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Effect of recombinant cytokines on leucocytes and physiological changes in bovine mammary glands during early involution

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We examined the effects of administering recombinant bovine cytokines to non-lactating dairy cows and measured mammary gland leucocytes and the involution process. After the final milking, groups of cows were given an intramammary infusion of cytokine in two quarters. These cytokines were recombinant bovine interleukin-2 (rboIL-2) (2×10^5 units, $n=6$), recombinant bovine granulocyte-macrophage colony stimulating factor (rboGM-CSF) (500 μg, $n=4$) and recombinant bovine interleukin-1β (rboIL-1β) (10 μg, $n=10$). Each animal also received an infusion of phosphate-buffered saline (PBS) in the other two quarters as controls. The rboIL-2 and rboGM-CSF were produced in a yeast expression system, while rboIL-1β was produced in *Escherichia coli*. Leucocyte numbers, bactericidal activity of leucocytes, and concentrations of citrate and lactoferrin in quarter secretion samples were monitored after infusion of cytokine or PBS. Infusion of rboIL-2 had minimal effect on leucocyte numbers and concentrations of citrate and lactoferrin. Both rboGM-CSF and rboIL-1β induced a rapid increase in the number of neutrophils and macrophages compared with control PBS quarters. Concentrations of lactoferrin in secretions were increased by rboGM-CSF and rboIL-1β compared with control PBS quarters. In addition, infusion of glands with rboIL-1β lowered the citrate : lactoferrin molar ratio compared with PBS control quarters. The results indicate that intramammary infusion of either rboGM-CSF or rboIL-1β at cessation of milking immediately increased the number of phagocytic cells in the gland. These cytokines, in particular rboIL-1β, also increased the rate of mammary gland involution during the early dry period.

Keywords: Mastitis, lactoferrin, citrate, dairy cow.

The mammary gland has a complex immune defence system against bacterial infection. Cellular defences within the mammary gland are regulated by cytokines, which have specific roles in modulating inflammatory and innate and acquired immune responses. Cytokines activate phagocytic cells such as neutrophils and macrophages, while cytokines such as interleukin-1β (IL-1β) play a key role in eliciting the acute phase response following bacterial invasion of the mammary gland, and promote accumulation of leucocytes at the sites of infection (Riollet et al. 2000). Recombinant cytokines enhance mammary cellular defence mechanisms when administered during lactation or in the dry period. Intramammary infusion of interleukin-2 (IL-2) and interferon-γ (IFN-γ) to lactating cattle improves protection of mammary glands against subsequent intramammary challenge with *Staphylococcus aureus* or *Escherichia coli* (Sordillo & Babiuk, 1991; Daley et al. 1993; Sordillo, 1995). Cytokines improve cure rates when administered in combination with antibiotics (Reddy et al. 1992; Daley et al. 1992; Dunney & Shuster, 1995; Erskine et al. 1998). During the non-lactating period the bovine mammary gland is highly susceptible to new intramammary infections especially during the transition from lactating to dry gland (Oliver, 1988). Recombinant IL-2, when administered intramammarily after the last milking prior to drying off, reduces alveolar epithelial and luminal areas and increases connective tissue stroma and degree of leucocytosis (Nickerson et al. 1993). Changes in composition of mammary secretion following intramammary infusion of recombinant IL-1β or IL-2 leads to an apparent increase in the rate of gland involution (Rejman et al. 1995). Since the involuted gland is more resistant to new infection, it may be possible to reduce the incidence

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of mastitis by administration of recombinant cytokines to the gland during the dry period.

The current study examined further the physiological response of mammary glands treated with recombinant bovine IL-2 (rboIL-2) and IL-1β (rboIL-1β) at the commencement of the dry period. To reduce the possibility of adverse side-effects, a lower dose of rboIL-2 was used in this study than was used in other studies (Nickerson et al. 1993; Rejman et al. 1995; Erskine et al. 1998). The dose of rboIL-2 used in the current trial has a therapeutic effect in lactating cows and is well tolerated (Wedlock et al. 2000). The effect of administering recombinant bovine granulocyte-macrophage colony stimulating factor (rboGM-CSF), a cytokine which has not been tested in non-lactating cows, was determined. Two of the cytokines (rboIL-2 and rboGM-CSF) were produced in a yeast expression system, *Pichia pastoris*, which has the potential advantages of absence of endotoxin and expression of recombinant proteins with increased bioactivity *in vivo* due to glycosylation. The recombinant cytokines were infused into gland quarters at the cessation of milking and their effects on leucocytes and bactericidal activity of leucocytes in the gland secretions were determined. Their effect on mammary gland involution was determined by measuring the concentrations of citrate and lactoferrin in gland secretions.

Materials and Methods

Animals

In Expt 1, eight Friesian Jersey crossbred dairy cows were selected from the Dexcel research herd. Bacteriological analysis of the foremilk prior to the experiment showed that two of the animals had a subclinical coagulase-negative staphylococcus (CNS) infection in one quarter. Infections were indicated by the isolation of the same pathogen at consecutive samplings and an individual quarter foremilk somatic cell count (SCC) >250 000/ml. The remaining quarters in each animal were free of infection and individual cow SCC averaged 30 000/ml before the start of the experiment. In Expt 2 and Expt 3, conducted concurrently, 14 Friesian-Jersey crossbred dairy cows were selected from the Dexcel research herd. Bacteriological analysis of the foremilk prior to the experiment showed that one animal had a CNS infection in one quarter. The remaining quarters were free of infection and individual cow SCC averaged 73 000/ml before the start of the experiments.

Recombinant cytokines

The rboIL-2 was produced in *Pic. pastoris* as described previously (Wedlock et al. 1999a, b) and shown to be biologically active using a bioassay as described by Wedlock et al. (1999a). The rboIL-1β was produced in *Esch. coli* as previously described (Wedlock et al. 1999c). Residual lipopolysaccharide was removed from the purified protein using AffinityPak™ Detoxi-Gel™ endotoxin removing gel (Pierce, Rockford, IL, USA). The amount of endotoxin in the rboIL-1β was <0·1 endotoxin units/μg protein as determined by a limulus amoebocyte lysate (LAL) assay (E. Toxate, Sigma kit No. 210, Sigma Chemicals, St Louis, MO, USA).

The rboGM-CSF was produced in *Pic. pastoris* as follows. The cDNA encoding the mature bovine GM-CSF (amino acids 18–143) was isolated by RT-PCR from bovine macrophages using primers 5′-TATCTCGAGAAAAGA**GC**-**A**CCTACTCGCCCACCCAA-3′ (sense) and 5′-ATGAATTC-A*ATGATGGTGATGATGGTG*CTTCTGGGCTGGTTCCCA-GC-3′ (anti-sense). The *Xho*I and *Eco*RI sites are underlined, the codon coding for the N-terminal amino acid (Ala) of the mature GM-CSF is in bold, and the sequence coding for a six histidine affinity tag is shown in italics. The cDNA was cloned into the *Xho*I and *Eco*RI sites of the expression plasmid pPIC9 (Invitrogen Corporation, Carlsbad, CA, USA) to produce a fusion between the GM-CSF and the yeast alpha mating factor leader (MF-α) sequence. The resulting construct was digested with *Sal*I, transformed into *Pic. pastoris* strain SMD1168 and hist+ transformants were selected and screened for production of rboGM-CSF. The rboGM-CSF was produced in batch cultures of a selected transformant and purified by immobilized metal-chelating affinity chromatography with Talon (Clontech Laboratories Inc., Palo Alto, CA, USA). Bioactivity of rboGM-CSF was assessed by measuring GM-CSF-induced proliferation of human TF-1 cell line (Kitamura et al. 1989). Triplicate wells in 96-well plates contained 10^4 cells in 200 μl of RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with penicillin and streptomycin, 2 mM-glutamine and 50 ml fetal calf serum/l and dilutions of rboGM-CSF. After the plates were incubated for 48 h, 0·25 μCi of [^{3}H]thymidine was added to each well and the cultures were harvested 5 h later. The amount of tritiated thymidine incorporated was determined using a liquid β-scintillation counter (Microbeta, Wallac, Finland). Recombinant human GM-CSF (R&D Systems Inc., Minneapolis, MN, USA) was used as a positive control. Specific activity of the rboGM-CSF on TF-1 cells was estimated as 1000 units/μg protein. The purified protein had <0·1 endotoxin units/μg protein as determined by a LAL assay (Sigma).

Experimental design and infusion of cytokines

Cytokines were administered at commencement of drying off by infusion through the teat canal after the last evening milking. Quarters were infused with recombinant cytokine in 10 ml of endotoxin-free phosphate-buffered saline (PBS; 0·01 M sodium phosphate–0·137 M NaCl, pH 7·4) containing low endotoxin bovine serum albumin (BSA) (ICP, Auckland, New Zealand) at 1 g/l. In Expt 1, six animals were given rboIL-2 (2×10^5 units) in two diagonally opposed quarters (left front and right hind). Remaining

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quarters were given 10 ml of PBS containing 1 g/l BSA as a control. Two additional animals were given PBS in two quarters only (left front and right hind). Infusions of rboIL-2 and PBS were distributed among the two quarters with a pre-existing CNS infection. In Expt 2, four animals were infused with rboGM-CSF (500 μg) in two diagonally opposed quarters and, in Expt 3, ten animals were given rboIL-1β (10 μg) in two quarters. Infusions were distributed equally between both diagonal pairs of quarters. All remaining quarters were given 10 ml of PBS containing BSA at 1 g/l. The quarter with a pre-existing CNS infection was given PBS. This infection persisted throughout the experiment and this quarter was excluded from analysis of the leucocyte bactericidal data. In the second and third experiments, no animals were given PBS alone as it was considered that PBS in two quarters of each cytokine-treated animal provided sufficient control for measuring the effects of cytokine. Animals in all experiments were checked for signs of systemic response at 3 h after infusion of cytokine or PBS and in the morning following infusion.

Doses of rboIL-2 and rboIL-1β used in these studies were based on the results of previous trials in lactating cattle. Cattle in lactation were infused with 2×10^5 units of rboIL-2 and this dose markedly enhanced bactericidal activity of neutrophils with only a transient elevation of SCC and no effect on milk yield (Wedlock et al. 2000). In a separate experiment, lactating cattle were infused with either 10 or 50 μg of rboIL-1β. Cows infused with either dose had markedly elevated SCC (unpublished observations). In the current experiments, cattle were infused with the lower dose to reduce the risk of causing a systemic response. The selection of an appropriate dose of rboGM-CSF was based on the finding that lactating cattle can tolerate infusion of up to 5 mg of rboGM-CSF and that a dose of 1 mg was effective in preventing *S. aureus* infection (Daley et al. 1993). Since the tolerance of glands to rboGM-CSF infused during the dry period was unknown, the cows were given a more conservative dose of 500 μg per quarter.

Determination of somatic cell counts, differential leucocyte counts and bacteriological status

Quarter foremilk samples were collected 7 d before the start of the experiments and on the day of administration of cytokines (day 0), for the determination of SCC and assessment of the presence of blood and/or clots. SCC was determined electronically using a Fossomatic 450 (Foss Electric DK-3400, Hillerød, Denmark). Quarter secretion samples were collected on days 2, 7 and 14 in Expt 1 and on days 1, 6 and 13 in Expt 2 and Expt 3 for determining the number of leucocytes by differential cell-counting after staining with Levowitz–Weber stain. Differential cell counts were not conducted on secretions before infusion of cytokine (day 0) because cell numbers were low prior to cessation of milking. In all experiments, additional secretion samples were collected aseptically at each time point for determination of bacteriological status. Secretion samples were streaked onto quarter-plates of Columbia blood agar containing 50 ml/l whole bovine blood and 1 g aesculin /l and the plates were incubated at 37 °C for 48 h. Presumptive identification of isolates was by colony morphology, Gram's stain, haemolysis, catalase production, and tube coagulase reaction (Harmon et al. 1990). Secretions were checked for the presence of clots.

Leucocyte bactericidal assay

Quarter secretion samples (200 ml) were obtained 7 d after infusion of rboIL-2 or PBS in Expt 1 or 6 d after infusion of cytokines or PBS in Expt 2 and Expt 3. Samples were centrifuged at 1500 ***g*** for 20 min to pellet the leucocytes, the cream and whey were discarded, and the cells washed three times with PBS. Leucocytes were resuspended at a concentration of 10^6/ml in RPMI medium with 100 ml fetal calf serum/l and viability checked with Trypan Blue. Bactericidal activity was assessed with a colourimetric method based on the reduction of 3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma) to formazan (Stevens et al. 1991). Briefly, opsonized *S. aureus* were incubated with leucocytes (10 : 1 ratio of bacteria to leucocytes) in 96-well plates. After incubation at 37 °C for 1 h, the leucocytes were lysed by addition of 2 g saponin/l (Sigma) and MTT was added to each well (100 μg/well). The ability of live bacteria to reduce MTT to purple formazan was measured by dissolving the formazan in 6·5 M-dimethylformamide (BDH Laboratory Supplies, Poole DH15 1TD, UK) containing 200 g sodium dodecyl sulphate/l (BDH), and measuring the optical density at 560 nm. The percentage killing of *S. aureus* was determined from a standard formazan curve relating optical density to known concentrations of bacteria as described previously (Wedlock et al. 2000). The *S. aureus* strain was isolated from a clinical case of bovine mastitis in New Zealand.

Measurement of citrate and lactoferrin in secretions

Quarter secretion samples (20 ml) were obtained on days 0, 2, 7 and 14 in Expt 1 and on days 1, 6, and 13 in Expt 2 and Expt 3. Samples were centrifuged at 1500 ***g*** for 20 min to remove fat and cellular debris. Concentration of lactoferrin in the skim samples was measured by ELISA using a bovine lactoferrin ELISA kit (Bethyl Laboratories Inc. Montgomery, TX, USA). Intra-assay and inter-assay CV was <10%. To measure citrate concentration, the skim samples were deproteinized by addition of an equal volume of 1 M perchloric acid. After neutralization of the deproteinized sample with 0·3 M potassium carbonate, the concentration of citrate was measured spectrophotometrically using a citric acid analysis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Intra-assay and inter-assay CV was <5%.

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Statistical analysis

Differential cell counts, citrate and lactoferrin concentrations were logarithmically transformed (ln) and analysed by repeated measures ante-dependence analysis of order 1 using Minitab (State College, PA, USA) after blocking on animal and front and hind quarters. Macrophage bactericidal measurements were analysed by one-way analysis of variance. Comparisons of means were performed by LSD.

Results

Effect of cytokine treatment on leucocytes

SCC in secretions from both PBS-and rboIL-2-treated quarters increased from day 0 to day 14 with no significant differences between the groups. Effects of intramammary infusion of rboIL-2 or PBS on leucocytes, determined by differential cell counts in quarter secretions are shown in Table 1. There were no significant differences between numbers of leucocytes in secretions from quarters infused with rboIL-2 and those infused with PBS. Two animals were given PBS in each of two quarters while the other two quarters received no treatment. In those animals, there were no significant differences at each time point between the mean leucocyte numbers in the secretions from PBS-treated quarters and quarters that received no treatment (results not shown). There were no visible signs of a systemic response in any of the animals infused with either rboIL-2 or PBS.

Effects of intramammary infusion of rboGM-CSF or rboIL-1β on differential leucocyte counts in quarter secretions are shown in Table 2. Infusion of rboGM-CSF caused a rapid increase in the number of neutrophils and macrophages in secretions. On day 1, the mean numbers of neutrophils and macrophages in quarters infused with rboGM-CSF were significantly greater than mean numbers in the control PBS quarters ($P<0{\cdot}01$). Numbers of macrophages remained elevated on day 6 with the mean number of macrophages in rboGM-CSF-treated quarters higher than the control PBS quarters ($P=0{\cdot}066$). On day 6, macrophages comprised 75±10% of the total leucocyte population in the rboGM-CSF infused quarters compared with 38±11% in PBS control quarters. On day 13, macrophages comprised 61±6% of the total leucocyte population in the rboGM-CSF infused quarters compared with 61±5% in PBS controls.

Infusion of rboIL-1β into quarters caused an immediate increase in leucocyte numbers (Table 2). On day 1, the mean numbers of lymphocytes ($P<0{\cdot}05$), neutrophils ($P<0{\cdot}01$) and macrophages ($P<0{\cdot}01$) in secretions from rboIL-1β-treated quarters were significantly higher than in secretions from control PBS quarters. On day 6, macrophages comprised 47±6% and 42±5% of total leucocytes in the rboIL-1β and PBS-infused quarters respectively, while on day 13, macrophages comprised 62±5% and 59±5% of total leucocytes in rboIL-1β and PBS-infused quarters respectively.

Table 1. Differential leucocyte counts in secretions from quarters of non-lactating dairy cows infused with recombinant bovine interleukin-2 or phosphate-buffered saline

Values are no. of cells ($\times 10^{-4}$)/ml; means±SE ($n=12$) for secretions collected on days 2, 7 and 14

Days after infusion of cytokine	Quarter treatment	Leucocytes		
		Lymphocytes	Neutrophils	Macrophages
2	PBS	29·0±10·5	113·8±22·8	56·9±26·8
	rboIL-2	29·9±8·1	191·2±48·6	77·9±23·5
7	PBS	23·5±8·7	248·8±47·9	180·8±69·1
	rboIL-2	30·3±5·9	282·9±54·5	169·3±31·6
14	PBS	53·2±9·6	291·0±45·8	235·9±27·7
	rboIL-2	44·5±8·0	263·1±30·7	213·2±28·8

PBS, phosphate-buffered saline; rboIL-2, recombinant bovine interleukin-2

None of the animals infused with rboGM-CSF or rboIL-1β showed visible signs of an adverse reaction 3 h after treatment or on the following day. On day 2, clots were visible in one quarter infused with rboIL-1β. On day 4, secretions from 6 of the 8 quarters infused with rboGM-CSF showed clots. None of these glands required treatment with antibiotics.

Effect of infusion of cytokines on citrate and lactoferrin in gland secretions

Infusion of rboIL-2 did not affect concentrations of citrate and lactoferrin in gland secretions during the experiment (Table 3). Concentrations of citrate in secretions from quarters infused with rboIL-2 or PBS decreased from day 0 to day 14, while lactoferrin levels increased during this period. Concentrations of citrate and lactoferrin at each time point did not differ between rboIL-2 and PBS-infused quarters. Citrate : lactoferrin molar ratios in both cytokine-treated and control quarters decreased from day 0 to day 14, and there were no significant differences between the two groups.

In Expts 2 and 3, lactoferrin and citrate were not measured on day 0 prior to infusion of cytokine or PBS. Results from the first experiment had shown that on day 0, there were no significant differences in the mean concentrations of lactoferrin and citrate in quarters that subsequently were infused with rboIL-2 or PBS. Secretions from quarters infused with rboGM-CSF had consistently lower citrate concentrations, higher lactoferrin concentrations and lower citrate/lactoferrin molar ratios on days 1, 6 and 13 after infusion compared with control PBS quarters (Table 4, Fig. 1). On day 6 after infusion of cytokine, concentration of lactoferrin was significantly higher in rboGM-CSF-treated quarters than in PBS control quarters ($P<0{\cdot}05$). Secretions from quarters infused with rboIL-1β had consistently lower

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Table 2. Differential leucocyte counts in secretions from quarters of non-lactating dairy cows infused with recombinant bovine granulocyte-macrophage colony stimulating factor, recombinant bovine interleukin-1β or phosphate-buffered saline

Values are number of cells ($\times 10^{-4}$)/ml; means ± SE for secretions collected on days 1, 6 and 13 after infusion. For GM-CSF and corresponding PBS, $n=8$; for IL-1β and corresponding PBS, $n=10$

Days after infusion of cytokine	Quarter treatment	Leucocytes		
		Lymphocytes	Neutrophils	Macrophages
1	PBS	3·0 ± 1·7	10·5 ± 6·0	23·0 ± 6·5
	rboGM-CSF	13·7 ± 5·6	318·8** ± 62·0	361·6** ± 82·5
	PBS	4·5 ± 4·2	24·4 ± 16·6	33·3 ± 26·4
	rboIL-1β	29·5* ± 10·5	662·0** ± 127·3	135·5** ± 35·7
6	PBS	20·2 ± 8·0	244·0 ± 60·7	169·0 ± 52·4
	rboGM-CSF	16·3 ± 7·3	165·6 ± 75·1	575·7 ± 139·8
	PBS	20·9 ± 7·7	89·1 ± 26·1	74·4 ± 16·2
	rboIL-1β	27·7 ± 5·8	113·9 ± 12·0	148·0 ± 28·4
13	PBS	38·3 ± 10·3	144·6 ± 28·8	355·0 ± 95·6
	rboGM-CSF	34·3 ± 11·1	108·8 ± 22·6	241·0 ± 44·4
	PBS	45·6 ± 10·0	159·8 ± 25·8	323·9 ± 62·3
	rboIL-1β	31·3 ± 9·5	124·6 ± 25·8	228·9 ± 30·3

PBS, phosphate-buffered saline; rboGM-CSF, recombinant bovine granulocyte-macrophage colony stimulating factor; rboIL-1β, recombinant bovine interleukin-1β

* Values significantly different from values of control PBS quarters ($P<0·05$)

**Values significantly different from values of control PBS quarters ($P<0·01$)

Table 3. Citrate and lactoferrin concentrations and molar ratios of citrate to lactoferrin in secretions from quarters infused with recombinant interleukin-2 or phosphate-buffered saline

Values are concentration (g/l) (citrate, lactoferrin) and molar ratios of citrate to lactoferrin for secretions collected from quarters on the day of infusion (day 0) and on days 2, 7 and 14 after infusion; mean ± SE for $n=12$

Days after infusion of cytokine	Citrate		Lactoferrin		Citrate/lactoferrin ratio	
	PBS	rboIL-2	PBS	rboIL-2	PBS	rboIL-2
0	1·20 ± 0·08	1·17 ± 0·07	0·10 ± 0·02	0·11 ± 0·02	7563 ± 1521	7078 ± 1810
2	1·22 ± 0·1	1·18 ± 0·09	0·33 ± 0·08	0·48 ± 0·11	2188 ± 360	1643 ± 341
7	0·99 ± 0·15	0·90 ± 0·09	6·08 ± 0·80	6·32 ± 0·96	102 ± 38	78 ± 15
14	0·56 ± 0·09	0·55 ± 0·07	10·22 ± 2·34	9·12 ± 1·35	34 ± 8	35 ± 9

PBS, phosphate-buffered saline; rboIL-2, recombinant bovine interleukin-2

citrate concentrations, higher lactoferrin concentrations and lower citrate/lactoferrin ratios on days 1 and 6 than control PBS quarters (Table 4). On day 1, the mean lactoferrin concentration in rboIL-1β infused quarters was higher than in PBS control quarters ($P<0·01$) and the citrate : lactoferrin molar ratio was lower than for PBS control quarters ($P<0·01$). On day 6, the mean citrate : lactoferrin molar ratio was lower in the IL-1β-treated quarters than in PBS control quarters ($P=0·077$).

Effect of cytokines on bactericidal activity of leucocytes

Leucocytes were isolated from secretions of quarters infused with cytokines or PBS. The leucocyte population comprised predominantly neutrophils and macrophages (Tables 1 and 2). The capacity of these leucocytes to kill *S. aureus* was determined using a bactericidal assay *in vitro*. Leucocytes isolated from quarters infused with rboIL-1β showed significantly higher bacterial killing than leucocytes from the PBS control quarters ($P<0·05$) (Table 5). Mean bactericidal activity of leucocytes from quarters treated with rboGM-CSF or rboIL-2 was not significantly different from the means for respective control PBS quarters.

Discussion

Administration of rboGM-CSF into mammary glands caused an immediate transient influx of neutrophils and macrophages into the treated quarters. Administration of rboIL-1β also caused an immediate influx of neutrophils, macrophages and lymphocytes into the glands leading to a

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Table 4. Citrate and lactoferrin concentrations in secretions from quarters infused with recombinant bovine granulocyte-macrophage colony stimulating factor, recombinant bovine interleukin-1β or phosphate-buffered saline

Values are concentration (g/l) (citrate, lactoferrin) for secretions collected from quarters on days 1, 6 and 13 after infusion; for GM-CSF and corresponding PBS, $n=8$; for IL-1β and corresponding PBS, $n=10$

Days after infusion of cytokine	Citrate				Lactoferrin			
	PBS	rboGM-CSF	PBS	rboIL-1β	PBS	rboGM-CSF	PBS	rboIL-1β
1	1·82 (±0·12)	1·48 (±0·23)	2·07 (±0·24)	2·02 (±0·25)	0·31 (±0·056)	0·37 (±0·067)	0·12 (±0·034)	0·19** (±0·037)
6	1·32 (±0·28)	0·96 (±0·25)	1·76 (±0·34)	1·01 (±0·21)	2·46 (±0·47)	3·02* (±0·52)	1·10 (±0·14)	1·70 (±0·32)
13	0·69 (±0·11)	0·64 (±0·10)	0·57 (±0·07)	0·65 (±0·09)	9·34 (±1·88)	10·56 (±2·26)	4·98 (±0·58)	5·36 (±1·02)

PBS, phosphate-buffered saline; rboGM-CSF, recombinant bovine granulocyte-macrophage colony stimulating factor; rboIL-1β, recombinant bovine interleukin-1β

*Value significantly different from value for control PBS group ($P<0·05$)

**Value significantly different from value for control PBS group ($P<0·01$)

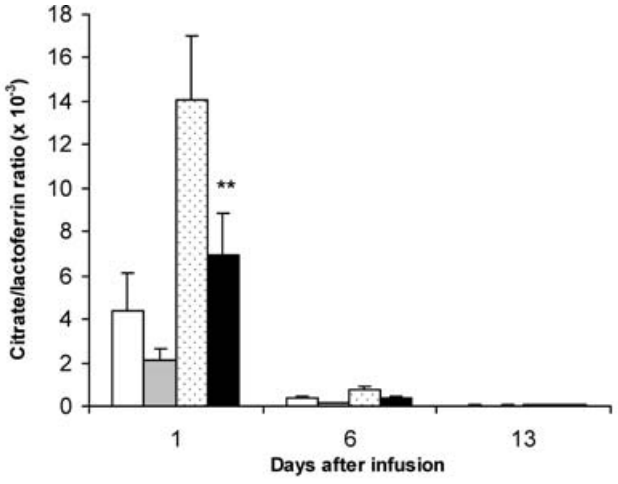

Fig. 1. Molar ratios of citrate to lactoferrin from quarters infused with recombinant bovine granulocyte-macrophage colony stimulating factor, recombinant bovine interleukin-1β or phosphate-buffered saline. For GM-CSF and corresponding PBS, $n=8$; for IL-1β and corresponding PBS, $n=10$. ▩, quarters infused with recombinant bovine granulocyte-macrophage colony stimulating factor; □, control quarters infused with phosphate-buffered saline; ■, quarters infused with recombinant bovine interleukin-1β; ⊡, control quarters infused with phosphate-buffered saline. **Mean significantly lower ($P<0·01$) than mean for control phosphate-buffered saline quarters.

large increase in numbers of neutrophils. It is known that administration of rboIL-1β during the early nonlactating period predominantly increases the number of neutrophils, while macrophages and lymphocytes predominate in IL-2-treated glands (Nickerson et al. 1993). In that study, glands were infused with 1 mg of IL-2 which, based on the reported specific activity, would equate to about 2×10^6 units of IL-2. In the present study a 10-fold lower dose of

Table 5. Bactericidal activity of mammary macrophages in secretions of non-lactating dairy cows after infusion with recombinant bovine interleukin-2, recombinant bovine granulocyte-macrophage colony stimulating factor, recombinant bovine interleukin-1β or phosphate-buffered saline

Values are percentages of *Staphylococcus aureus* killed; means ± SE. For IL-2 and corresponding PBS, $n=12$; for GM-CSF and corresponding PBS, $n=8$; for IL-1β, $n=9$, and for corresponding PBS, $n=8$

Cytokine	Control quarters treated with PBS	Quarters treated with cytokine
rboIL-2	22·3 ± 4·1	25·7 ± 2·4
rboGM-CSF	29·3 ± 7·9	34·3 ± 8·5
rboIL-1β	13·5 ± 3·9	31·4* ± 7·0

PBS, phosphate-buffered saline; rboIL-2, recombinant bovine interleukin-2; rboGM-CSF, recombinant bovine granulocyte-macrophage colony stimulating factor; rboIL-1β, recombinant bovine interleukin-1β

*Mean significantly greater ($P<0·05$) than mean for control PBS treated quarters

2×10^5 units of rboIL-2 had a minimal effect on the leucocyte population within the gland. A low dose of IL-2 was used in the current study because an association between intramammary infusion of IL-2 into glands during the dry period and an increased incidence of abortion can occur with higher doses of 1 mg (Erskine et al. 1998). The low dose of rboIL-2 given to non-lactating cattle in the current study did not enhance bactericidal activity of mammary leucocytes isolated from the secretions, which contrasted with a previous finding that a similar dose of rboIL-2, administered by the intramammary route to lactating cows, significantly increased the ability of neutrophils to kill *S. aureus* (Wedlock et al. 2000). The bactericidal assay in the current study measured activity of both neutrophils and macrophages as significant numbers of both these cells were present in dry secretions when the assay was conducted.

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Administration of rboIL-1β to the mammary glands of cows at commencement of drying-off appeared to enhance the capacity of leucocytes to kill *S. aureus* but the effect was relatively weak. Results of the current study confirm that during involution of the gland, macrophages become the numerically dominant cell type in gland secretions. Macrophages play a key role in early and innate defences in the gland and in events during acquired immune responses such as antigen presentation to T cells (Riollet et al. 2000). In the rboGM-CSF-infused quarters there were higher proportions of macrophages to neutrophils at the time the bactericidal assay was conducted. Administration of rboGM-CSF did not appear to have a major effect on the bactericidal capacity of these cells. However, leucocytes from PBS control quarters of rboGM-CSF-treated animals showed higher bactericidal activity than leucocytes isolated from PBS control quarters of rboIL-1β-infused animals. Administration of rboGM-CSF to two quarters in each animal may have caused a systemic response resulting in activated leucocytes being present in the PBS control quarters. Such a response would have masked rboGM-CSF-induced stimulation of leucocyte bactericidal activity in quarters treated with this cytokine. There were marked differences in the concentrations of both citrate and lactoferrin in the PBS-infused control quarters of rboGM-CSF-treated animals compared with the PBS-infused quarters of the rboIL-1β-treated animals on days 1 and 6, further suggesting that rboGM-CSF had induced a systemic effect. We have shown that stimulation of macrophage cultures with rboGM-CSF *in vitro* can enhance killing of *S. aureus* (our unpublished observations). GM-CSF is known to increase chemotactic and bacteridical activities of mammary neutrophils *in vitro* (Sordillo et al. 1992).

Effects of infusing recombinant cytokines into the mammary gland at the cessation of milking on the involution process were determined by measuring concentrations of citrate and the milk protein, lactoferrin, in quarter secretions. The low dose of rboIL-2 used in the current study affected neither citrate nor lactoferrin concentrations in the quarter secretions and was insufficient to enhance gland involution. This result contrasts with earlier studies which showed that administration of 1 mg of rboIL-2 accelerates the rate of mammary gland involution (Nickerson et al. 1993; Rejman et al. 1995). Further studies may determine a more optimal amount of IL-2, either administered as a single dose or as multiple doses, for enhancing cellular defences and involution of the glands with minimal induction of adverse side-effects such as abortion. However, our results suggest that IL-2 may be of limited use as a therapeutic agent for mastitis during the dry period. Both rboGM-CSF and rboIL-1β increased the concentration of lactoferrin in cytokine-treated glands. Citrate : lactoferrin molar ratios indicate the degree of bovine mammary involution (Oliver & Smith, 1982a, b) and decrease as involution proceeds. Infusion of rboGM-CSF into the glands may have enhanced the early involution process, although interpreting the results was difficult owing to the likely systemic effects caused by this cytokine. Infusion of rboIL-1β into the glands at the commencement of the dry period enhanced early involution, as indicated by a significant reduction of the citrate : lactoferrin molar ratio on day 1 and a lower ratio on day 6 compared with control quarters. A previous study showed that administering rboIL-1β into non-lactating glands at a similar dose used in the current study also significantly reduces the citrate : lactoferrin ratio (Rejman et al. 1995). This effect was evident on day 1 after cytokine treatment, while by day 2 there was no significant difference in citrate : lactoferrin ratio between robIL-1β-treated and control glands. From these studies it can be concluded that rboIL-1β had an immediate effect on the involution process. The rboIL-1β-induced acceleration of involution appeared to be relatively short-lived when the cytokine is infused as a single dose. Application of multiple doses of rboIL-1β to glands or slow-release of cytokine might enhance this effect on mammary gland involution.

Our studies showed that infusion of either rboGM-CSF or rboIL-1β into the mammary gland at the cessation of milking increased the number of phagocytic cells in the gland during the early dry period. An increase in phagocyte concentration during the early phase of drying-off may enhance the gland's defences against infection. Infusion of these cytokines, in particular rboIL-1β, resulted in a rapid increase in the rate of mammary gland involution during the early period following cessation of milking. Earlier involution may also result in increased resistance of the gland to new intramammary infections. The gland may also be brought back into production more quickly, so potentially increasing milk yield during the following milking season. The present results suggest that rboIL-1β and rboGM-CSF could have therapeutic use for preventing new intramammary infections during the dry period. Alternative immunomodulators or adjuvants could be administered, which enhance levels of these cytokines in the mammary gland. Future studies will be directed towards determining the efficacy of these cytokines in protecting the gland against experimental challenge with *Streptococcus uberis*.

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