

## Bovine Milk Enhances the Oxidative Burst Activity of Polymorphonuclear Leukocytes in Low Concentrations

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**ABSTRACT.** Bovine milk contains various immunoreactive components, and the activation of polymorphonuclear leukocytes (PMNLs) function in breast-fed infants has been reported. In this study, the effect of milk on the oxidative burst of bovine PMNLs was investigated *in vitro*. When PMNLs were incubated with 0.1% colostrum or normal milk, the oxidative burst induced by serum-opsonized *Staphylococcus aureus* was enhanced, and the enhancement declined dose-dependently. The enhancement of the oxidative burst by milk was not due to opsonins but the priming activities. Also, the phorbol 12-myristate 13-acetate (PMA)-induced oxidative burst increased after incubation with 0.1% colostrum, but the colostrum enhancement of the oxidative burst was unaffected by the incubation time. These results suggest that bovine milk contains oxidative burst promoting factor(s).

**KEY WORDS:** bovine, colostrum, normal milk, oxidative burst, polymorphonuclear leukocyte.

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Bovine milk is a critical source of nutrition and passive immunity for the newborn [21]. The biodefense system of newborns is immature at birth. In particular, bovine colostrum is important for newborns to protect them from infectious diseases [2, 3, 15, 16, 18]. In addition, an increase in PMNL phagocytic activity in breast-fed infants has been reported [13, 17]. Phagocytes, especially neutrophils, have an important role in innate immunity. Bovine colostrum enhances phagocytic activity *in vitro* [23]. Ultrafiltered bovine whey product enhances neutrophil function [20]. Prior exposure of neutrophil to a certain cytokine greatly amplifies the magnitude of response to an activating agonist. This phenomenon is known as priming [1, 14, 22]. Interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-alpha), which are priming agents have been detected in bovine colostrum [8, 9]. These results suggest that milk enhances the function of neutrophils.

The aim of this investigation was to study the effect of milk on the oxidative burst of bovine PMNLs *in vitro*. In this study we demonstrated that bovine colostrum and normal milk enhance the oxidative burst activity of PMNL in low concentrations.

### MATERIALS AND METHODS

**Colostrum, normal milk and serum samples:** Bovine colostrum was obtained from 10 clinically healthy Holstein cows that had just delivered calves. Bovine normal milk was obtained from 10 clinically healthy Holstein cows, which were three days or more (a maximum of seven months) past parturition day. Milk samples were centri-

fuged at  $40,000 \times g$  for 30 min at 4°C. After removing the lipid and cellular layer, the aqueous layer of the colostrum was removed. Bovine sera were collected from 5 clinically healthy Holstein cows. Milk and serum samples were pooled and stored at –80°C until use.

**Isolation of PMNLs:** Peripheral blood was taken from the same clinically healthy 5 Holstein cows from which serum was collected by heparinized vacutainer. The blood sample was diluted with a 1.6-fold volume of PBS. Four milliliters of diluted blood sample were layered onto the same volume of LYMPHOPREP (Nycomed Pharma, Oslo, Norway). After centrifugation at  $750 \times g$  for 20 min, the fraction containing PMNLs and erythrocytes was isolated. Erythrocytes were lysed by adding 10 ml of lysis buffer (8.26 g NH<sub>4</sub>Cl, 1.19 g NaHCO<sub>3</sub>, 0.0378 g EDTA2Na, pH 7.3, and 1,000 ml distilled water). PMNLs were pelleted by centrifugation at  $430 \times g$  for 10 min, and washed twice with cold PBS. PMNLs were then stained with Turk solution and counted with a hemocytometer. The final cell density was adjusted to a concentration of  $1 \times 10^7$  cells/ml in Hanks balanced salt solution without Ca<sup>2+</sup>, Mg<sup>2+</sup> (HBSS (–)) (Gibco, Gaithersburg, MD). The cell viability assessed by trypan blue dye exclusion was always more than 95%.

**Opsonization of bacteria:** *Staphylococcus aureus* (ATCC 25923) was grown in brain-heart infusion broth (Nissui, Tokyo, Japan) at 37°C for 12 hr. Subsequently the bacteria were heat-killed at 70°C for 30 min and centrifuged at  $8,000 \times g$  for 30 min. After washing three times with PBS, the bacteria were adjusted to approximately  $1 \times 10^{10}$  CFU/ml in HBSS (–). The bacterial suspension was incubated with the culture medium containing 25% of the sample (colostrum, normal milk or serum) at 37°C for 30 min in a test tube. After washing three times with PBS, the bacteria were adjusted to  $1 \times 10^{10}$  CFU/ml in HBSS (–) and stored at –80°C.

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**Assay of oxidative burst:** Oxidative burst of PMNLs was measured by luminol dependent chemiluminescence (LDCL). PMNLs ( $1 \times 10^5$  cells), luminol (Wako Pure Chemical Industries, Osaka, Japan) (final conc.  $80 \mu\text{g/ml}$ ) and colostrum, normal milk or serum in different concentrations (0.1%, 0.5%, 1%, 10% and 50%) were prepared in each test tube and the total volume was adjusted to  $400 \mu\text{l}$  of HBSS (-). After 10 min incubation, the PMNLs suspension was stimulated by adding  $100 \mu\text{l}$  of *S. aureus* (final conc.  $5 \times 10^8$  CFU/ml) or phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co, St. Louis, MO) (final conc.  $100 \text{ng/ml}$ ), then LDCL from each test tube was measured for 30 min. The LDCL from each test tube was measured by a LB953 (EG&G Berthold, Wildbad, Germany) at  $37^\circ\text{C}$ . The integral value of LDCL intensity was expressed as stimulation index (SI). SI was derived from the equation:  $\text{SI} = \frac{\text{integral of chemiluminescent intensity with sample}}{\text{integral of chemiluminescent intensity without sample}}$  (SI=1).

**Statistical analysis:** Student's *t*-test was used for statistical analysis of the data.

## RESULTS

The oxidative burst of PMNLs stimulated with *S. aureus* was measured in the presence of colostrum, normal milk or serum (Fig. 1). When PMNLs were incubated with colostrum, the oxidative burst was enhanced in comparison to the control with concentrations up to 10%, but it was inhibited at 50%. The incubation with normal milk or serum enhanced the oxidative burst of PMNL at all concentrations. No significant difference was found between the enhancement of the oxidative burst activities due to colostrum and normal milk. When PMNLs were incubated with 0.1% colostrum or normal milk, the oxidative burst was strongly enhanced by the stimulation of the serum-opsonized *S. aureus* (SOSA), but the enhancement of the oxidative burst was not induced by the stimulation of colostrum-opsonized *S. aureus* (COSA) or normal milk-opsonized *S. aureus* (MOSA) (Fig. 2). After 10 min incubation with 0.1% colostrum, the oxidative burst activity of PMA-stimulated PMNLs was slightly enhanced (Fig. 3). When PMNLs were preincubated with colostrum for several minutes, the colostrum enhancement of the oxidative burst was not dependent on the preincubation time (Fig. 4).

## DISCUSSION

We found that low concentrations (0.1%) of colostrum and normal milk enhanced the oxidative burst activity of PMNL more than serum did. The oxidative burst promoting activity in colostrum and normal milk declined as their concentrations increased. This suggests that bovine colostrum and normal milk include both LDCL inhibitory and activating factors that are attributable to the oxidative burst. We speculate that the inhibitory factor is an anti-oxidant. When the concentration of colostrum or normal milk is high, anti-oxidant activity is stronger than the oxidative burst promot-

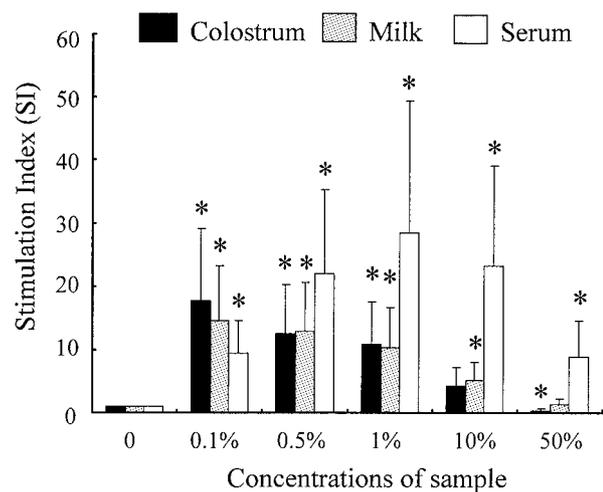


Fig. 1. Oxidative burst of PMNLs incubated with different concentrations of colostrum, milk or serum. The stimulation index (SI) shows the means  $\pm$  S.D. of the results from five calves. \* Significantly different ( $p < 0.05$ ) in SI-values between preincubation with and without the sample.

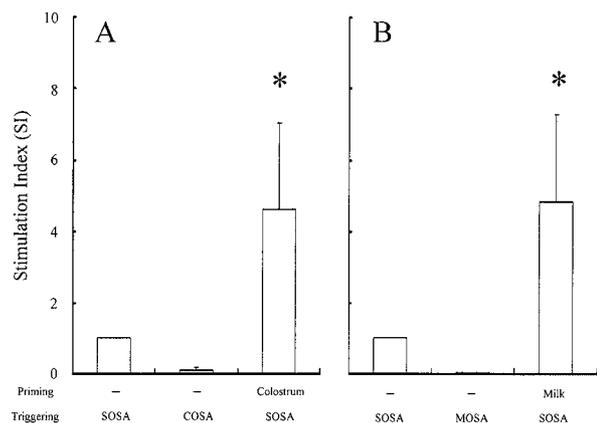


Fig. 2. Serum-opsonized *S. aureus* (SOSA), Colostrum-opsonized *S. aureus* (COSA) and normal Milk-opsonized *S. aureus* (MOSA) induced oxidative burst of PMNLs after exposure to 0.1% colostrum (A) or milk (B). The stimulation index (SI) shows the means  $\pm$  S.D. of the results from five calves. \* Significantly different ( $p < 0.05$ ) in SI-values between preincubation with and without the sample.

ing activity. On the other hand, in the case of low concentrations, the oxidative burst promoting activity behaves as a superior factor. IL-1 beta, IL-6, and TNF-alpha have been identified as oxidative burst promoting substances in bovine milk. These cytokines are known to have the priming effect [1, 14, 22, 27]. Anti-oxidant substances in bovine colostrum have been reported as vitamins A and E [10, 11]. Bovine milk whey and casein are also known to be anti-oxidants [4, 25]. Alpha-lactalbumin, beta-lactoglobulin, bovine serum albumin and lactoferrin have also been reported as anti-oxi-

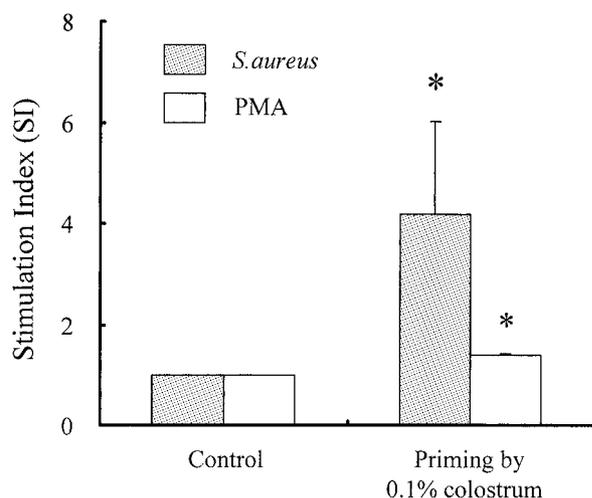


Fig. 3. PMA or SOSA-induced oxidative burst of PMNLs after preincubation with 0.1% colostrum. The stimulation index (SI) shows the means  $\pm$  S.D. of the results from five calves. \* Significantly different ( $p < 0.05$ ) in SI-values between preincubation with and without the sample.

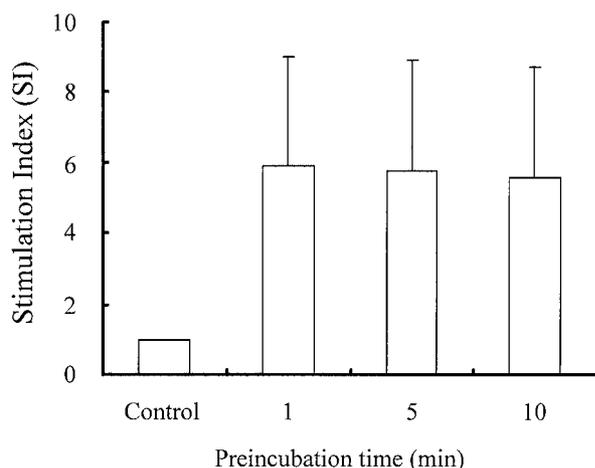


Fig. 4. Effect of pre-incubation time on the oxidative burst in the 0.1% colostrum. The stimulation index (SI) shows the means  $\pm$  S.D. of the results from five calves. \* Significantly different ( $p < 0.05$ ) in SI-values between preincubation with and without the sample.

dants in bovine milk. These materials would affect the antioxidant potencies of colostrum and normal milk. In serum, the promoting effect of oxidative burst fell at 1%. This could have been caused by anti-oxidants such as vitamin A, C and E contained in serum.

The triggering stimulation of COSA and MOSA did not enhance the oxidative burst, while the SOSA-induced oxidative burst of PMNLs was strongly enhanced in the presence of 0.1% colostrum or normal milk. Therefore, the enhancement of the oxidative burst by milk was not due to opsonins but the priming activities. Furthermore, PMA-induced oxidative burst of PMNLs was also enhanced by

preincubation with 0.1% colostrum. PMA is a strong activator of protein kinase C (PKC). PKC directly phosphorylates p47<sup>phox</sup> that is essential for the activation of superoxide-generating NADPH oxidase [6, 19]. It has been reported that the priming effect changes according to the kind of stimulating agent; i.e. the PMNL primed by TNF- $\alpha$  or GM-CSF enhanced fMLP-induced oxidative burst but the enhancement was not caused by PMA-stimulation [12, 24]. Therefore, the colostrum priming-like effect activated an alternative signaling pathway from PKC to p47<sup>phox</sup>. In addition, the colostrum priming effect of the oxidative burst was unaffected by the incubation time. The priming effect of human recombinant IL-8 depended on preincubation time [27]. Further studies are needed to define the mechanism of rapid colostrum oxidative burst enhancement.

In this study, we found that bovine colostrum and normal milk enhanced the oxidative burst of PMNL *in vitro*, so we believe that the oxidative burst promoting factor(s) surely exist in bovine milk. Unlike IgG1, the oxidative burst enhancement effect was not a specific characteristic of colostrum. Wong *et al.* reported that ovine whey protein concentrates have an enhancing effect on superoxide production in a dose-dependent manner [26]. These substances may enhance newborn's PMNLs function. However, the responsiveness in PMNLs differs between newborns and adults [5, 7]. Therefore, though the results of this investigation cannot be extrapolated directly to newborns, we did show that both bovine colostrum and normal milk have oxidative burst enhancement properties. In addition, bovine milk must contain various kinds of immunoactive substances. Investigation into the effect of milk on phagocytes will contribute to the understanding of the development of newborn immunity.

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