Effects of Vitamin B\textsubscript{2} on Neutrophil Functions in Cattle
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ABSTRACT. Vitamin B\textsubscript{2} was intramuscularly administered to Holstein cattle and the ensuing changes in peripheral blood neutrophil function were investigated. The neutrophil count displayed a significant increase at 1–2 days after administration, while nitroblue tetrazolium reducing activity and phagocytic bactericidal activity were enhanced at 1–4 days after administration in calves and at 1–6 days after administration in adult cows. The increases in the neutrophil count and the activation of neutrophil functions were observed, being manifested at dosages of 10 mg/kg or greater for calves and 5 mg/kg or greater for adult cows.—\textit{Key words:} cattle, neutrophil, vitamin B\textsubscript{2}

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Neutrophils are cells which constantly phagocytize and kill bacteria or foreign substances invading the body, and these cells play an extremely important role in host defense mechanisms. In recent years, a serious problem has been posed by the increased incidence of opportunistic infections in cattle due to stress-associated immunosuppression, depression of neutrophil or macrophage function caused by bovine immunodeficiency virus infection [16], or genetic defects [4]. The production of neutrophils is regulated by factors such as granulocyte colony-stimulating factor (G-CSF) or granulocyte—macrophage colony-stimulating factor (GM-CSF) [5, 11, 13], and the clinical application of these cytokines has been performed in humans. However, almost no studies have been performed of such an approach in the case of domestic animals. With the aim of applying immunologically active substances to the treatment of cattle, we previously explored the possibility of administering dihydroheptaprenol [14, 15] or active egg white products [6], and each of these substances was shown to enhance peripheral blood neutrophil function.

Vitamin B\textsubscript{2} (VB\textsubscript{2}) was found to enhance non-specific host defense mechanisms against a variety of bacterial infections in mice by stimulating the generation of neutrophils and also enhancing neutrophil functions [1]. In the present study, VB\textsubscript{2}, the role of which in immunological function is virtually unknown, was administered intramuscularly to cattle and changes in the neutrophil count were observed. Nitroblue tetrazolium (NBT) reduction and phagocytic killing of \textit{Staphylococcus aureus} were also assessed to confirm the effects of VB\textsubscript{2} on neutrophil function.

VB\textsubscript{2} (riboflavin) was prepared as a microemulsion with lecithin at a 5% concentration. This placebo (vehicle) alone has been reported not to increase peripheral blood leukocytes and not to augment neutrophil function in adult cows [15]. Nine healthy female Holstein calves aged 21–63 days and six healthy Holstein cows aged 3–6 years were used. All of the calves and cows were raised on a farm attached to the university. The procedure for VB\textsubscript{2} administration to the calves consisted of dividing the nine animals into three groups of three, and giving single intramuscular VB\textsubscript{2} injections of 5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively. The six adult cows were divided into two groups of three, and received single intramuscular doses of VB\textsubscript{2} at 2 mg/kg and 5 mg/kg, respectively. Blood was collected before and after VB\textsubscript{2} administration for the assessment of NBT reduction and bacterial phagocytosis.

The peripheral blood leukocyte count was determined with an automatic hemocytometer and the neutrophil count was estimated from the differential count determined with a Giemsa-stained blood smear. The neutrophils for functional tests were isolated from calf blood specimens by Ficoll-Paque (Pharmacia Fine Chemicals, Sweden) density centrifugation with a modification of the method of Roth and Kaebere [6, 9, 14]. But for adult cow blood specimens, centrifugation was performed not only with Ficoll-Paque but also with Ficoll-Hypaque (specific gravity: 1.135 g/cm\textsuperscript{3}, Sigma Chemical Co., U.S.A.) in order to remove contaminating eosinophils. Then the final neutrophil concentration was adjusted to 5 × 10\textsuperscript{6} cells/ml with Hanks' balanced salt solution (Sigma Chemical Co., U.S.A.). Viability of the cells was more than 95% as detected by trypan blue (0.5%) exclusion test. The rate of neutrophils was about 90%. Testing of neutrophil function by NBT reduction involved stimulating the neutrophils with Zymosan A (Sigma Chemical Co., U.S.A.) to cause the reduction of NBT to formazan. After coloration of the formazan with dimethyl sulphoxide, the change in absorbance (OD) at 565 nm was measured and taken as the index of NBT reducing activity [14].

As described previously [6, 14], phagocytic bactericidal activity was tested by shaking the culture of a neutrophil suspension with 2.5 × 10\textsuperscript{7} cells/ml of \textit{S. aureus} 209P at 37°C for 90 min, followed by incubation in ordinary agar medium at 37°C for 48 hr. The residual viable bacterial count was expressed as a percentage of the count prior to shaking the culture, and the changes in this percentage were regarded as an index of phagocytic activity.

Data were analysed statistically by Student’s paired t-test.

As regards the peripheral blood neutrophil count after the administration of VB\textsubscript{2} to calves, the 5 mg/kg group showed no significant changes (Fig. 1). On the other hand,
the neutrophil count in the 10 mg/kg group prior to administration was 2,438±631/μl, as compared with 3,544±774/μl one day after administration, representing a significant increase (P<0.05). In the 20 mg/kg group, the pre-injection count was 2,500±566/μl and the count on the second day after administration was 3,700±668/μl, also a significant increase (P<0.05). In each group the leukocyte count displayed almost the same trend as the changes in the neutrophil count.

With regard to adult cows, the neutrophil count in the 2 mg/kg group displayed a slight increase on the second day after VB2 administration (Fig. 2). In the 5 mg/kg group, the neutrophil count was 1,680±211/μl prior to VB2 administration, but displayed a distinct increase to 4,251±1,706/μl by 2 days after administration (P<0.05). Additionally, the leukocyte count displayed a similar pattern to the changes in the neutrophil count. Within the body, VB2 is converted to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are known to act physiologically as coenzymes for redox reactions [7]. The action of VB2 as a coenzyme with respect to the NADPH activity involved in the production of superoxide (O₂⁻), an essential factor in the bactericidal functions of leukocytes, has also been demonstrated [3], but no reports have appeared concerning the effects of VB2 on the peripheral neutrophil count or neutrophil function. The present study revealed for the first time that the peripheral blood neutrophil count increases after the intramuscular administration of VB2 to cattle. Moreover, a dose of 10 mg/kg of VB2 calves or half of this dose (i.e., 5 mg/kg) in adult cows induced a significant increase in the peripheral blood neutrophil count by 1-2 days after administration. The principal actions of phagocytic cells such as neutrophils include migration, phagocytosis, and a bactericidal effect, so the influence of VB2 on these functions was also investigated. It was found that in the group of calves treated with 5 mg/kg of VB2, NBT reducing activity as well as bactericidal phagocytic activity against S. aureus displayed no clear enhancement, like the changes in the neutrophil count (Fig. 1). However, in the 10 mg/kg and 20 mg/kg groups, both NBT reducing and bactericidal phagocytic activity were distinctly enhanced at 1-4 days after administration. The activation of neutrophil functions was delayed relative to the increase in the neutrophil count. In the 20 mg/kg group, activation of neutrophils tended to be more pronounced than in the 10 mg/kg group. Next, as regards the adult cows, tests of
neutrophil function revealed no clear activation in the group receiving VB₂ at 2 mg/kg (Fig. 2). On the other hand, the 5 mg/kg group displayed an increased peripheral blood neutrophil count on the second day, significant enhancement of NBT reducing activity from 3-6 days after administration, and significant enhancement of bactericidal phagocytic activity from 1-6 days after administration (P<0.01 or P<0.05). Thus, the present study revealed the interesting fact that intramuscular injection of VB₂ to cattle not only increased the peripheral blood neutrophil count but also enhanced neutrophil activation.

The peak values in NBT reducing activity of neutrophils from calves and cows were observed at different days, as shown in Figs. 1 and 2. The reason for this was unclear, and further studies will be required to clarify whether this difference between calves and cows may be due to the difference in age.

Activation of neutrophils is believed to involve cytolytic products created at sites of inflammation as well as circulating factors such as IL-2 [12]. Inflammation-related proteins are also known to stimulate cells such as monocyte / macrophages [13], fibroblasts [5], and endothelial cells [11], thereby promoting the production of G-CSF. In turn, G-CSF mobilizes mature neutrophils from the bone marrow, enhances neutrophil development from bone marrow precursor cells and activates mature neutrophils [12]. Administration of G-CSF is reported to rapidly increase the peripheral blood neutrophil count, as well as enhancing superoxide production and neutrophil migration [2]. Moreover, prolonged administration of G-CSF is reported to increase the bone marrow stem cell population and induce a sustained increase in peripheral blood neutrophils in a dose-dependent manner [2]. The mechanism underlying the activation of neutrophil function following VB₂ administration in the present study is not clear. It was shown that the serum level of VB₂ after injection was correlated with the activation of neutrophils (data not shown); however, the fact that both an increased peripheral neutrophil count and activation of neutrophils were observed after VB₂ injection, suggests the involvement of G-CSF rather than a direct mechanism. When VB₂ was administered intramuscularly, VB₂ induced mild inflammation at the injection site, and this inflammation disappeared 2 or 3 days after injection. Though the inflammation always seems not to induce an increase in host resistance, the mild inflammation caused by VB₂ may be, at least in part, involved in G-CSF production. However, when mice were given riboflavin sodium phosphate intravenously without inflammation of the injection site, VB₂ was able to induce augmentation of host resistance to bacterial infections (data not shown). Further studies concerning G-CSF appear important with a view to understanding the mechanism of action of VB₂.

In recent years, the incidence of infectious diseases such as pneumonia, diarrhea, and mastitis has been increasing in cattle, and various antibiotics or corticosteroids are being employed for the treatment and prophylaxis of these infections. However, a depressive effect of such agents on nonspecific immunity has been documented [8, 10]. The present study clearly demonstrated that the administration of natural VB₂ to cattle activated peripheral blood neutrophils, suggesting that the application of VB₂ to the treatment of various bovine infections may be quite promising.

REFERENCES