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Comparing chemiluminescent response and intracellular bactericidal activity of bovine blood neutrophils and monocytes against *Salmonella* Dublin and *Escherichia coli*

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Abstract

Chemiluminescent (CL) response and the intracellular killing activity of polymorphonuclear neutrophils (PMNs) and monocytes (MOs) from healthy calves were evaluated to clarify the responsiveness of phagocytes to *Salmonella* Dublin (*S.* Dublin) and *Escherichia coli* (*E. coli*). CL responses of PMNs and MOs against *S.* Dublin and *E. coli* were significantly lower ($P < 0.05$) than that with opsonized zymosan. The intracellular bactericidal activity of PMNs and MOs was found to be effective against *E. coli* but not against *S.* Dublin. These findings indicated that the bactericidal activity of bovine PMNs and MOs against *S.* Dublin was impaired in innate immune response and that a decreased killing activity against *S.* Dublin could be a contributing factor for chronic salmonellosis in cattle.

Key Words: bovine monocytes, intracellular bactericidal activity, *Salmonella* Dublin

Gram-negative bacteria *Escherichia coli* and *Salmonella* spp. are major pathogens that cause diarrhea, enteritis, and mastitis in cattle[5,6,20)]. It is well known that *Brucella* spp.[19)], *Salmonella* spp.[4,14)], *Haemophilis sommnus*[7,18)], and *Mycobacterium* spp.[25)] can survive within macrophages/mononuclear phagocytes.[16,17)]

As the use of bactericidal drugs on gram-negative bacteria is not effective, it was selected to cull infected animals from the herds on the farm. The control of *E. coli* and *Salmonella* spp. infections in farm animals is necessary for good

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dairy management practices to improve cattle environments in farms. Further, prudent use of antimicrobial drugs is required to avoid drug residues in animals, and to make safe and high-quality animal products on farm[1].

PMNs and macrophages/mononuclear phagocytes play an essential role in host defense against invading pathogens in innate immune response[15,27]. Leukocytes effectively kill microorganisms and eliminate them in phagocytic vacuoles. Studies on the pathogenicity of Salmonella spp. have been conducted mainly by using macrophages, epithelial cells, and lymphoid cells as effector cells[4,8,13]. To the best of the author's knowledge, little data is known about the phagocytic-killing activity of bovine PMNs and MOs from peripheral blood against *Salmonella* spp.[8,12]. This study evaluated the functional response induced by phagocytic activity and intracellular killing activity of PMNs and MOs from healthy calves against *S.* Dublin compared with that of *E. coli*.

S. Dublin and *Escherichia coli* (*E. coli*) (wild type, isolated from diarrhea from a calf, Rakuno Gakuen University) were used as target microorganisms. Bacteria were cultured overnight in trypto-soya broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and washed (1800 x g, 15 min) twice in sterile physiological saline. Their concentrations were adjusted to 4×10^7/ml viable bacteria. Serial, 10-fold dilutions in saline were made to 10^{-3}, and the number of colonies that grew overnight following incubation in blood agar was then determined.

Peripheral blood was collected from the jugular vein of five healthy Holstein calves, 3–5-months-old, into a tube containing heparin (20 IU/ml). PMNs were isolated from density gradients followed by hypotonic red blood cell lysis as described[22]. Isolated PMNs were washed twice with phosphate-buffered saline (PBS) and resuspended in Hanks' balanced salt solution (HBSS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to a concentration of 5×10^6 cells/ml. The cell populations comprised 90%–95% PMNs as determined by morphological evaluation and 95% viability as assessed by trypan-blue exclusion.

MOs were isolated from mononuclear cell suspensions according to the procedure described previously[23]. Isolated MOs were washed (300 x g, 3 min) twice with PBS (pH 7.2) and resuspended in HBSS (12.5 mM-HEPES-buffered) containing 5% fetal calf serum (FCS) to a concentration of 1×10^7 cells/ml. The purity of the MOs was 80%–90% as assessed by neutral-red staining and 97%–99% viability as assessed by trypan-blue exclusion[22]. MOs were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 20% heat-inactivated FCS and antibiotics (100 IU penicillin/ml and 100 µg streptomycin/ml).

The cell samples of *S.* Dublin-phagocytosed PMNs were fixed with half-strength Karnovsky for 90 min. After several rinses with 0.1 M phosphate buffer, the cell samples were post-fixed with 1% osmium tetroxide for 30 min. The samples were dehydrated in an ethanol gradient from 30% to 100% for 10 min in every step. Samples were embedded in epoxy resin. Ultrathin sections were obtained using an ultramicrotome and double-stained with uranyl acetate and lead citrate. The samples were examined using a transmission electron microscope (TEM)(JEM-1220, JEOL, Tokyo, Japan) at 80 kV.

The chemiluminescent (CL) responses of bovine PMNs and MOs were measured according to the procedure described previously[21]. A PMN and MOs suspension (150 µl, 2×10^6 cells/ml) was incubated at 37°C for 10 min in a luminometer (JNR AB2100, Atto Co., Ltd., Tokyo, Japan), and then 10 µl luminol (final 10^{-4} mol/l, Sigma Chemical Co., St. Louis, MO, USA) was added. The cell suspensions were set up in triplicate and equilibrated for 5 min, and then 20 µl opsonized zymosan (OPZ) (Sigma Chemical Co., St. Louis, MO, USA) that was made by adding pooled fresh bovine serum to zymosan A (*Saccharomyces cerevisiae* yeast) and incubated for 30 min at 37°C. OPZ was prepared by centrifugation at 500 x g for 20 min and was used as 10mg/ml[23].

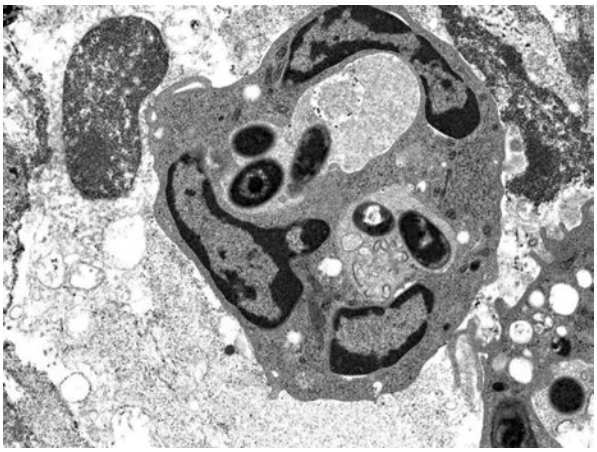

Fig. 1. Transmission electron microscope photograph of S. Dublin-phagocytosed bovine neutrophils. Bar: 1 μm.

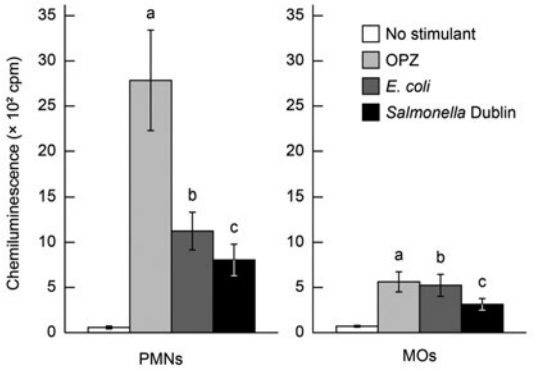

Fig. 2. Comparison of chemiluminescence response of polymorphonuclear neutrophils (PMNs) and monocytes (MOs) stimulated with opsonized zymosan, *E. coli*, and *S.* Dublin. Values were expressed as mean ± SE of 5 calves. Significant difference: PMNs (a–c, $P < 0.01$; b–c, $P < 0.05$); MOs (a–c, b–c, $P < 0.05$).

Isolated phagocytes were stimulated with OPZ, *E. coli* and *S.* Dublin, respectively. The peak CL response was recorded as counts per minute (cpm), and values were logarithmically transformed for analysis.

Intracellular phagocytic-killing of microorganisms by bovine PMNs and MOs was determined as follows: leukocyte suspension (200 μl, 2×10^5 cells/ml) in 0.1% gelatin-Hanks were incubated with 100 μl (1×10^6 CFU) of *S.* Dublin and *E. coli* were incubated for 25 min at 37°C. Antibiotics (gentamycin, final concentration 50 μg/ml) were added to the test tubes to kill extracellular bacteria at 37°C for 20 min, and then solutions were washed twice with PBS at 500 x g for 5 min. Serial dilutions with Hanks were made, and samples (100 μl) were taken for plating in nutrient agar to determine the CFU after 24 hr at 37°C. CFUs were enumerated before incubation (A: 0 min) and after incubation (B: 30 and 60 min). The intracellular bactericidal activity (% survival) was calculated as follows: B/A × 100.

The values were expressed as the mean ± standard error. Additionally, the values of CL response and intracellular killing activity of PMNs and MOs from calves were analyzed using the Kruskal–Wallis test for comparison between groups. $P < 0.05$ was considered significantly different.

The phagocytic-killing of *S.* Dublin by PMNs was evaluated by TEM (Fig. 1). Visualizing *S.* Dublin-phagocytosed PMNs by TEM, we observed short rod-shaped and oval structure of ingested microbes in phagosomes of the PMNs (Fig. 1). The cell wall of ingested microbes was surrounded by phagosomes, and their cytoplasmic granules were observed in microbes. However, these findings could not reveal any difference in morphologic characteristics of living or dead *S.* Dublin. Several S. Dublin were also observed in membrane-bound vacuoles in PMNs. However, this finding could not reveal any difference in morphology of dead or living *S.* Dublin.

CL responses of bovine PMNs and MOs stimulated with OPZ, *E. coli*, and *S.* Dublin were compared (Fig. 2). The CL responses of PMNs when stimulated with OPZ, *E. coli*, and *S.* Dublin were 2–5-fold higher than those of MOs stimulated with the same stimulants. Conversely, CL responses of PMNs and MOs stimulated with *S.* Dublin were significantly lower ($P < 0.05$) than those stimulated with *E. coli* (Fig. 2).

Intracellular killing activities of bovine PMNs and MOs against *E. coli* and *S.* Dublin were determined at 0-, 30-, and 60-min incubation times (Fig. 3). The mean (±standard error [SE]) of *E. coli* in PMNs and MOs incubated for 0, 30,

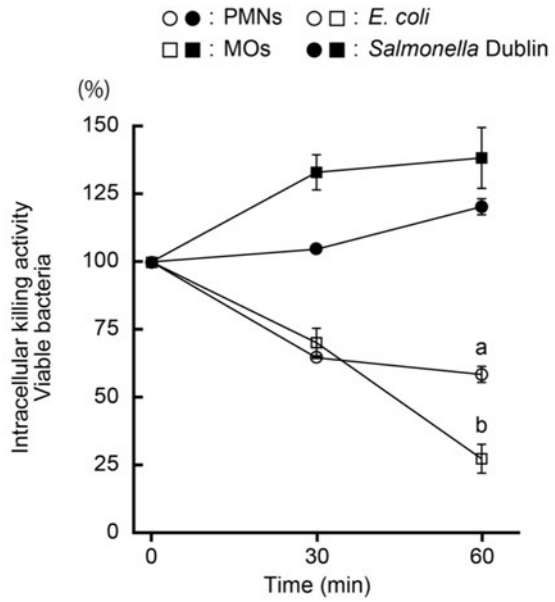

Fig. 3. Comparison of intracellular bactericidal activities of polymorphonuclear neutrophils (PMNs) and monocytes (MOs) against *E. coli* and *S.* Dublin.
Intracellular killing activity of PMNs and MOs against *E. coli* and *S.* Dublin.

Values were expressed as mean ± SE of 5 calves.
Significant difference from the incubation time at 0 min (a: $P < 0.05$; b: $P < 0.01$).

and 60 min were 49.1 (±6.1), 31.6 (±4.7), and 28.4 (±5.0) × 10^2 CFU/ml in PMNs, and 21.3 (±5.0), 15.0 (±3.6), and 5.8 (±1.9) × 10^2 CFU/ml in MOs, respectively. The percentage of viable *E. coli* was decreased in PMNs and MOs during the incubation period, and a significant ($P < 0.05$) decrease in *E. coli* was found in PMNs and MOs after 60 min incubation compared with those before incubation.

The mean number (±SE) of *S.* Dublin in bovine PMNs and MOs incubated for 0, 30, and 60 min were 47.9 (±6.7), 49.9 (±6.4), and 57.3 (±11.4) × 10^2 CFU/ml in PMNs, and 18.5 (±4.2), 24.6 (±5.3), and 25.5 (±4.6) × 10^2 CFU/ml in MOs, respectively. The percentages of viable *S.* Dublin in bovine PMNs and MOs were gradually increased during incubation.

Acute salmonellosis in calves is associated with lymphocyte depletion in Peyer's patch follicles and necrosis in chronic stages of infection. Nunes et al[24] studied the role of PMNs in response to *Salmonella typhimurium*-induced enteritis in healthy calves and calves with adhesion molecules CD18-deficiency and found a clear difference in morphologic changes of complete absence of lesions in Peyer's patches in healthy calves compared with those in CD18-deficient calves showing atrophy and fusion of villi. Changes in villi morphology in salmonellosis appeared to have been caused by epithelial invasion in the absence of neutrophilic inflammation[24]. Intracellular survival bacteria could escape phagocytic-killing activities, humoral antibodies, extracellular drugs, and strong resistance to host defense mechanisms[12]. Impaired intracellular bactericidal activity and CL response of bovine PMNs and MOs against *S.* Dublin was demonstrated in this study. *E. coli* and *S.* Dublin-stimulated CL responses of bovine PMNs and MOs were significantly lower than of OPZ-stimulated responses. CL response of bovine PMNs stimulated with OPZ, *E. coli*, and *S.* Dublin were significantly higher than those found in bovine MOs. The phagocytic killing response involving the recognition of microorganisms by bovine PMNs and MOs appeared to be lower than that stimulated with fresh pooled serum OPZ, which is recognized by inactivated complement 3b and Fc fragment of immunoglobulin G[3,26]. Additionally, a significant decrease in CL response by bovine PMNs and MOs was found when stimulated with *S.* Dublin than with *E. coli*, indicating that recognition and phagocytic ingestion of S. Dublin by bovine PMNs and MOs were lower and stimulus-dependent CL response was decreased. This demonstrated that *S.* Dublin could be associated with characteristics of ingestion of *S.* Dublin and its signal transduction in these cells[28]. The decrease in CL response to *S.* Dublin by PMNs and MOs suggests that superoxide production is weakly activated by the exposure of such organisms, as was suggested[9]. As shown in Fig. 3, of the intracellular killing activities of bovine PMNs and MOs against *E. coli* and *S.* Dublin, *E. coli* was significantly decreased

compared with *S*. Dublin during the incubation period. *E. coli* in bovine PMNs and MOs were significantly decreased at 60 min incubation compared with that without incubation. In contrast, *S*. Dublin increased during the incubation period from 30 to 60 min. This finding could be associated with chronic salmonella infection and difficulty in eliminating *Salmonella* spp. from the infected animals.

The pathogenicity of Salmonella spp. known as Salmonella pathogenicity island (SPI)[4,11,14]. SPI 1 and 2 are associated for the injection of virulence proteins into host cells, SPI 1 for host cell invasion and inflammation, and SPI 2 for intracellular survival and replication in phagocytes[4,14]. Previous studies have shown that Salmonella spp. can inhibit the fusion of phagosomes and lysosomes in salmonella-phagocytosed macrophages, which leads to impaired phagolysosome formation, and can escape from phagocytosed cells in salmonella-containing vacuoles [9,10,13,28]. They remodel their phagosomes in macrophages by inhibiting phagosome maturation, which may be associated with the escape of the bactericidal mechanisms[2,29]. The exact mechanism of intracellular phagocytic-killing activity of bovine PMNs and MOs against *Salmonella* spp. remains to be elucidated. In therapeutic trials, it is a rationale that phagocytic cells have higher permeability to antibiotics; thus, engulfing *Salmonella* spp. would be based on a reasonable selection of antibiotics. Further studies are required to elucidate the characteristics of survival of *Salmonella* spp. in phagocytes in bovine system using various infection.

In summary, bovine PMNs and MOs generated a less pronounced CL response to *E. coli* and *S*. Dublin than OPZ. Furthermore, the intracellular killing activity of bovine PMNs and MOs to *S*. Dublin was impaired compared with that of *E. coli*, which was effectively killed. Thus, this study revealed that the intracellular bactericidal activity of bovine phagocytic leukocytes to *S*. Dublin is impaired and may be associated with chronic salmonellosis in animals.

Potential conflicts of interest

The authors have nothing to disclose.

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