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Effect of Combination Therapy with Lactoferrin and Antibiotics against Staphylococcal Mastitis on Drying Cows

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ABSTRACT. We examined combination therapy with both lactoferrin (Lf) and antibiotics on clinical mastitis due to *Staphylococcus aureus* (*S.aureus*) on drying cows. The clinical symptoms of mastitic quarters were cured 81% of combination therapeutic quarters at 7 days post injection (dpi). Moreover, most of mammary gland secretions (MGSs) in combination therapeutic quarters were normal at 7 days after parturition. In the quarters with combination therapy, *S.aureus* counts, Lf concentrations and content rate of concanavalin A (Con A) low-affinity Lf decreased and were lower than in the quarters treated with Lf or antibiotics alone. The mRNA expression of tumor necrosis factor α (TNFα) of the quarters with combination therapy also decreased and was lower than that of the Lf or antibiotics treated. The mRNA expression of pro-inflammatory cytokines and chemokines in bovine mammary gland epithelial lined cells (BMEC) stimulated with Lf were lower than those of Con A low-affinity Lf stimulated BMEC. Moreover, Lf showed an inhibitory effect to the induction of pro-inflammatory cytokine mRNA expression when co-stimulated with Lf and Con A low-affinity Lf. Nuclear factor kappa B (NFκB) activation was also induced with Con A low-affinity Lf, and the inhibitory effects of Lf were also confirmed on BMEC co-stimulated with Lf and Con A low-affinity Lf. These results indicated that the efficacy of combination therapy with antibiotics and Lf caused antibacterial effect of antibiotics and inhibition of pro-inflammatory cytokine and chemokine production with Lf via the inhibition of NFκB activation.

KEY WORDS: lactoferrin, mastitis, nuclear factor κ B, *Staphylococcus aureus*.

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Staphylococcus aureus (*S.aureus*) is the most important etiological agent of bovine acute and chronic mastitis [14, 31, 40]. Therefore, the many therapeutic methods have been reported, such as antibiotics [8, 28, 29], cytokines [30, 35], immunoglobulin [1], vaccines [27, 34], disinfectants [10, 11, 38] and biologically active substrates [16, 19].

Non-specific anti-infectious agent of lactoferrin (Lf) have various physiological functions, such as anti-bacterial [2, 9, 39], antiviral [15, 33] and anti-inflammatory effects [3], the inhibition of nuclear factor kappa B (NFκB) activation [13] and chelating of iron ion [23]. Therefore, Lf was used for bovine mastitis therapy and recent studies have reported the efficacy of Lf therapy against subclinical mastitis in the lactating period with the antibacterial activity of Lf [19] and chronic mastitis in the dry period with alternative complement activation by Lf [16]. However, the sensitivity of the antibacterial effect with Lf was differed according to the strain of *S.aureus* from bovine mastitis [6, 26]. Moreover, the combination of both Lf and antibiotics was more effective than Lf or antibiotics alone in an *in vitr*o examination [5, 7].

In the dry period, protective agents, such as lysozyme, Lf, complement and immunoglobulin, and the phagocytic activity increased in mammary gland secretions (MGSs) [17]. On the other hand, we found the concanavalin A (Con A) low-affinity Lf molecules in staphylococcal mastitic MGSs during lactation and the early dry period [20]. The Con A low-affinity Lf is a small molecule of 37 kDa, and it shows a decreasing antibacterial effect and chelating of the iron ion [20]. Moreover, Con A low-affinity Lf has inflammatory effects and induces inflammatory cytokines, chemokines, superoxide and leukocyte infiltration into the bovine mammary gland [20]. Then, the content rate of Con A low-affinity Lf increases, following the appearance of mastitic symptoms, and the content rate of Con A low-affinity Lf could be used as a diagnostic indicator of mastitis in drying cows [21].

In this study, we attempted combination therapy with both Lf and antibiotics against *S.aureus* bovine mastitis. Then, we examined the mechanisms of the anti-inflammatory effect of Lf in the *S. aureus* bovine mastitis during the dry period in dairy cows.

MATERIALS AND METHODS

Bovine lactoferrin (Lf) and anti-serum: Bovine Lf was purchased from Morinaga milk industry Co., Ltd., (Tokyo, Japan) and used for a Lf injection test. Anti-bovine Lf rabbit

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serum and anti-bovine Lf rabbit IgG were purchased from YAGAI, Co. (Yamagata, Japan) and were used for concanavalin A (Con A) two dimensional immuno-electrophoresis. The Con A low-affinity Lf was isolated and purified as described previously [20].

Experimental cows: The 22 dry cows from 4 dairy farms were 3 to 6 years old and 1 to 10 days after the onset of the dry period. These cows suffered from clinical mastitis with *S.aureus*. These mastitic cows showed systemic clinical symptoms (pain and fever) and local clinical symptoms (swelling), *S.aureus* counts in mammary gland secretion (MGS) were higher than 200 CFU/m*l*, and somatic cel-counts (SCC) in MGS were more than 500,000 cells/m*l*.

Injection of Lf into mammary glands (MGs): In the early dry periods, from 7 to 10 days after the cessation of milking, the control group (n=6) was intra-mammary injected with a cephalosporin-based antibiotics that contained 250 mg of sodium cefazolin, CEFAZORIN DC (Nippon Zenyaku Kogyo, Fukushima, Japan) per quarter according to the instructions on the package. The Lf injected cows (n=10) were intra-mammarily injected in the mastitic quarters with 200 mg of Lf per quarter in 10 m*l* of sterile phosphate buffered saline (PBS) pH7.2 through the teat canal [16]. In the cows with combination therapy of both Lf and antibiotics (n=9), Lf was injected into the mastitic MG at 3 days after antibiotics injection (0 days post injection (dpi)).

Mammary gland secretions (MGSs): MGS samples were collected before injection (0dpi), 3 and 7 dpi respectevely. MGSs were measured for each SCC, *S.aureus* counts, the concentration of Lf, content rate of Con A low-affinity Lf and the mRNA expression of tumor necrosis factor α (TNFα) in MG cells. The causative staphylococci in mastitic MGSs were isolated with staphylococcus No.110 agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the *S.aureus* counts were measured as described by Kai *et al.* [16, 17]. The coagulase production was determined by tube test of rabbit plasma (Eiken Chemical Co., Ltd., Tokyo, Japan). Isolated staphylococci were identified with a commercial kit (Api staph system, bioMe'rieux sa., Marcyl'Etoile, France). The concentration of Lf was measured by the single radial immunodiffusion (SRID) method (Eco-Biosystem Institute, Co., Furukawa, Japan) [18]. The mRNA expression of TNFα on MG cells was analyzed with the reverse transcription (RT)- polymerase chain reaction (PCR) method. The data were expressed as the mean of the decreasing index on MG cells at 0 dpi.

Examination of cured quarters: To determine whether quarters were cured, the clinical symptoms and MGSs were diagnosed at 7 days after parturition. MGSs were examined for the presence of clots, the bacterial number and modified California Mastitis test (CMT; P.L.tester, Nippon Zenyaku Kogyo) [17].

Con A two-dimensional immunoelectrophoresis: The carbohydrate structure of each Lf fraction was analyzed by Con A two-dimensional immunoelectrophoresis as described previously [20]. First-dimension electrophoresis was performed in a heated agar solution containing 0.02 M Tris-HCl (pH 7.2), 1.2% agarose (SeaKem ME Agarose, FMC BioProduct, Rockland, MA) and 0.1% Con A (Seikagaku Corporation, Tokyo, Japan). The samples (30 μg of Lf/well) were run at 9 V/cm for 1.5 hr. Second-dimension electrophoresis was performed in a heated agar solution containing 0.02 M Tris-HCl (pH 7.2), 0.5% anti-bovine Lf rabbit serum and 6% α-D methylmannoside (SIGMA-Aldrich., Co., Ltd., St Louis, MO), and run at 1.5 V/cm for 15 hr. After drying, the gels were stained with Coomassie Brilliant Blue R-250. The concentration of Con A low-affinity Lf was calculated as described previously [20], and the content rate of Con A low-affinity Lf was calculated with the total Lf concentration with the SRID method.

Cells: Bovine mammary gland epithelial lined cells (BMECs) [32] were adjusted to 4×10^5 cells/m*l* in culture medium. The culture medium was Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Co., Ltd.) including 20% fetal calf serum (FCS), 5 mM sodium acetate, and 10 μg/m*l* of apo-transferrin (SIGMA-Aldrich, Co., Ltd., St. Louis, Mo). The culture medium was changed every 3 days. BMEC were stimulated in the culture medium containing Lf (10 μg/m*l*), Con A low-affinity Lf (10 μg/m*l*), and both Lf (10 μg/m*l*) and low Con A affinity Lf (10 μg/m*l*). Stimulated BMEC were analyzed for the mRNA expression of cytokines and chemokines, and nuclear factor κ B (NFκB) activation.

Assay of cytokine and chemokine mRNA expression: Cytokine mRNA expression in MG cells and BMEC was examined by RT-PCR as described previously [20]. Specific primers for TNFα, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 were made, as reported previously [4, 41]. The mRNA expression levels for each cytokine and chemokine are presented as relative units after normalization to the observed glyceraldehydes phosphate dehydrogenase (GAPDH) [20] level.

Assay of NFκB activation: NFκB activation was analyzed with a transcription factor assay kit (TransAM NFκB/p65; Active Motif North American Carlsbad, CA) according to the manufacturer's instructions as described previously for bovine retinal microvascular endothelial cells [24]. The peroxidase reduction was quantified at 450 nm with a reference wavelength of 655 nm on the microplate reader. The tests were replicated 3 times. The NFκB activation levels are presented as relative units after normalization to the observed NFκB activation level of un-treated (medium) BMEC.

Statistical analysis: Data were expressed as the mean ± standard errors of the means (SEM). *In vitro* experiments were conducted at least three times to confirm the reproducibility of the results. The statistical significance of differences was evaluated by one-way layout ANOVA and a multiple test using the Bonferroni test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Clinical effects of lactoferrin (Lf) injection on mastitis

Table 1. Efficacy of combination therapy with Lf and antibiotics to the clinical mastitis on drying cows

	injected with	0 dpi	3 dpi	7 dpi	7 days after parturition
Cured mastitic quarters[a)] (%)	Antibiotics	0[c]	0	53.6	
	Lf	0	0	52.0	
	Antibiotics and Lf	0	44.4	80.7	
Normal MGS[b)] (%)	Antibiotics				33.3
	Lf				20.0
	Antibiotics and Lf				77.8
SCC ($\times 10^4$/m*l*)	Antibiotics	100 ± 28[c)]			280 ± 77[A]
	Lf	429 ± 98			253 ± 96[A]
	Antibiotics and Lf	446 ± 158[A]			44 ± 14[B,d),e)]

a) "Cured" was defined as the disappearance of local clinical signs (swelling and firmness) of quarters.
b) Normal MGSs contained no clots and its CMT resulted negative.
c) Somatic cell counts (SCC) were analyzed by Flow cytometry.
d) SCC values in combination therapeutic group with different letters (A, B) differ significantly ($P<0.01$).
e) SCC values at the 7 days after parturition with different letters (A, B) differ significantly ($P<0.01$).

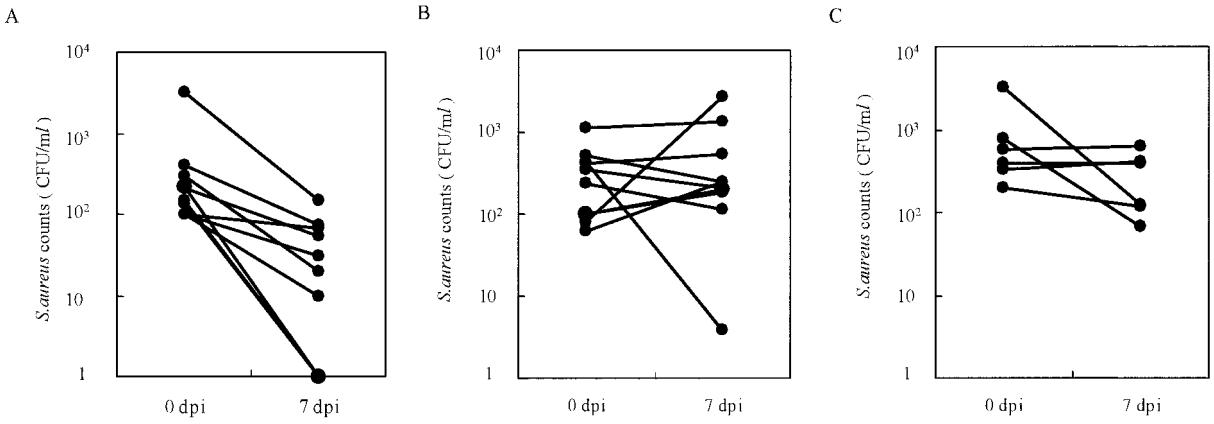

Fig. 1. *S.aureus* counts in each treated drying cow. The mastitic mammary gland secretions (MGSs) were collected at 0 dpi (before injection) and 7 dpi. These MGSs were isolated *S.aureus* with staphylococcus No.110 agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the *S.aureus* counts were measured as described previously (A; combination therapy of Lf and antibiotics, B; Lf injection, C; antibiotics injection) [20].

with S.aureus during the dry period: After injection, the local clinical symptoms (swelling and/or firmness) were cured at a rate of approximately 81% in antibiotics and Lf combination therapeutic quarters at 7 days post injection (dpi). However, the antibiotics or Lf injected quarters showed a low rate of cured quarters (approximately 50 to 55%) at 7 dpi (Table 1). Table 1 also showed the percentage of normal mammary gland secretions (MGSs) at 7 days after parturition in the three treatment groups. Many MGSs in the combination therapeutic quarters are normal (77.8%). But in the other two groups, normal MGS rates are low (antibiotics only; 33.3%, Lf only; 20%). In addition, the somatic cell counts (SCC) of the antibiotics and Lf combination therapeutic mastitic quarters ($44 \pm 14 \times 10^4$/m*l*) was lower than antibiotics injected ($279 \pm 76 \times 10^4$/m*l*) or Lf injected ($253 \pm 96 \times 10^4$/m*l*) quarters at 7days after parturition. The decreases of *S.aureus* counts in MGS are shown in Fig. 1. In the combination therapeutic quarters with both Lf and antibiotics, the decreasing effect on all therapeutic quarters (10/10) at 7 dpi was confirmed. However, the therapeutic effects of each Lf (4/10) and antibiotics therapy (3/6) were lower than combination therapy at 7dpi.

The changes of Lf concentration and the Concanavalin A (Con A) low-affinity Lf content rate: The Lf concentration in MGS increased in Lf and both Lf and antibiotic injected quarters. The maximum value was 35.6 ± 6.4 mg/m*l* in MGS of Lf injected quarters, and 36.8 ± 4.4 mg/m*l* in MGS of Lf and antibiotic injected quarters at 7 dpi. However, we could not confirm the increasing Lf concentration in MGSs in antibiotics injected quarters (Fig. 2A). On the other hand, the content rate of Con A low-affinity Lf in MGS decreased in each injected quarter. We were able to confirm the significant differences in comparison with the content rate of Con A low-affinity Lf in MGS at 0 dpi and 7dpi after Lf and antibiotics injection ($P<0.01$) (Fig. 2B).

Tumor necrosis factor α (TNFα) mRNA expression after

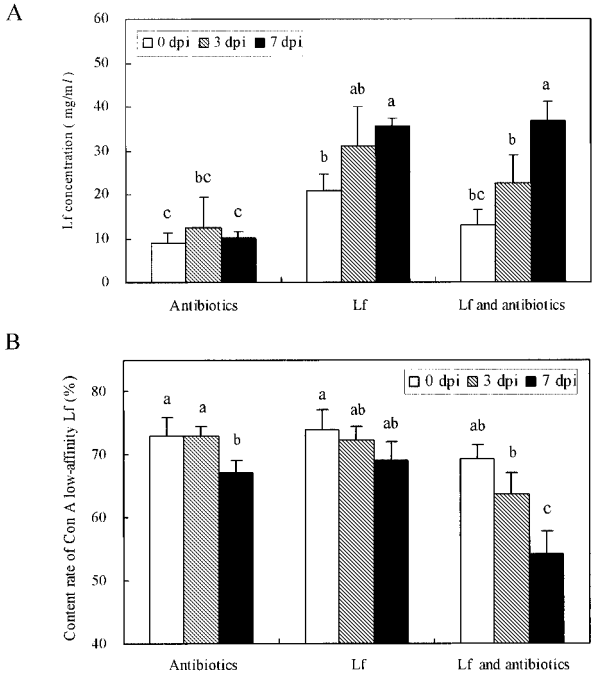

Fig. 2. Concentration of Lf in mammary gland secretions (MGSs) and Con A low-affinity Lf concent rate after injection in MGSs. MGSs were collected from each injected group at 0 dpi (before injection; □), 3 dpi (▨) and 7 dpi (■), respectively. The total concentration of Lf in MGSs (A) was measured by the SRID method. The concentration of low Con A affinity Lf was calculated with the total Lf concentration and the height of the peaks on Con A two-dimensional electrophoresis (B) [20]. The data are expressed as the means of the decreasing index ± SEM. The values of each tests with different letters (a, b, c) differ significantly (P<0.05).

injection: The mRNA expression of TNFα on MG cells after the combination therapy (polymerase chain reaction (PCR) index at 7dpi; 0.5 ± 0.07) decreased in comparison with the PCR index of TNFα at 0 dpi. We confirmed the significant differences at 7 dpi in comparison with the PCR index of TNFα on mammary gland (MG) cells at 0 dpi. On the other hand, we could not confirm the decrease of mRNA expression of TNFα on MG cells in both the antibiotics and Lf injected quarters (Fig. 3).

mRNA expression of cytokine and chemokine on Lf treated bovine mammary gland epithelial lined cells (BMECs): Lf stimulated BMECs increased the expression of the pro-inflammatory cytokine mRNA of both IL-6 and TNFα in comparison with the non-treated (medium) BMECs. The greatest increased level of mRNA expression was Con A low-affinity Lf stimulated BMECs (PCR index of IL-6; 1.8 ± 0.5, PCR index of TNFα; 1.7 ± 0.3) in comparison with the non-treated BMECs. Moreover, the mRNA expression of both IL-8 and MCP-1 also increased in BMECs stimulated with Con A low-affinity Lf (PCR index of IL-8; 1.1 ± 0.5, PCR index of MCP-1; 1.8 ± 0.6) in comparison with the non-treated BMECs (PCR index of IL-8;

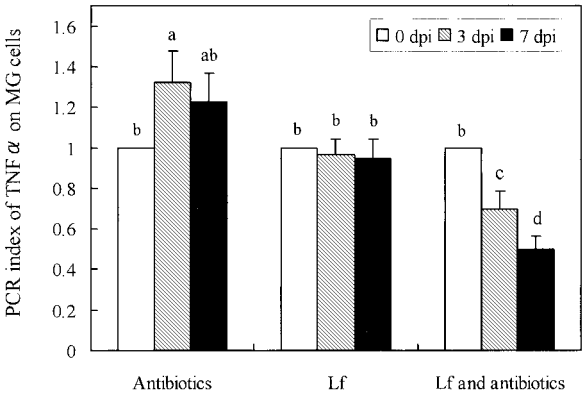

Fig. 3. mRNA expression of TNFα on mammary gland (MG) cells after injection. The mRNA expression of TNFα on MG cells (0 dpi (before injection), 3 dpi and 7 dpi) was analyzed by RT-PCR. PCR index are expressed as the means of the decreasing index ± SEM on MG cells at 0 dpi (before injection; □), 3 dpi (▨) and 7 dpi (■). The values of each tests with different letters (a, b, c, d) differ significantly (P<0.05).

0.6 ± 0.2, PCR index of MCP-1; 3.0 ± 0.7). We confirmed the low increased mRNA expression level of MCP-1 (PCR index; 1.8 ± 0.6) on Lf stimulated BMEC in comparison with non-treated BMECs. However, we were able to confirm the increased mRNA expression of IL-8 (PCR index; 0.60 ± 0.20) (Fig. 4). On the other hand, mRNA expression of each cytokine and chemokine decreased on co-stimulation with both Lf and Con A low-affinity Lf in comparison with Con A low-affinity Lf stimulation.

Nuclear factor κ B (NFκB) activation on Lf treated BMECs: Con A low-affinity Lf (NFκB Index; 7.5 ± 0.9) showed a higher level of NFκB activation than Lf stimulated BMECs (NFκB Index; 1.7 ± 0.5) (P<0.01). Although, Lf and Con A low-affinity Lf co-stimulation on BMECs (NFκB Index; 3.6 ± 0.8) showed a higher level than Lf stimulated BMECs (P<0.01), it showed a lower level than Con A low-affinity Lf stimulation (P<0.01). We confirmed the inhibitory effect of NFκB activation by Lf on co-stimulation of Lf and Con A low-affinity Lf stimulation.

DISCUSSION

The number of quarters in which the local clinical symptoms and mammary gland secretions (MGSs) were cured was approximately 2 times in comparison with antibiotics or lactoferrin (Lf) therapy alone. Moreover, all mammary gland (MG) treated with the combination therapy of both Lf and antibiotics decreased the causative bacteria counts in MGS. In the case of antibiotics therapy, half of MG had decreased the causative bacterial counts in MGS. We tried the combination therapeutic pretests such as Lf injection at 3 days before antibiotics injection, both Lf and antibiotics injection at same time, and Lf injection at 3 days after antibiotics injection in the mastitic cows with *S.aureus*. Then, we confirmed that the most effective combination therapy

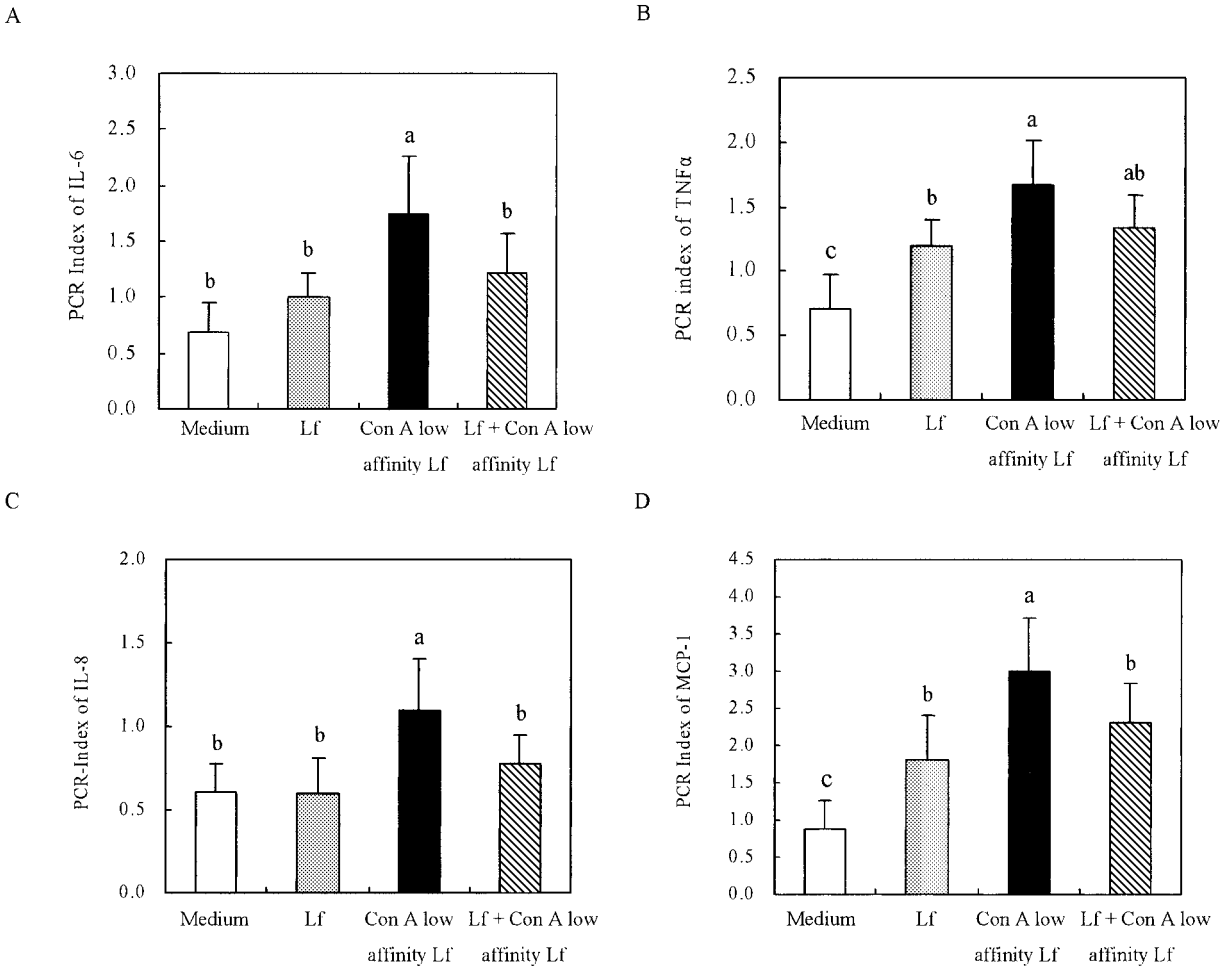

Fig. 4. mRNA expression of IL-6, TNFα, IL-8 and MCP-1 in Lf stimulated bovine mammary gland epithelial lined cells (BMECs). Each non-treated BMEC (medium; □), Lf (10 μg/ml; ▨), Con A low-affinity Lf -stimulated BMEC (10 μg/ml; ■), and Lf and Con A low-affinity Lf co-stimulated BMEC (each 10 μg/ml; ▩) were incubated at 37°C for 4 hr. The mRNA expression of GAPDH, IL-6 (A), TNFα (B), IL-8 (C) and MCP-1 (D) were analyzed by RT-PCR. The mRNA expression levels IL-6, TNFα, IL-8 and MCP-1 for each cytokine are presented as relative units after normalization to the observed GAPDH level. The data are expressed as the means of PCR index ± SEM. a, b, c: Different letters indicate significant differences ($P<0.05$).

was Lf injection at 3 days after antibiotic injection (data not shown). Bovine Lf has antibacterial effects and we attempted to treat coliform mastitis and subclinical mastitis in lactating and drying cows [16, 19, 22]. However, each report indicated a low antibacterial effect of Lf and/ or the neutralizing effect of lipopolysaccharide (LPS) than antibiotics on coliform mastitis [25].

S.aureus causes intractable mastitis, including acute and chronic mastitis [12, 40]. Moreover, staphylococcal mastitic MG cells express pro-inflammatory cytokines [31]. In these experiments, the mRNA expression of tumor necrosis factor α (TNFα) in MG cells confirmed the lower level following combination therapy. On the other hand, we isolated the concanavalin A (Con A) low-affinity Lf molecule from staphylococcal mastitic MGSs. Con A low-affinity Lf has previously been shown to have an inducing effect of pro-inflammatory cytokines and chemokines on bovine mammary gland epithelial lined cells (BMEC) [20], and induces the inflammatory reactions such as leukocyte infiltration with mammary gland infusion [20]. In this study, the content rates of Con A low-affinity Lf in MGSs treated with combination therapy showed lower levels in comparison with both Lf and antibiotics therapeutic quarters. Moreover, Con A low-affinity Lf showed a higher inducing effect of pro-inflammatory cytokines and chemokines, such as IL-6, TNFα, IL-8 and MCP-1 comparison with Lf. On the other hand, Lf showed an inhibitory effect to the induction of pro-inflammatory cytokine and chemokine by Con A low-affinity Lf on co-stimulation with Lf and Con A low-affinity Lf on BMEC. Lf shows the down-regulation of TNFα and IL-6 in LPS induced endotoxemia in mice, based on the inhibition of nuclear factor κ B (NFκB) activation caused by the

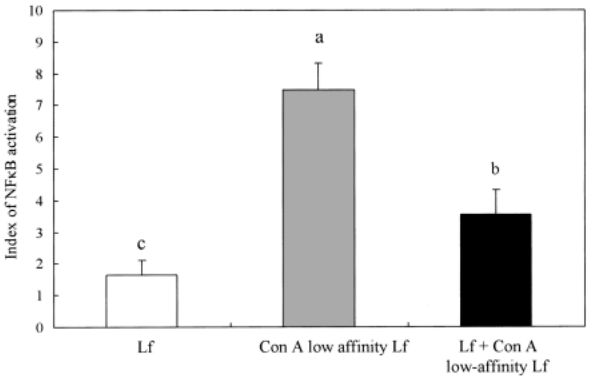

Fig. 5. Activation of NFκB in Lf stimulated bovine mammary gland epithelial line cells (BMECs). The BMEC were stimulated with Lf (□), Con A low-affinity Lf (▩), and Lf + Con A low-affinity Lf (■) at 37°C for 1 hr. NFκB activity measured with ELISA [24]. The data express the index of NFκB activation in comparison with non-treated BMEC. a, b, c: Different letters indicate significant differences (P<0.05).

binding activities of Lf and LPS [42]. Therefore, we examined the inhibitory effect of NFκB activation on Lf stimulated BMEC. Lf stimulated BMEC showed the lower level of NFκB activation in comparison with Con A low-affinity Lf stimulation. Moreover, Lf showed an inhibitory effect of NFκB activation with Con A low-affinity Lf stimulation on BMECs. Therefore, these results suggested that the efficacy of combination therapy with antibiotics and Lf caused both an anti-bacterial effect due to antibiotics and an anti-inflammatory effect of Lf based on the inhibition of NFκB activation.

It was reported that combination therapy with penicillin G and Lf is effective to the infection with *S.aureus* in mouse mammary gland epithelial cells [5, 7]. On the other hand, Lf has low anti-bacterial activity against the some strains of *S.aureus* that were isolated from bovine mastitic MGSs [29]. Therefore, these reports indicated that Lf acted with penicillin G to enhance the phagocytes of *S.aureus* by bovine polymorphonuclear neutrophils and to decrease the invasion of mammary epithelial cells [7]. In this experiment, Lf had an anti-bacterial effect, however, this physiological effect had an activity in comparison with the anti-bacterial effect of the antibiotics. On the other hand, Lf showed an anti-inflammatory effect to the intractable mastitis with *S.aureus* in drying cows. Moreover, the major target cells of Lf, such as macrophages and monocytes, having Lf receptor increases and being activated during the dry period [36, 37]. Therefore, the combination of antibiotics and Lf was an effective therapy for intractable mastitis due to *S.aureus* in drying cows.

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