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Lactoferrin Stimulates A *Staphylococcus aureus* Killing Activity of Bovine Phagocytes in the Mammary Gland

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***Abstract*: Lactoferrin (Lf) may play a key role in the clearance of microorganisms from a host. To study *in vitro* the bactericidal mechanisms of Lf during nonlactating periods, we investigated whether the effects of Lf were influenced by bovine mammary gland secretory cells (MGSC) and fresh normal bovine serum (NBS) as a source of complement. Phagocytic killing tests demonstrated that a phagocytic mixture of unopsonized *Staphylococcus aureus* (*S. aureus*) and MGSC in the presence of Lf reduced bacterial growth, compared with that of unopsonized *S. aureus* and MGSC without Lf. The opsonization with Lf and fresh NBS together resulted in more than a 95% reduction in CFU. The activation of complement induced by Lf also resulted in increased deposition of C3 on *S. aureus*, and the phagocytic activity of MGSC was augmented by opsonization with Lf and fresh NBS. Inhibition of C3 deposition by Lf was not induced in the presence of Mg-EGTA, but was induced by the addition of bovine Lf antiserum. These results strongly suggest that Lf induces the activation of complement in fresh NBS mainly through an alternative pathway. The results demonstrated a Lf-dependent, antibody-independent and complement-mediated phagocytic killing of *S. aureus*, and implied that Lf was synergistically capable of activating both the alternative pathway of the bovine complement cascade and phagocytosis by phagocytes.**

***Key words*: Lactoferrin, Alternative pathway, *Staphylococcus aureus*, Phagocyte**

Bovine Lf, an 80 kDa iron-binding glycoprotein, is present in milk and specific granules of polymorphonuclear leukocytes (12). Lf is thought to contribute to the prevention of microbiological infectious diseases (3, 6, 27, 29). The physiological concentration of Lf increases in mammary secretions after the cessation of milking and reaches a high during the mid-nonlactating period (31). In this period, from 21 to 35 days after all milking has stopped, it is known that host defence against infection increases (17).

The direct bactericidal activity of Lf differs among strains (1). Lf can be resistant (1) or sensitive (2) to *Staphylococcus aureus*. In our preliminary study, it was found that Lf could not kill *S. aureus* (ATCC25923) at concentrations less than 1 mg/ml. Therefore, we tried to investigate the host immune mechanism by which Lf induced the killing of *S. aureus* at physiological concentrations in bovine mammary gland during nonlactating periods.

Both the amount and the hemolytic activity of bovine complement were low (22, 24) or undetectable (23), during the lactating period. The hemolytic activity increases in mammary gland secretions at the start of a nonlactating period after milking has ceased (19, 24), and remains high until the production of colostrum (25).

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Abbreviations: C3, the third component of the complement system; C1q, a sub component of the first component of the complement system; CR, complement receptor; EDTA, ethylenediaminetetraacetate; EGTA, ethylene-glycol-bis-aminomethyl-ether-*N*-*N*'-tetraacetate; GVB, gelatin veronal buffer; Lf, lactoferrin; MGSC, mammary gland secretory cells; NBS, normal bovine serum.

Bovine Lf-coated *Streptococcus agalactiae* activates the classical complement pathway in fresh bovine agammaglobulinaemic serum (20). On the other hand, Lf can not block the deposition of C1 or C4, but blocks the formation of EAC142, as C3 convertase, resulting in an inhibition of C3 deposition (10, 11). Lf is known to bind many ligands, although its biological function in complement is not yet known. To study *in vitro* the bactericidal mechanisms of Lf during nonlactating periods, we investigated whether it induced the activation of complement through an alternative pathway in fresh normal bovine serum (NBS) and opsonized the bacteria. Also, we tried to demonstrate that the bacteria opsonized by both Lf and complement were reduced by phagocytes from bovine mammary gland.

Materials and Methods

Lactoferrin. Lactoferrin (Lf) from bovine colostrum was purchased from Wako Pure Chemicals (Osaka, Japan).

Bacteria. Staphylococcus aureus (ATCC25923) was obtained from the American Type Culture Collection (Rockville, Md., U.S.A.).

MGSC separation. MGSC (Mammary gland secretory cells) were separated according to the procedure of Jensen and Eberhart (8). Briefly, udders were not treated with antibiotics at the end of lactation. Mammary glands were considered normal if bacterial counts in all milk samples were less than 250 CFU/ml. Twenty-three samples of mammary secretions were obtained aseptically from 8 glands during the early nonlactating period, the first 10 to 21 days after the cessation of milking. Samples were centrifuged at $1,000 \times g$, for 20 min at 4 C to remove fat and whey, then washed three times with saline containing 1% heat-inactivated fetal bovine serum. After a wash, MGSC were suspended in RPMI 1640 medium containing 5% fetal calf serum (Life Technologies, New Zealand) and adjusted to a concentration of 2×10^6 cells/ml. The MGSC comprised 61.0% polymorphonuclear leukocytes, 4.3% macrophages and 46.7% cells positive for CD11b, also known as complement receptor 3 and involved in phagocytosis, as determined by microscopic assay (8), and flow cytometry using bovine CD11b monoclonal antibody (VMRD, Pullman, Wash., U.S.A.) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Bethyl Laboratories, Montgomery, Ala., U.S.A.). The characterization of MGSC during the early nonlactating period indicated typical phagocyte-rich population.

Buffers. Gelatin veronal buffer (GVB), pH 7.4, contained 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin. GVB supplemented with 33 mM ethylene-glycol-bis-aminomethylether-*N*-*N'*-tetraacetate (EGTA; Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 4.9 mM $MgCl_2$ (Mg-EGTA), was used for the assay of the alternative complement pathway (26). GVB supplemented with 10 mM ethylenediaminetetraacetate (EDTA; Sigma) was used to stop the reaction of both the classical and alternative pathway.

Opsonization of S. aureus. *S. aureus* were grown overnight in Trypticase soy broth (Nissui, Tokyo). FITC-labeled *S. aureus* (approximately 2×10^7 CFU) were washed with saline containing 0.2% human albumin (Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands) and were incubated with bovine lactoferrin at a concentration of 0.01 to 10 mg/ml in 10% fresh or heat-inactivated NBS containing GVB or Mg-EGTA. After the mixture had been incubated for 1 hr at 37 C, opsonized *S. aureus* were washed three times with saline containing 0.2% human albumin.

Assay for growth inhibition of S. aureus. To assess the MGSC-mediated inhibition of *S. aureus* growth, live bacteria were enumerated. Phagocytic mixtures comprised 50 µl of either an intact or opsonized bacterial suspension (4×10^5 colony formation units [CFU]), 500 µl of MGSC suspension (1×10^6 cells), and 100 µl of Lf diluted RPMI 1640 containing 5% heat-inactivated fetal bovine serum (final concentration, 0.01−10 mg/ml). Control tubes contained increasing concentrations of Lf, or 5% heat-inactivated fetal bovine serum in medium alone. After the mixtures had been incubated for 6 hr at 37 C, portions of the suspended mixtures were diluted in saline. Bacterial counts in diluted mixtures were made by enumerating CFU on plate agar, using *Staphylococcus* agar No.110 (Nissui) after incubation for 48 hr at 37 C. In this experiment, the limit of detection was 2×10^3 CFU/ml.

Microscopic assay of phagocytosis. After incubation for 30 min at 65 C, heat-killed *S. aureus* (approximately, 5×10^8 CFU) were labeled with 250 µg/ml of FITC in 0.1 M sodium carbonate buffer (pH 9.6) for 1 hr at 37 C and washed four times with phosphate-buffered saline (pH 7.2). The FITC-labeled *S. aureus*-ingested MGSC (2×10^4 cells) were made to adhere to slides, using Cytospin (Shandon Southern Instruments, Sewickley, Pa., U.S.A.). After air drying, the slides were stained with May-Grünwald-Giemsa reagents and observed under an oil immersion objective, using a light and fluorescent microscope system, the BX-60 (Olympus, Tokyo). To visualize the relative distribution of ingested FITC-labeled *S. aureus* and the morphology of the cells, the same range of views was collected in each photograph.

Phagocytosis of FITC-labeled bacteria by MGSC. FITC-labeled *S. aureus* (approximately, 10^7 CFU) were

incubated with MGSC (10^6 cells/ml) in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum for 30 min at 37 C, with continuous agitation. The phagocytosis of FITC-labeled *S. aureus* was examined by FACSCalibur analysis, using forward and side scatter parameters to gate macrophages and polymorphonuclear leukocytes. The results were expressed as the mean fluorescence intensity (MFI) of 20,000 cells.

Preparation of complement reagents. Normal bovine serum (NBS) was obtained by centrifugation of blood collected from five clinically healthy cows and stored at −80 C. These sera were used as a source of complement and were virtually devoid of antibodies against *S. aureus*, as ascertained by enzyme-linked-immunosorbent assay (ELISA) using *S. aureus*-immobilized microtiter plates (23) (results not shown). Bovine C3 was isolated according to a modification of the procedure of Kai et al. (9). Briefly, 100 ml of serum was left to react with 60 mg of zymosan A (Sigma) for 1 hr at 37 C. The precipitate was washed four times with GVB, and resuspended in 2% methylamine-HCl (Wako) containing GVB to disrupt covalent binding (9). C3 was separated from zymosan particles with the methylamine-HCl containing buffer for 1 hr at 37 C. To remove zymosan particles, zymosan was precipitated by centrifugation for 10 min, $3{,}000 \times g$, and supernatants were stored at −80 C. The purity of C3 was checked by precipitation reaction in gel (22). Antisera against bovine complement C3 were fostered in Japanese white rabbits. For anti-C3, rabbits were injected intramuscularly once a week for 6 weeks with 1 mg of purified C3 in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) and bled 14 days after the last injection. Antisera were heat-inactivated for 30 min at 56 C. Anti-C3 rabbit sera were partially purified to IgG, using a DE52 cellulose (Whatman, England) column (1.8×7.9 cm) equilibrated with 0.02 M Tris. The column was eluted with 200 ml of 0.02 M Tris, 0.05 M NaCl. Contaminated anti-bovine immunoglobulins were removed by passing them twice through the bovine IgG1, IgG2 or IgM bound to cyanogen bromide (CNBr)-activated sepharose 4B (Pharmacia Biotech AB, Sweden)-column (1.8×4.0 cm) equilibrated with 0.02 M Tris. Anti-C3 rabbit IgG (1 ml) was absorbed twice for 2 hr at 37 C and once overnight at 4 C with 10^{10} cells of heat-killed *S. aureus*. Anti-C3 rabbit IgG, subsequently, was checked by precipitation reaction in gel (results not shown).

Complement deposition on S. aureus. To study all the pathways of complement activation, heat-killed *S. aureus*, treated for 30 min at 65 C, (10^7 CFU) were incubated with increasing concentrations of bovine Lf for 30 min at 37 C. In some experiments, diluted (1:100) $F(ab)_2$ fragments of rabbit anti-bovine Lf antiserum (Bethyl) were added to the mixture. After a wash in GVB, the Lf-bound *S. aureus* was incubated with 10% NBS in the presence of either GVB or Mg-EGTA for 1 hr at 37 C. To detect the deposition of the complement component, C3, opsonized *S. aureus* were incubated with anti-C3 rabbit IgG for 30 min on ice, and incubated with 1:100-diluted FITC-conjugated goat anti-rabbit IgG secondary antibody (Bethyl). After each incubation, the bacteria were washed twice with saline. Immuno-stained C3 deposited on *S. aureus* was determined by FACSCalibur analysis, using forward and side scatter parameters to gate on *S. aureus*. The results were expressed as the mean fluorescence intensity (MFI) of 50,000 bacteria.

Statistical analysis. Differences between the mean values for unopsonized *S. aureus* and opsonized *S. aureus*, as well as for the presence of Lf and the absence of Lf, were analyzed using the Student's *t*-test. A P value less than 0.05 was considered significant.

Results

Opsonization of S. aureus with Lf and NBS Inhibits S. aureus *Growth*

The aim of these experiments was (i) to investigate whether Lf enhanced the opsonic activity of serum and increased the phagocytic activity of MGSC, (ii) to determine which pathway would induce deposition of complement at the site where Lf bound to *S. aureus*.

To that end, the effect that opsonizing bacteria with Lf, NBS and Lf plus NBS have on their growth was studied in medium containing MGSC alone (Fig. 1A) or MGSC and Lf (Fig. 1B). It was clearly shown that the growth of *S. aureus* was inhibited significantly in the MGSC suspension when the bacteria were opsonized with Lf plus NBS (Fig. 1, A and B). Treatment with either Lf or NBS was less effective on the growth inhibition than that with Lf plus NBS. The inhibition of *S. aureus* growth was ineffective when complement was heat-inactivated (Fig. 1). Surprisingly, chelation of the divalent ions of fresh NBS with Mg-EGTA, which is used as an inhibitor of the calcium-dependent activity of C1 (21) and the calcium-dependent binding activity of mannose binding lectin to carbohydrate (13), did not affect the inhibition of *S. aureus* growth caused by opsonization with fresh NBS and 1 mg/ml of Lf together (Fig. 1, A and B). In inhibiting *S. aureus* growth, the opsonization with fresh NBS and Lf in the presence of 10 mM EDTA had a similar effect on the opsonization with inactivated NBS and Lf (results not shown). These results indicate that the inhibition of bacterial growth may be due to ingestion by MGSC, because of opsonization of the bacteria by Lf and complement in NBS. In addition, these results suggest that the opsonic activity by the complement system can

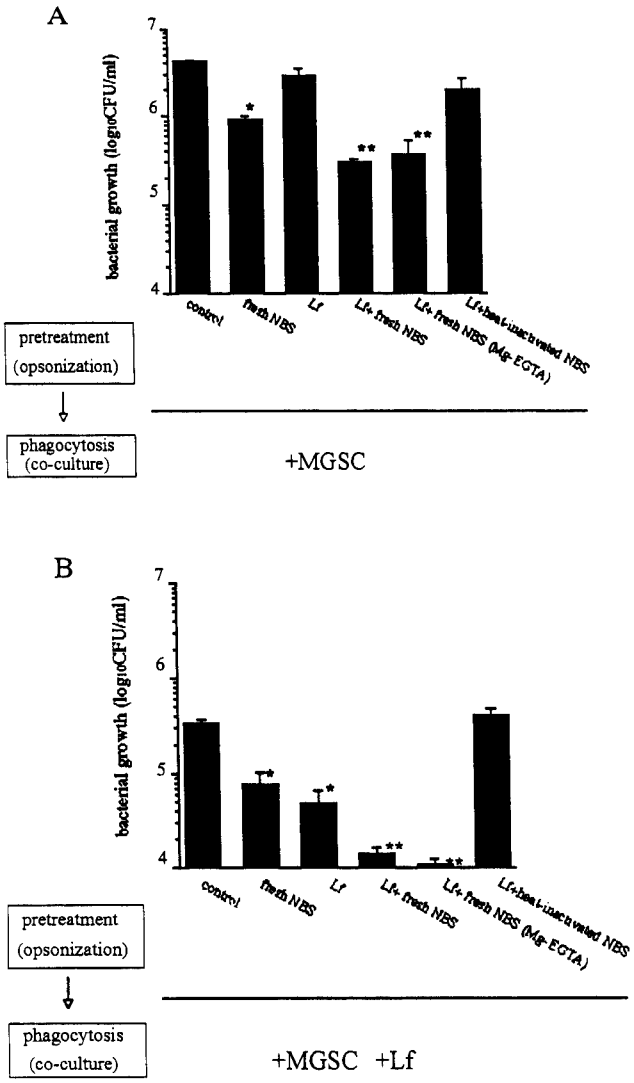

Fig. 1. Lf induces inhibition of *S. aureus* growth. *S. aureus* was opsonized with 10% fresh NBS diluted in GVB or Mg-EGTA, in the presence or absence of Lf (1 mg/ml) for 1 hr at 37 C. Controls included the unopsonized *S. aureus*, and *S. aureus* opsonized with Lf alone, fresh NBS alone, or Lf plus heat-inactivated NBS. After opsonization, treated *S. aureus* (4×10^5 CFU) were co-cultured with MGSC (1×10^6 cells) in the absence (A) or presence (B) of Lf (1 mg/ml) for 6 hr at 37 C. Data are means for three separate experiments $\pm$ standard deviation. *, $P<0.05$; **, $P<0.01$.

be obtained through the activation of complement, via not the classical or lectin pathway, but the alternative pathway. The phagocytotic activity of bovine MGSC activated by the incubation of a phagocytic mixture with 1 mg/ml of Lf (Fig. 1B) was greater than that of unstimulated intact MGSC incubated without Lf (Fig. 1A).

Lf Induces Deposition of Complement Component on S. aureus

To confirm further the effect of Lf on the activation of complement, we tested whether Lf can increase the

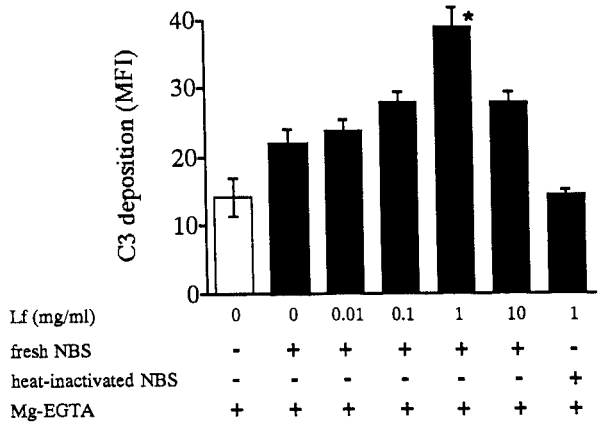

Fig. 2. Lf increases C3 deposition on *S. aureus* via the alternative complement pathway. Heat-killed *S. aureus* were incubated in 10% fresh or heat-inactivated NBS with increasing concentrations of Lf in the presence of Mg-EGTA for 1 hr at 37 C (solid bar). Deposits of C3 on *S. aureus* were detected with bovine C3 anti-rabbit IgG and goat anti-rabbit IgG FITC labeling, and determined by FACSCalibur. Background levels indicated the mixture contained *S. aureus* without NBS in the presence of Mg-EGTA (open bar). Data are MFI for three separate experiments $\pm$ standard deviation. *, $P<0.05$.

deposition of a complement component on *S. aureus* by flow cytometry. The contribution of the activation to the Lf-promoted deposition of C3 was determined by using bovine C3 anti-rabbit IgG. Figure 2 shows that Lf increases C3 deposition on *S. aureus* in the presence of Mg-EGTA and fresh NBS, which is devoid of anti-*S. aureus* antibodies. These results suggest that Lf increases C3 deposition on *S. aureus* through the alternative pathway of the complement cascade.

S. aureus (16) and coagulase negative staphylococci (CNS) (15) express the Lf receptor which is known as at least two proteins of 67 and 92 kDa. But it is not clear whether the bacterial killing by Lf results from the interaction of Lf and Lf receptor. To investigate the effect of Lf on the bacterial surface, *S. aureus* were bound to Lf, washed, and opsonized with fresh NBS. Lf increased C3 deposition on *S. aureus* in 10% NBS with GVB (Fig. 3). These results indicated that Lf could bind to *S. aureus*. Inhibition of C3 deposition by Lf was not induced in the presence of Mg-EGTA, but did occur on addition of bovine Lf antiserum. Heat-inactivation, which destroyed factor B of the alternative pathway and C1q of the classical pathway, abolished the deposition. The C3 deposition was not induced on addition of Lf to fresh NBS in the presence of 10 mM EDTA (results not shown). Taken together, these results suggest that the binding of *S. aureus* to Lf activates the alternative pathway of the bovine complement cascade through the interaction of C3 with Lf bound to the bacteria.

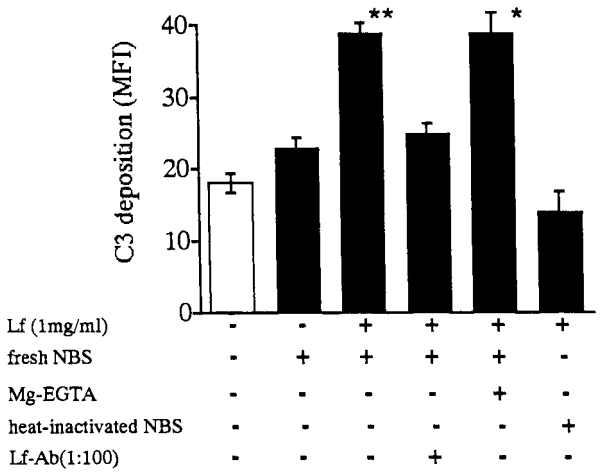

Fig. 3. Lf promotes the deposition of complement component on *S. aureus* in the opsonic mixtures containing fresh NBS. Heat-killed *S. aureus* were incubated in either GVB or Mg-EGTA with or without Lf (1 mg/ml) for 30 min at 37 C (solid bar). Then, an opsonic mixture was added, either an anti-rabbit serum to bovine Lf (diluted 1:100) or buffer alone, for 30 min at 37 C. After a wash, Lf-bound *S. aureus* were incubated with 10% fresh NBS diluted in either GVB or Mg-EGTA for 1 hr at 37 C. Deposits of complement components were detected with bovine C3 anti-rabbit IgG and goat anti-rabbit IgG FITC labeling, and determined by FACSCalibur as described in "Materials and Methods." Background levels indicated the mixture contained *S. aureus* plus GVB (open bar). Data are MFI for three separate experiments ± standard deviation. *, $P<0.05$; **, $P<0.01$.

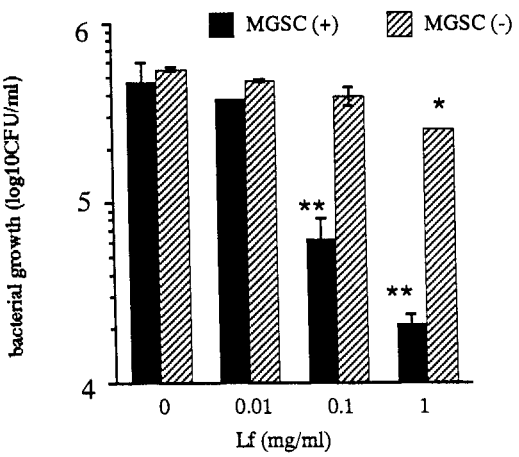

Fig. 4. Lf inhibits bacterial growth in the presence of MGSC. *S. aureus* (5×10^4 CFU) were incubated with MGSC (1×10^6 cells) or without MGSC in the presence of increasing concentrations of Lf in a phagocytic mixture for 6 hr at 37 C. The increase in colony forming units per ml (CFU/ml) was used to evaluate bacterial growth. Data are means for three separate experiments ± standard deviation. *, $P<0.05$; **, $P<0.01$.

Lf Inhibits S. aureus *Growth in the Presence of MGSC*

MGSC activated by Lf inhibited effectively the growth of *S. aureus* in bacteria culture, as indicated in Fig. 1, A and B. Bovine Lf promoted the phagocytosis of FITC-labeled latex beads by human neutrophils, at concentrations from 0.05 to 0.25 mg/ml (14). Therefore, we examined whether Lf could inhibit the growth in the presence of MGSC rich in phagocytes (8), such as polymorphonuclear leukocytes, macrophages and monocytes during nonlactating periods.

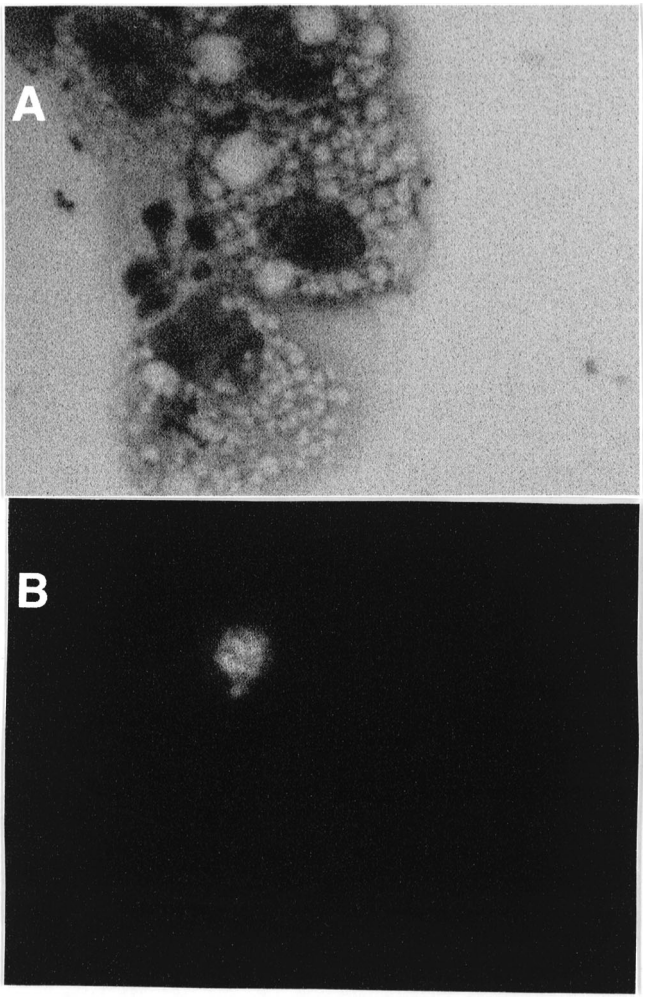

Fig. 5. Phagocytosis of *S. aureus* by bovine MGSC via complement receptor. FITC-labeled *S. aureus* ingested by MGSC, were stained with May-Grünwald Giemsa, represented in (A) and (B), as described in "Materials and Methods." FITC-labeled *S. aureus* in MGSC are represented in (A) and Giemsa-stained MGSC are represented in (B). Magnification, ×1,000.

To this end, unopsonized *S. aureus* were incubated with increasing concentrations of Lf with or without MGSC for 6 hr at 37 C. As shown in Fig. 4, *S. aureus* could multiply more in the culture medium with Lf in the absence than the presence of MGSC. A concentration of 0.1 mg/ml was sufficient to kill or arrest the growth of the bacteria in the presence of MGSC, while even 2 mg/ml of Lf could not exert the same effect in the absence of MGSC (results not shown). These results suggest that Lf

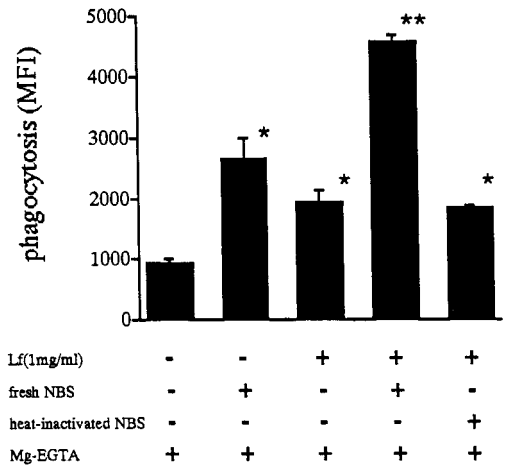

Fig. 6. Lf promotes the phagocytosis of *S. aureus* by MGSC after opsonization with fresh NBS in the presence of Mg-EGTA. FITC-labeled *S. aureus* were opsonized under various conditions in the presence of Mg-EGTA. The opsonized *S. aureus* were incubated with MGSC for 30 min at 37 C. Phagocytosis of bacteria was assessed by FACSCalibur analysis, using forward and side scatter parameters to gate granulocytes and macrophages. Data are MFI for four separate experiments ± standard deviation. *, $P<0.05$; **, $P<0.01$.

induces the inhibition of *S. aureus* growth in culture through the MGSC.

Lf Increases Opsonophagocytosis of S. aureus *by MGSC*

Since the opsonization by Lf and fresh NBS together inhibits effectively *S. aureus* growth in the presence of MGSC, we speculated that Lf could enhance the opsonophagocytosis by MGSC. To test this possibility, the *S. aureus* ingested by MGSC were explored by a microscopic assessment of phagocytosis after the incubation of *S. aureus* with Lf and fresh NBS together. After opsonization in the presence of Mg-EGTA, *S. aureus* was co-cultured with MGSC for 30 min at 37 C. If phagocytosis accompanied the inhibition of *S. aureus* growth, then opsonized *S. aureus* ingested by MGSC should be present. Figure 5 shows that *S. aureus* was ingested by MGSC. The phagocytosis assay, which has proved useful in the study of the opsonization of *S. aureus*, was used to assess the various treatments for opsonization. Also, the contribution of the alternative pathway in the presence of Mg-EGTA after treatment with fresh NBS alone compared with fresh NBS plus Lf was examined using FITC-labeled *S. aureus*. Figure 6 shows that an apparent increase in the phagocytosis of *S. aureus* opsonized with 10% fresh NBS alone in the presence of Mg-EGTA was obtained. Addition of Lf to the opsonized mixture together with fresh NBS resulted in the highest level of phagocytosis of all the treatments. Taken together, these results suggest that bacteria opsonized with Lf plus fresh NBS as a source of complement potentiate the phagocytosis of live bacteria through interaction with C3 on *S. aureus* and the complement receptors of phagocytes.

Discussion

Lf may play a key role in the clearance of microorganisms from hosts. Therefore, we investigated whether Lf enhanced the killing of *S. aureus* in the presence of MGSC from the bovine mammary gland during nonlactating periods. Without opsonization, *S. aureus* growth was inhibited effectively by MGSC in the presence of Lf. However, the level of bactericidal activity in this experiment was lower than that of the preliminary study in which Lf had been infused in bovine mammary gland *in vivo*, during a nonlactating period (unpublished data).

Since Lf binds to bacteria, without antibody, and promotes the deposition of C1q on *Streptococcus agalactiae* (*S. agalactiae*) (20), we speculated that it contributed to the deposition of complement and enhancement of phagocytic killing. The deposition of C1q on *S. agalactiae* required Lf at a concentration of 0.01 to 0.04 mg/ml, which is the physiological concentration range in milk during periods of lactation. Surprisingly, an assay of C3 deposition revealed that 1 mg/ml of Lf activated complement in the presence of Mg-EGTA to block both the classical and lectin pathway. Therefore, we demonstrated that Lf could activate the alternative pathway. At less than 0.1 mg/ml of Lf, this effect was weak.

It is known that the physiological concentration of Lf in the bovine mammary gland during nonlactating periods is more than 0.25 mg/ml. To inhibit the formation of EAC142 (9, 10), Lf at 1 mg/ml may act directly to bind C3, without using the classical pathway initiated by the deposition of C1q or C4. Also, this result was similar to the finding that Lf promoted both the deposition of C3 and bacterial killing after opsonization of *S. aureus*. From these results, it is evident that at concentrations less than 0.1 mg/ml, Lf could not activate the alternative complement pathway. These results, therefore, suggest that the activation of the alternative pathway occurs at high concentrations of Lf, such as in mammary gland secretions after the infusion of Lf or after cessation of milking.

In this study, 10 mg/ml of Lf most activated the complement, as assessed by hemolytic assay (results not shown). However, the extent of C3 deposition at this concentration of Lf in the presence of Mg-EGTA was low. These results suggest that 10 mg/ml of Lf might inhibit the deposition of C3 on *S. aureus*, since an excess of Lf depleted most of the C3 in fresh NBS as a result of the activation of complement.

The assay of C3 deposition showed that the presence of Mg-EGTA had little influence on the efficacy. It was concluded that the main effect of Lf was to facilitate the deposition of C3 on the bacteria and to activate the alternative complement pathway. Bacterial counts of live *S. aureus* were not affected by opsonization (results not shown). The extent of the inhibition of *S. aureus* growth was similar to the extent of the phagocytosis. Therefore, the reduced effect of live *S. aureus* opsonized with Lf and complement together suggests that complement receptors, such as complement receptor 1 (CR1) which can bind to C3b (4), or CR3 which can bind to iC3b (28), contribute to phagocytosis.

The bactericidal activity of Lf against *S. aureus* ATCC 25923 in the presence of MGSC was stronger than that of the cell-free medium. Furthermore, to determine if phagocytic activities were enhanced by Lf, MGSC were preincubated with Lf, washed, and then cultured with FITC-labeled *S. aureus* in the absence of Lf. The phagocytic activity of the MGSC was enhanced by a preliminary incubation of these cells with Lf for more than 30 min (results not shown). Also, it is known that Lf induces the enhancement of phagocytic activity by pretreating human neutrophils with Lf (14). This evidence suggests that Lf directly promotes the enhancement of phagocytic activity of MGSC. Rat macrophages treated with Lf at concentrations of 0.01 to 1 mg/ml increased their production of NO, which is indispensable to kill the bacteria (30). Bovine Lf at 0.03 mg/ml inhibits the growth of *Candida albicans* in the presence of human neutrophils (18). From these reports, it was suggested that the apparent killing activity by Lf was caused not only by the effect of iron chelation but also by the induction of innate immunity (7). Based on these results, it was considered that phagocytes were activated by Lf, which increased the phagocytic activity (14, 18, 30).

The percentage of unopsonized *S. aureus* in the co-culture with Lf and MGSC dropped to 5%, compared with that in the culture with MGSC alone. Furthermore, the number of *S. aureus* opsonized by Lf plus fresh NBS dropped to 1/20, compared with that of unopsonized *S. aureus*.

Unlike in the efficacy of complement, the pretreatment of *S. aureus* with Lf alone was more effective on the growth inhibition of *S. aureus* than that with untreated *S. aureus*. However, the addition of heat-inactivated NBS to this mixture abolished the effect of inhibition on bacterial count. These results suggest that (i) the binding of Lf to *S. aureus* induces MGSC to take up *S. aureus* through Lf receptor on the surface of MGSC, and (ii) heat-inactivated serum contains a substance which interferes with the binding between Lf-bound *S. aureus* and Lf receptor of MGSC. Since neutrophils may ingest Lf-coated latex beads via the contact between the Lf receptor of the neutrophils and Lf on the surface of latex beads (14), the phagocytes in MGSC may promote ingestion of the Lf-bound *S. aureus* by the contact between phagocytes and *S. aureus*. It is known that proteins of high-electric points, such as lactoperoxidase or lysozyme inhibit the binding between Lf and Lf receptor on macrophages (5). If the interfering substances exist in heat-inactivated serum, Lf-coated *S. aureus* may be inhibiting the binding to Lf receptor by the effect of interference. Therefore, the addition of heat-inactivated serum to the mixture containing Lf and *S. aureus* in the pretreatment may abolish the induction of the phagocytosis by the interference between Lf and Lf receptor on the surface of MGSC. Similar results were obtained by the culture of the phagocytic mixture containing heat-inactivated serum with MGSC for 6 hr. To decrease the bacterial count of Lf pretreating *S. aureus* by the addition of exogenous Lf into the phagocytic mixture, an excess amount of Lf may inhibit the effect of interference between Lf and Lf receptor. Since the presence of Mg-EGTA in the mixture containing heat-inactivated serum abolished the effect of interference between Lf and Lf receptor, this effect of interference in heat-inactivated serum might depend on the calcium level in the mixture.

The bacterial growth inhibition test suggested that Lf enhanced the opsonization of the bacteria and the phagocytic activity of MGSC. The results of this study demonstrated Lf-dependent and antibody-independent complement-mediated phagocytic killing of *S. aureus*, and implied that Lf was capable of activating both the alternative pathway of bovine complement and phagocytosis by MGSC. No attempt was made in this study to characterize the complement receptor (CR1,CR2,CR3 and CR4) bound to opsonized *S. aureus*. However, the deposition of C3 on *S. aureus* in the absence of specific antibody was promoted by Lf, which is an important effector in bovine innate immunity. Furthermore, the ability of Lf to promote the phagocytic killing of *S. aureus in vitro* suggests that Lf, phagocytes and complement provide important protection against infection by *S. aureus* in the bovine mammary gland during nonlactating periods.

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