***In vitro* and *in vivo* Effects of Riboflavin Sodium Phosphate on the Phagocytic Activity of Peritoneal Macrophages in Mice**

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Abstract The *in vitro* and *in vivo* effects of riboflavin sodium phosphate (vitamin B₂ sodium phosphate : VB₂Na) on host defense mechanisms, especially macrophage function, were investigated in mice. VB₂Na (100 mg/kg) was injected intraperitoneally or intravenously at 1 day before the phagocytosis assay. After intraperitoneal administration, the phagocytic index of peritoneal macrophages in the control and VB₂Na-treated groups was $8.3 \pm 1.6\%$ and $34.6 \pm 6.8\%$, respectively, with the index being about 4-fold higher in the treated mice ($p < 0.01$). After intravenous administration, the phagocytic index of peritoneal macrophages was $6.6 \pm 2.1\%$ and $15.4 \pm 3.7\%$ in control and VB₂Na-treated mice, respectively, and the treated mice again showed a significantly higher index ($p < 0.01$). Intravenous administration of VB₂Na showed as effective as intraperitoneal administration. In a separate investigation of the direct effect of VB₂Na on macrophage activity, we determined the *in vitro* phagocytic index of mouse peritoneal macrophages for latex particles in the presence of various concentrations of VB₂Na. Addition of VB₂Na at a concentration of more than $25 \mu\text{g/ml}$ increased the phagocytic index. Thus, our results suggest that VB₂Na enhances macrophage function both *in vitro* and *in vivo*.

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Key words : Immunopotentiator, Mouse, Peritoneal macrophage, Phagocytic activity, Riboflavin sodium phosphate (VB₂Na)

It is generally accepted that the development of disease in domestic food animals, such as respiratory disease in cattle and pigs, involves the interaction of host exposure to stress combined with viral or bacterial challenge. Indeed, several studies have shown that stress- and pathogen-induced immunosuppression contributes to the development of bovine respiratory disease⁷⁾. If such immunosuppression in livestock is related to abnormal immune regulation, then methods of modulat-

ing the immune response should be of benefit.

Enhancing both specific and nonspecific defense mechanisms against infection is of interest to veterinarians. Many biological response modifiers (e.g., physiological products, microbial substances, and synthetic compounds) have been tested for use as immunopotentiators or immunomodulators in domestic food animals^{7,9,14)}. So far, immunomodulators appear to have the greatest potential for the prevention and/or treatment of

early infections associated with immuno-suppression⁹). With the aim of applying immunologically active substances to the treatment of domestic food animals, we have previously explored the possibility of administering dihydroheptaprenol^{1,2,8,11,15,19-21} or fermented egg white powder^{3,4,12,13,16}, and both substances were shown to enhance neutrophil and macrophage function.

Riboflavin (vitamin B₂ : VB₂) has been found to enhance nonspecific host defense mechanisms against a variety of bacterial infections in mice by stimulating the generation of neutrophils and by enhancing macrophage function⁵). In addition, VB₂ has been reported to stimulate neutrophil function in calves and cows¹⁷).

In the present study, to elucidate the mechanism of action of riboflavin sodium phosphate (vitamin B₂ sodium phosphate : VB₂Na), we investigated its *in vitro* effect on the phagocytic activity of mouse peritoneal macrophages, and also performed an *in vivo* investigation in mice to determine whether intraperitoneal or intravenous administration could modulate peritoneal macrophage activity.

Materials and Methods

Animals : Outbred male mice (slc : ICR) were obtained from Japan SLC, Inc, and were used for these studies at 5-6 weeks of age when they weighed 28-32 g. The mice were maintained at 22±2°C with a 12-h light-dark cycle, and were given standard laboratory chow (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*.

Preparation of VB₂Na : VB₂Na (Wako-Pure Chemical Co., Tokyo, Japan) was prepared as a 0.5% w/v solution in sterile water for injection (Otsuka Pharmaceutical Co., Tokushima Japan), and then passed through a 0.22-μm membrane filter (Millipore Bedford, MA, U.S.A.) before use. Endotoxin was not detected in this preparation by the *Limulus* amoebocyte lysate assay (*Limulus* J Single Test Wako,

Wako-Pure Chemical Co., Tokyo, Japan).

Preparation of peritoneal macrophages : Mice were killed by exsanguination via the carotid artery under ether anesthesia and peritoneal macrophages were harvested after the intraperitoneal injection of 5 ml of cold Hanks' balanced salt solution (HBSS ; Gibco Laboratories, U.S.A.). The cell suspension obtained from the peritoneal cavity was washed twice with cold HBSS by centrifugation for 10 min at 1,000 rpm, and the resultant cells consisted exclusively of macrophages. These cells were then suspended at 1×10⁶/0.5 ml in Eagle's minimal essential medium (Nissui, Pharmaceutical Co., Tokyo, Japan) containing 15% inactivated murine serum.

***In vivo* study :** Groups of 5 mice received an intraperitoneal or intravenous injection of VB₂Na at a dose of 100 mg/kg. Control mice were given the same volume of vehicle (sterile water for injection) alone. The phagocytic activity of peritoneal macrophages was determined at 1 day after injection.

***In vitro* study :** Macrophages were harvested from 10 untreated mice and pooled. Then the macrophages (1×10⁶ cells in 0.5 ml of Eagle's minimal essential medium with 15% inactivated murine serum) were treated with various concentrations of VB₂Na or 100 μg/ml muramyl dipeptide (MDP ; a reference drug), and were incubated for 24 h at 37°C in slide chambers (four-compartment type ; Lab-Tek Products, Inc., Naperville, Ill., U.S.A.) with 5% CO₂. Phagocytic activity was determined at the end of this period incubation.

Phagocytic activity assay : Fluorescent monodispersed carboxylated microspheres (Polyscience, Inc., Warrington, PA) were suspended (1×10⁷/0.05 ml) in 1 ml of 15% inactivated murine serum in Eagle's minimal essential medium and added to a macrophage suspension (1×10⁶ cells in 0.5 ml of Eagle's minimal essential medium with 15% inactivated murine serum). Then the cells were incubated for 1 h at 37°C in a slide chamber with 5% CO₂. The

slide chamber was washed 5 times with warm HBSS, and adherent cells were stained with Giemsa solution for observation under a microscope. The phagocytic index was calculated as follows : phagocytic index (%) = (number of cells incorporating 8 or more latex particles / 200 cells) $\times$ 100.

Statistical analysis : Results were analysed statistically using Student's *t*-test.

Results and Discussion

We previously reported that a lecithin VB₂ emulsion increased host resistance to bacterial infection⁵. Therefore, the effect of water-soluble VB₂Na on host defense mechanisms, especially macrophage function, was investigated in the present study.

The phagocytic index of peritoneal macrophages in the control and VB₂Na-treated groups was $8.3 \pm 1.6\%$ and $34.6 \pm 6.8\%$, respectively, after intraperitoneal administration, and the phagocytic index was about 4-fold higher in the treated mice ($p < 0.01$) (Fig. 1).

The phagocytic index of peritoneal macrophages was $6.6 \pm 2.1\%$ and $15.4 \pm 3.7\%$ in the

control and VB₂Na-treated mice, respectively, after intravenous injection, and the treated mice again showed a significantly higher phagocytic index ($p < 0.01$) (Fig. 2).

In a separate experiment performed to evaluate the direct effect of VB₂Na on macrophage activity, we determined the *in vitro* phagocytic activity of mouse peritoneal macrophages for latex particles in the presence of various concentrations of VB₂Na. Addition of VB₂Na at concentrations of more than $25 \mu\text{g/ml}$ enhanced phagocytic activity (Fig. 3).

These results suggested that VB₂Na could enhance macrophage function both *in vitro* and *in vivo*.

We have previously reported that intramuscular injection of VB₂ at doses of 12.5–100 mg/kg enhanced host defences against experimental *Escherichia coli* infection in mice. VB₂ also exhibited a protective effect against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Actinobacillus pleuropneumoniae*. The mechanism by which VB₂ enhances resistance in mice may be related to the stimulation of neutrophil and monocyte

Fig. 1. *In vivo* effect of intraperitoneal (i.p.) injection of VB₂Na on the phagocytosis of latex particles by mouse peritoneal macrophages. Groups of 5 mice were injected with VB₂Na (100 mg/kg, i.p.). Results are expressed as the mean and standard deviation.

** : $p < 0.01$ (Student's *t*-test).

VB₂Na : Vitamin B₂ sodium phosphate.

Control : Vehicle only.

Fig. 2. *In vivo* effect of intravenous (i.v.) injection of VB₂Na on the phagocytosis of latex particles by mouse peritoneal macrophages. Groups of 5 mice were injected with VB₂Na (100 mg/kg, i.v.). Results are expressed as the mean and standard deviation.

** : $p < 0.01$ (Student's *t*-test).

VB₂Na : Vitamin B₂ sodium phosphate.

Control : Vehicle only.

Macrophage Activation by VB₂Na

Fig. 3. *In vitro* effect of VB₂Na on the phagocytosis of latex particles by mouse peritoneal macrophages.

VB₂Na : Vitamin B₂ sodium phosphate.

MDP : Muramyl dipeptide

Control : Vehicle only.

proliferation as well as to the activation of macrophages⁵⁾.

Osame *et al.*¹⁷⁾ reported the marked enhancement of nitroblue tetrazolium reduction and phagocytic killing by peripheral neutrophils obtained from calves and cows at 1–6 days after the intramuscular injection of VB₂ (5–20 mg/kg).

The present study demonstrated that intraperitoneal or intravenous injection of water-soluble VB₂Na was also able to activate macrophages. In addition, VB₂Na directly activated macrophages *in vitro*. However, it remains unclear whether the *in vivo* effects of VB₂ or VB₂Na are due to a direct action on neutrophils or an indirect action via the stimulation of macrophages or other cells to produce soluble mediators, such as colony stimulating factors, interferons, and interleukins.

A major function of neutrophils and macrophages is the phagocytosis, digestion, and destruction of invading microorganisms, and a congenital or acquired defect of phagocytic activity will result in enhanced susceptibility to bacterial pathogens⁶⁾. Therefore, phagocytes play a pivotal role in protecting the host against microbial infection¹⁸⁾. Thus, our

findings suggest that VB₂Na might be useful as a nonspecific immunopotentiator for preventing opportunistic infection in domestic animals. Further investigation of the effect of VB₂Na on host defence mechanisms is currently in progress at our laboratories.

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マウスの腹腔マクロファージの食食能に及ぼすリン酸リボフラビン ナトリウムの *in vitro* および *in vivo* での影響

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マウスの腹腔マクロファージ(M ϕ)の食食能に及ぼすリン酸リボフラビンナトリウム(VB₂Na)の影響を検討した。*in vivo* 試験では, 1群5匹のマウス(ICR, ♂, 5週齢)にVB₂Naを注射用蒸留水で溶解し, 100 mg/kgを腹腔又は静脈内に投与した。投与後1日目に腹腔内からM ϕ を採取し, M ϕ とラテックス粒子をラプテックチャンバー内で37°C, 5% CO₂ の条件下で24時間培養し, 食食試験を実施した。*in vitro* 試験は, 腹腔内より常在M ϕ を採取し, M ϕ と各種濃度のVB₂Na(最終濃度200 μ g/ml)又はムラミルジペプチド(MDP, 最終濃度100 μ g/ml)をラプテックチャンバー内で37°C, 5% CO₂ の条件下で24時間培養し, その後, ラテックス粒子を添加して食食試験を実施した。VB₂Na腹腔内投与群のラテックス粒子に対するM ϕ の食食率は, 34.6 $\pm$ 6.3%であり, 対照群の8.3 $\pm$ 6.3%と比較して約4倍の上昇を示し, その差は統計学的にも有意であった($p<0.01$)。また, VB₂Naの静脈内投与群の食食率は15.4 $\pm$ 3.7%であり, 対照群の6.6 $\pm$ 2.1%と比較して統計学的にも有意な上昇を示した($p<0.01$)。さらに, *in vitro* でもVB₂Naは, 25 μ g/ml以上の濃度で腹腔M ϕ の食食率を亢進した。なお, 陽性対照に用いたMDPもM ϕ の食食率を亢進した。以上の結果から, VB₂Naは*in vitro* および*in vivo* においてM ϕ の機能を直接的に増強することが示唆された。

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