Effects of Recombinant Bovine Granulocyte-Macrophage Colony-Stimulating Factor on Bovine Peripheral Blood Neutrophil Functions *in vitro* and *in vivo*

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ABSTRACT. Effects of recombinant bovine granulocyte-macrophage colony-stimulating factor (rboGM-CSF) on bactericidal activity of bovine peripheral blood neutrophils *in vitro* and *in vivo* were studied. In *in vitro* experiment, bovine blood neutrophils were cultured for 9 hr in media containing 0.005, 0.05 or 0.5 µg/ml of rboGM-CSF. Neutrophils treated with rboGM-CSF showed significantly higher luminol-dependent chemiluminescence (LDCL) than control cells. In *in vivo* experiment, neutrophils isolated from cows injected 5.0 µg/kg of rboGM-CSF showed significantly higher Nitrobluetetrazolium (NBT) reduction value than that from control cows 24 hr post injection. Total leukocyte counts of cows injected rboGM-CSF sharply decreased 6 hr post injection and recovered to normal level 2 days post injection. Body temperature of these cows rose 6 hr post injection and back to normal level at 24 hr post injection. It was suggested that rboGM-CSF enhanced bactericidal activity of bovine neutrophils both *in vitro* and *in vivo*.—**KEY WORDS:** bactericidal activity, neutrophil, recombinant bovine GM-CSF.

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein involved in the proliferation and differentiation of normal haematopoietic cells. This factor is not only regulating the myeloid precursor cell proliferation and differentiation, but also enhancing mature neutrophil, eosinophil and monocyte/macrophage functions [4, 8, 9, 16, 18, 24, 29]. Recombinant human GM-CSF (rhuGM-CSF) has been used for treatment of leukopenic patients with aplastic anemia or acquired immunodeficiency syndrome [3, 15, 17]. Reddy *et al.* [23] and Sordillo *et al.* [26] reported that bovine neutrophils treated with recombinant bovine (rbo) GM-CSF *in vitro* showed higher bactericidal activity than non-treated control cells. Neutrophil functions are closely related with outcome of bovine infectious bacterial diseases, such as mastitis [1, 5, 14]. However, bactericidal activity of bovine mammary gland neutrophils which infiltrate from blood was lower than that of peripheral blood neutrophils [7, 21]. In the case of bovine acute clinical mastitis, the bactericidal activity of blood neutrophils decreased at the onset or initial period of inflammation, and increased during recovery [20]. These reports suggested that enhancement of blood neutrophil bactericidal activity by rboGM-CSF may have preventive or therapeutic effect on bovine mastitis. However, only few experiments have been done about *in vivo* effects of rboGM-CSF on neutrophil functions in luminants [6, 12, 25], and the results were not consistent. In the present study, *in vitro* and *in vivo* effects of rboGM-CSF produced in Baculovirus expression system [10] were investigated.

The recombinant virus infected serum free culture fluid was used as the rboGM-CSF source, after removal of viral particles by ultra centrifugation and ultra filtration. Wild type virus infected cell culture fluid was used as placebo.

For *in vitro* experiment, venous blood samples were collected once from eleven Holstein cows (12–39 weeks after parturition, 2–6 years old). In *in vivo* experiment,

eleven Holstein cows (11–43 weeks after parturition, 2–5 years old) were assigned randomly to three treatment groups of four and three cows. Two groups of four cows received single subcutaneous injection of rboGM-CSF at doses of 5.0 µg/kg body weight and placebo in the cervical region, respectively. One group of three cows received single subcutaneous injection of rboGM-CSF at doses of 2.5 µg/kg body weight. Venous blood samples were collected before the treatment and 6, 12, 24 hr, 2, 3 and 7 days post injection of rboGM-CSF or placebo.

Neutrophils were isolated from the heparinized blood by Ficoll-Conray density gradient centrifugation [19].

In *in vitro* experiment, neutrophils isolated from each cow were incubated with Hanks Balanced Salt Solution supplemented with 0.005, 0.05 or 0.5 µg/ml of rboGM-CSF (5×10^6 cells/ml) in 24-well plates (Becton Dickinson and Co., U.S.A.) for 9 hr at 37°C in 5% CO₂. Untreated neutrophils served as a control.

Luminol-dependent chemiluminescence (LDCL) assay and Nitrobluetetrazolium (NBT) reduction assay were performed according to the method described previously [20, 28] with slight modification in reagent concentration, respectively.

In *in vitro* experiment, neutrophils treated with 0.05 and 0.5 µg/ml of rboGM-CSF showed higher peak LDCL response from start (just after treatment) to 9 hr after treatment than untreated control cells. Neutrophils treated with 0.005 µg/ml of rboGM-CSF showed less significant, however only just after treatment, neutrophil activities showed statistically higher value than control (Table 1).

In *in vivo* experiment, positive reactions of NBT reduction had been seen until 2 days post injection by all three groups. Among them, the values of 5.0 µg/kg injected cows were significantly high (Fig. 1). However, peak time was varied among four cows in this group, i.e. 6 hr post injection in two cows, 12 hr in one cow and 24 hr in one cow. At six

Table 1. Changes in peak LDCL response ($\times 10^6$ CPM) of neutrophils treated with rboGM-CSF

GM-CSF concentration ($\mu\text{g/ml}$)	Incubation time (hours)			
	0	3	6	9
0.5 (n=11)	3.863* (0.612)	4.644* (0.978)	4.972** (0.990)	5.187* (1.013)
0.05 (n=11)	3.859** (0.535)	4.467* (0.937)	4.840* (1.018)	5.098* (1.131)
0.005 (n=9)	3.966** (0.683)	4.395 (0.993)	4.809 (1.004)	5.002 (1.178)
0.0 (n=11)	3.321 (0.709)	3.873 (0.782)	4.095 (0.697)	4.423 (0.583)

Each value in parenthesis is SD. Higher value compared with control (0.0 $\mu\text{g/ml}$ of GM-CSF concentration) in each incubation time by the *t*-test (* $P < 0.05$, ** $P < 0.01$).

hours post injection, total leukocyte counts in peripheral blood from cows injected with 5.0 or 2.5 $\mu\text{g/kg}$ of rboGM-CSF decreased significantly (data not shown). Body temperature of these cows rose until 24 hr (data not shown). Both total leukocyte counts and body temperature were not affected by placebo injection. At sites of injection, no clinical side effects as swelling or induration were observed.

In vitro effects of rboGM-CSF on bovine neutrophil functions were previously reported [23, 25]. In these studies, neutrophils treated with 0.005 $\mu\text{g/ml}$ of rboGM-CSF for 9 to 12 hr showed significantly higher phagocytosing or bactericidal activities than control cells. In the present study, neutrophils treated with 0.5 $\mu\text{g/ml}$ of rboGM-CSF showed the highest peak LDCL response among 0.005, 0.05 and 0.5 $\mu\text{g/ml}$ in culture medium. Sordillo *et al.* [26] reported that bactericidal activity of neutrophils treated with 0.005 $\mu\text{g/ml}$ of rboGM-CSF was not significantly higher than that of control cells until 9 hr of incubation. In the present study, bactericidal activity of neutrophils treated with rboGM-CSF was significantly higher than control cells just after incubation. These differences may be due to the differences of the recombinant cytokines. Former works have been done with *E. coli* expressed rboGM-CSF, on the other hand, our works have been done with Baculovirus expressed one. There are some differences of post-translational modification between them. There may be some quantitative differences of biological activity as well. *In vivo* effects of rboGM-CSF on neutrophil functions in cattle were rarely reported. Daley *et al.* [6] showed that intramammary infusion of rboGM-CSF resulted in enhancement of inducible oxygen radical formation and phagocytosis in milk neutrophils. Kehrli [12] reported that no enhancement of various neutrophil or lymphocyte functional activities was detected with repeated systemic administration of rboGM-CSF. On the other hand, Sordillo [25] reported enhancement of NBT reduction value of neutrophils in goats.

In the present study, it was demonstrated that bactericidal activity of peripheral blood neutrophils was enhanced with

Fig. 1. NBT reduction value post injection of rboGM-CSF. *: $P < 0.05$ significant difference compared with control value by the *t*-test.

subcutaneous injection of rboGM-CSF. This result suggested the possibility of rboGM-CSF for clinical use to protect or treat bacterial infections such as mastitis in cattle. Dulin *et al.* [7] and Nickerson [21] demonstrated that phagocytic and bactericidal activities of bovine mammary gland neutrophils were lower than those of blood neutrophils due to lack of an energy source, decrease in opsonins and interference by casein and fat. It makes possible to survive some kind of bacteria as *Staphylococcus aureus* (*S. aureus*) in these cells after phagocytizing [1, 2]. Lohuis *et al.* [14] showed that preinfection bactericidal activity of blood neutrophils was negatively correlated with counts of *E. coli* in milk and with losses in milk production in experimental *E. coli* mastitis. Enhancement of neutrophil bactericidal activity is indispensable to prevent or treat infectious diseases, especially bovine mastitis. On the other hand, side effects of rboGM-CSF must be noticed. In this study, transient decrease in blood leukocyte counts and fever were observed by administration of rboGM-CSF. These phenomena were also observed in previous reports [11, 12, 25, 27]. Decrease in blood leukocyte counts may be due to the margination of neutrophils, because it was reported that rhuGM-CSF alter the expression of adhesive receptors on phagocytes [30].

Our results in this report, together with data of other groups, indicated that rboGM-CSF is one of the good candidate for therapeutic agent in cattle, though the side effects i.e. elevation of body temperature was observed.

In case of recombinant granulocyte colony-stimulating factor (G-CSF), some promising results for the protective effects against bovine bacterial infections were reported [13, 22].

Therefore, comparative studies of rboGM-CSF and rboG-CSF may required to elucidate protective efficacy against bovine bacterial diseases and side effects.

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