies[15,16] conducted to evaluate the effects of human G-CSF or bG-CSF indicate significant increases in absolute neutrophil counts could be achieved with daily administration of these cytokines at doses ranging from 1 to 5 µg/kg. Polyethylene glycol conjugation of proteins increases the duration of activity primarily by reducing first-pass renal clearance.[17] In the present study, absolute neutrophil counts in the peripheral circulation were increased for at least 14 days after 2 injections of PEG–bG-CSF at 10 and 20 µg/kg administered approximately 1 week apart. Suppression of neutrophil function in periparturient cattle persists for several weeks.[5] Polyethylene glycol–conjugated bG-CSF provides an extended duration of activity, which should improve administration convenience.

The incidence of clinical mastitis during the periparturient period was assessed by observing the condition of the mammary glands and milk at each milking during the first 28 days of lactation. This procedure is consistent with National Mastitis Council guidelines for diagnosis of clinical mastitis and consistent with practices used at commercial dairies. Approximately one-third of the cattle treated with sterile saline solution had at least 1 case of clinical mastitis during the first 28 days of lactation. Approximately one-third of the milk samples from these clinical mastitis cases did not contain recoverable microbial isolates. These results are consistent with those of a previous study[18] that indicate a significant proportion of milk samples collected from clinical cases of mastitis yield negative culture results. Most milk samples from clinical mastitis events containing bacterial isolates included gram-positive and gram-negative bacteria consistent with pathogens typically present for the commercial farm where the present study was conducted.[e]

Administration of PEG–bG-CSF at 10 and 20 µg/kg resulted in a lower incidence of clinical mastitis on a per-animal basis (9/54 and 5/53, respectively), relative to that of animals treated with sterile saline solution (18/53). Similarly, PEG–bG-CSF significantly reduced the number of mammary glands with clinical mastitis (10 and 7 for 10 and 20 µg/kg, respectively), relative to that for animals treated with sterile saline solution (22). These results are consistent with those of a previous study[19] that indicate administration of recombinant human G-CSF prevents the occurrence of *Staphylococcus aureus*–induced mastitis by use of an intramammary challenge-exposure technique.

Administration of PEG–bG-CSF did not significantly alter the severity of clinical score at the time each clinical mastitis event was diagnosed. In addition, the duration of each clinical mastitis event was not significantly different among treatments. It is reasonable to hypothesize that neutrophils primed for improved microbicidal activity by exposure to PEG–bG-CSF in the circulation may be better able to eliminate infections before an inflammatory response is apparent. However, once the immune response is overwhelmed and an acute inflammatory response is apparent, the severity of the inflammatory response and amount of time required for resolution remain unchanged. In the study reported here, cattle were exposed to environmental conditions that are known to result in mastitis. To confirm whether neutrophils from PEG–bG-CSF are more effective at killing invading bacteria, milk samples for microbiological testing would need to be collected immediately before and after signs of clinical mastitis are apparent.

Generating and maintaining immune competence require energy. It is possible that administration of PEG–bG-CSF to alter neutrophil numbers and function during the periparturient period could divert energy from milk production in healthy cows. Administration of PEG–bG-CSF had no significant effect on daily milk production in cows with healthy mammary glands.

Results of milk SCC assays indicated that cattle with healthy mammary glands were similar among groups. These results indicated that the increased numbers of circulating neutrophils in cattle treated with PEG–bG-CSF were not associated with elevated numbers of somatic cells in healthy mammary glands. In vitro exposure of bovine neutrophils to bG-CSF results in increased expression of adhesion molecules CD18 and CD11b, which play a role in extravasation of the cells.[8] In the present study, milk SCC was not determined at the time clinical mastitis was diagnosed. Therefore, it is not possible to determine whether cattle treated with PEG–bG-CSF had a more robust influx of neutrophils into an affected mammary gland early in the disease process.

Administration of PEG–bG-CSF did not significantly alter concentrations of fat, protein, lactose, or milk solids in healthy mammary glands. This suggested that administration of PEG–bG-CSF to cattle will not negatively impact processing of milk-derived products such as cheese and yogurt.

The impairment of neutrophil function in pregnant cattle during the periparturient period could be an adaptation to practices associated with the

husbandry of cows for milk production. Therefore, it is possible that altering neutrophil function during the periparturient period could have negative consequences for the ability of dams to maintain pregnancy late in gestation or could impair the survival of calves in utero. Administration of the first dose of PEG–bG-CSF before parturition had no significant effect on the duration of gestation or percentage of live births. These data suggested that manipulation of neutrophil numbers and function during late gestation had no negative effects on the dam's ability to maintain pregnancy or on the viability of calves in utero.

Analysis of the results of the present study indicated that administration of PEG–bG-CSF reduced the incidence of naturally occurring clinical mastitis in periparturient dairy cows and heifers. The mechanism of action by which PEG–bG-CSF was able to resolve impaired neutrophil function during a time when cattle are more susceptible to disease represents a novel approach to controlling mastitis and is worthy of further investigation.

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Footnotes

a. Quartermaster DC, Zoetis, Florham Park, NJ.
b. ADVIA, Siemens, Munich, Germany.
c. Dairy Food Safety Lab, Veterinary Medicine Teaching and Research Center, University of California-Tulare, Tulare, Calif.
d. SAS, version 9.2, SAS Institute Inc, Cary, NC.
e. TeVelde B, Lone Oak Farms, Hanford, Calif: Personal communication, 2014.

References

1. Sordillo LM. Factors affecting mammary gland immunity and mastitis susceptibility. *Livest Prod Sci* 2005;98:89–99.
2. Oliver SP, Sordillo LM. Udder health in the periparturient period. *J Dairy Sci* 1988;71:2584–2606.
3. Paape MJ, Bannerman DD, Zhao X, et al. The bovine neutrophil: structure and function in blood and milk. *Vet Res* 2003;34:597–627.
4. Sordillo LM, Shafer-Weaver K, DeRosa D. Immunobiology of the mammary gland. *J Dairy Sci* 1997;80:1851–1865.
5. Kehrli ME, Nonnecke BJ, Roth JA. Alterations in bovine neutrophil function during the periparturient period. *Am J Vet Res* 1989;50:207–214.
6. Nagata S. Gene structure and function of granulocyte colony stimulating factor. *Bioessays* 1989;10:113–117.
7. Nomura H, Imazeki I, Oheda M, et al. Purification and characterization of human granulocyte colony stimulating factor (G-CSF). *EMBO J* 1986;5:871–876.
8. Heidari M, Harp JA, Kehrli ME. Expression, purification and in vitro biological activities of recombinant bovine granulocyte-colony stimulating factor. *Vet Immunol Immunopathol* 2001;81:45–57.
9. Kehrli ME, Goff JP, Stevens MG, et al. Effects of granulocyte colony-stimulating factor administration to periparturient cows on neutrophils and bacterial shedding. *J Dairy Sci* 1991;74:2448–2458.
10. Molineux G. PEGylation: engineering improved biopharmaceuticals for oncology. *Pharmacotherapy* 2003;23:3S–8S.
11. Molineux G. The design and development of pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). *Curr Pharm Des* 2004;10:1235–1244.
12. Kimura K, Goff JP, Canning P, et al. Effect of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil number and function in periparturient dairy cows. *J Dairy Sci* 2014;97:4842–4851.
13. Cho H, Daniel T, Buechler YJ, et al. Optimized clinical performance of growth hormone with an expanded genetic code. *Proc Natl Acad Sci U S A* 2011;108:9060–9065.
14. Basu S, Dunn A, Ward A. G-CSF. Function and modes of action. *Int J Mol Med* 2002;10:3–10.
15. Cullor JS, Fairley N, Smith WL, et al. Hemogram changes in lactating dairy cows given recombinant granulocyte colony stimulating factor (r-MethuG-CSF). *Vet Pathol* 1990;27:311–316.
16. Cullor JS, Smith W, Zinki JG, et al. Hematologic and bone marrow changes after short- and long-term administration of two recombinant bovine granulocyte colony-stimulating factors. *Vet Pathol* 1992;29:521–527.
17. Harris JM, Martin NA, Modi M. PEGylation: a novel process for modifying pharmacokinetics. *Clin Pharmacokinet* 2001;40:539–551.
18. Hogan JS, Smith JL, Hoblet KH, et al. Field survey of clinical mastitis in low somatic cell count herds. *J Dairy Sci* 1989;72:1547–1556.
19. Nickerson SC, Owens WE, Watts JL. Effects of recombinant granulocyte colony-stimulating factor on *Staphylococcus aureus* mastitis in lactating dairy cows. *J Dairy Sci* 1989;72:3286–3294.