

Highly Purified Vitamin B2 Presents a Promising Therapeutic Strategy for Sepsis and Septic Shock

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Highly purified vitamin B2 (riboflavin 5'-sodium phosphate; purity > 97%) treatment by intravenous infusion at doses above those used clinically to treat vitamin B2 deficiency showed therapeutic effects in mice not only in cases of endotoxin- and exotoxin-induced shock but also in cases of gram-negative and gram-positive bacterial infection even after the toxemia had already begun.

Despite the advances in antibiotic therapy and intensive care medicine, sepsis still has a high mortality rate (1, 6, 8, 18). Considering that etiological pathogenesis is complicated (7, 14) and that patients are very heterogenous regarding underlying illness, site of infection, microbiological etiology, and

physiological derangement (2), it is not surprising that strategies only block each detrimental factor and that treatment with drugs that were discovered by pretreatment in a single sepsis model has been shown to have disappointing outcomes (5, 9, 13, 16). Apart from these approaches it has been previously

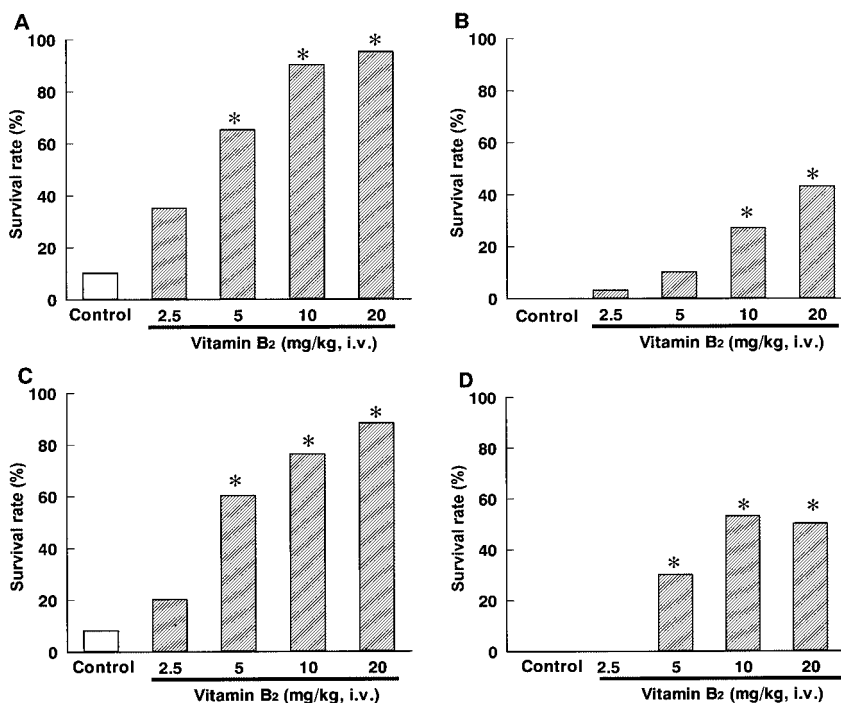


FIG. 1. Effects of intravenous (i.v.) bolus injection of highly purified vitamin B2 on levels of mouse mortality caused by toxin-induced shock and bacterial infection. (A) LPS-induced shock; (B) SEB-induced shock; (C) *E. coli* infection; (D) *S. aureus* infection. *, $P < 0.05$ versus the results for the control group (Steel test).

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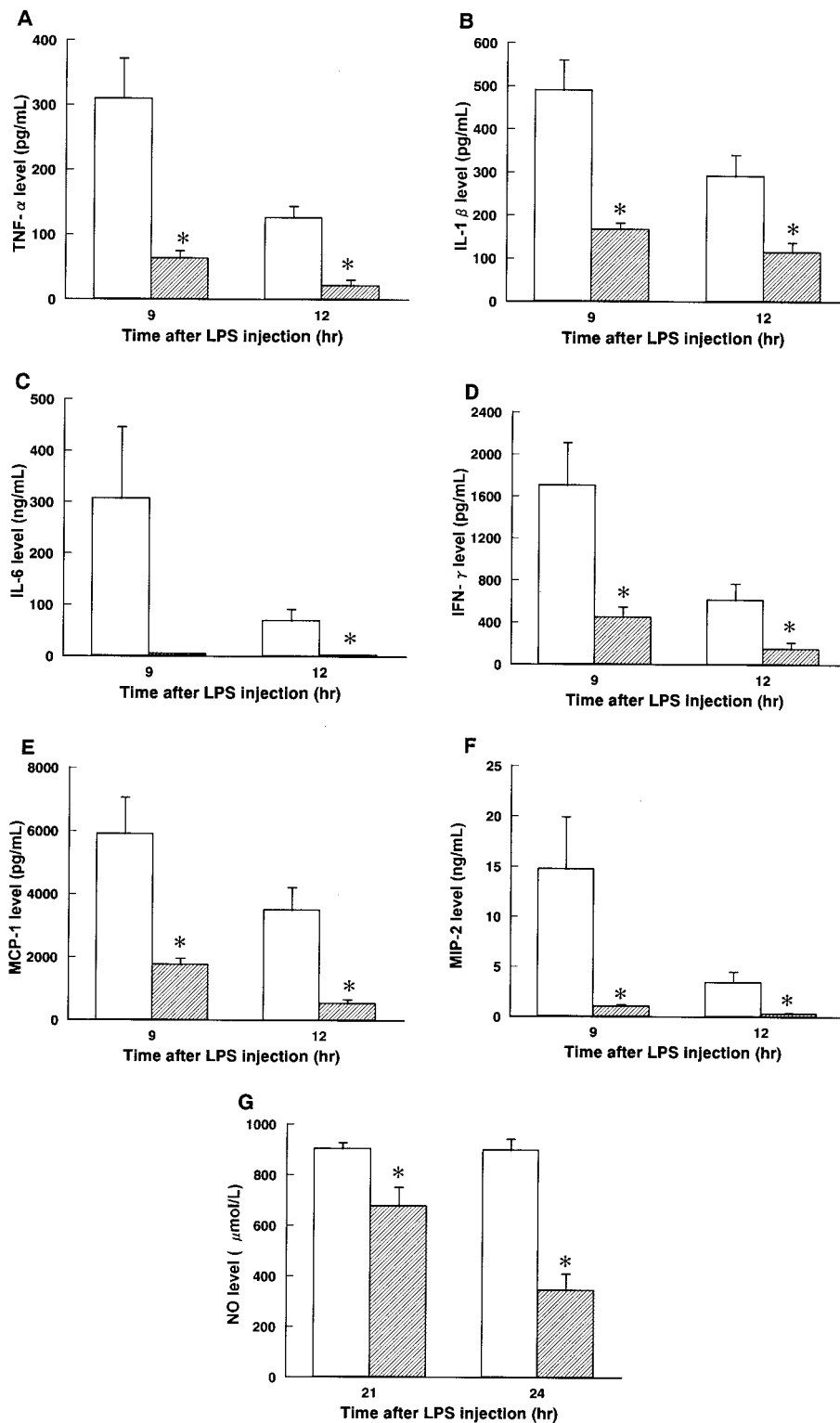


FIG. 2. Effect of highly purified vitamin B2 on plasma cytokine and NO levels in cases of LPS-induced shock. (A) Tumor necrosis factor alpha (TNF- α); (B) IL-1 β ; (C) IL-6; (D) gamma interferon (IFN- γ); (E) monocyte chemoattractant protein 1 (MCP-1); (F) macrophage inflammatory protein 2 (MIP-2); (G) NO. Open columns and hatched columns indicate saline (control)- and vitamin B2-treated groups, respectively. Data are expressed as means \pm standard errors of the means ($n = 6$). *, $P < 0.05$ versus the results for the control group (unpaired t test).

reported that vitamin B2 enhanced host resistance to infection (3), stimulated neutrophil functions (17), and boosted macrophage function (15). The purpose of this study was to evaluate (on the basis of a new immunotherapeutic concept) the therapeutic effects of highly purified vitamin B2 on levels of mouse mortality caused by toxin-induced shock and bacterial infection.

Highly purified vitamin B2 (riboflavin 5'-sodium phosphate; purity > 97%) was purchased from Sigma (St. Louis, Mo.) or was synthesized at Kashima Plant, Eisai Co., Ltd. Human activated protein C (Anact C [APC]) was purchased from Teijin (Tokyo, Japan). Endotoxin (*Escherichia coli* O111:B4 lipopolysaccharide [LPS]; Sigma), gram-positive bacteria (a clinical isolate of *Staphylococcus aureus* E311122), and gram-negative bacteria (a clinical isolate of *E. coli* E01292) were intravenously injected into male ICR mice (6 weeks of age), and exotoxin (*S. aureus* enterotoxin B [SEB]; Toxin Technology, Inc., Sarasota, Florida) was intraperitoneally injected into BALB/c mice (6 weeks of age). Saline (control), vitamin B2, and APC were intravenously given through a tail vein without anesthesia. Survival rates were determined at 7 days after LPS and SEB injection and *E. coli* infection. Survival rates were determined at 14 days after *S. aureus* infection. All experiments were approved by the Animal Care and Use Committee of Eisai.

Vitamin B2 was administered with a bolus injection 6 h after LPS injection. The survival rate of the control group was 10% (2 of 20). At doses of vitamin B2 of 2.5, 5, 10, and 20 mg/kg of body weight, survival levels were 35% (7 of 20), 65% (13 of 20 [$P < 0.05$]), 90% (18 of 20 [$P < 0.05$]), and 95% (19 of 20 [$P < 0.05$]), respectively (Fig. 1A). Moreover, treatment with vitamin B2 at 20 mg/kg 6 h after LPS injection not only lowered levels of excessive plasma proinflammatory cytokines, including tumor necrosis factor alpha (Fig. 2A), interleukin-1 beta (IL-1 β) (Fig. 2B), IL-6 (Fig. 2C), gamma interferon (Fig. 2D), monocyte chemotactic protein 1 (Fig. 2E), and macrophage inflammatory protein 2 (Fig. 2F), but also decreased NO levels (Fig. 2G).

At 6 h after injection of SEB-D-galactosamine, vitamin B2 was administered with a bolus injection. The survival rate of the control group was 0% (0 of 30). The survival rates of the groups treated with vitamin B2 at 2.5, 5, 10, and 20 mg/kg were 3% (1 of 30), 10% (3 of 30), 27% (8 of 30 [$P < 0.05$]), and 43% (13 of 30 [$P < 0.05$]), respectively (Fig. 1B).

Vitamin B2 was given 24 h before *E. coli* inoculation (2.07×10^8 CFU). The survival rate of the control group was 8% (2 of 25). The survival rates of the groups treated with vitamin B2 at 2.5, 5, 10, and 20 mg/kg were 20% (5 of 25), 60% (15 of 25 [$P < 0.05$]), 76% (19 of 25 [$P < 0.05$]), and 88% (22 of 25 [$P < 0.05$]), respectively (Fig. 1C). In addition, vitamin B2 administered at 20 mg/kg 24 h before *E. coli* inoculation significantly reduced levels of the bacteria in the blood (Fig. 3).

Vitamin B2 was given with a bolus injection immediately and 1 and 2 days after *S. aureus* inoculation (1.69×10^8 CFU). The survival rate of the control group was 0% (0 of 30). The survival rates of the groups treated with vitamin B2 at 2.5, 5, 10, and 20 mg/kg were 0% (0 of 29), 30% (9 of 30 [$P < 0.05$]), 53% (16 of 30 [$P < 0.05$]), and 50% (15 of 30 [$P < 0.05$]), respectively (Fig. 1D).

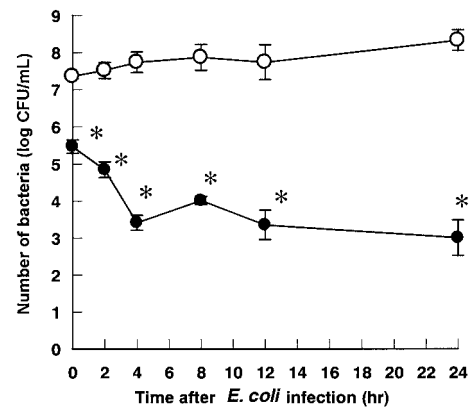


FIG. 3. Effect of highly purified vitamin B2 on the clearance of *E. coli* from blood in mice. Open and closed circles represent saline (control)- and vitamin B2-treated groups, respectively. Each value represents the mean \pm standard error of the mean ($n = 5$). *, $P < 0.05$ versus the results for the control group (unpaired t test).

Furthermore, we investigated the therapeutic effect of vitamin B2 in long-term intravenous treatment by infusion using an infusion pump (Natsume, Tokyo, Japan). Infusion of vitamin B2 for 6 h (*E. coli* infection) or 12 h (*S. aureus* infection) was begun 1 h after inoculation with bacteria. In the *E. coli* infection experiments, the survival rate of the control group was 0% (0 of 30). Survival rates of the groups treated with vitamin B2 at 5, 10, 20, and 40 mg/kg/6 h were 20% (2 of 10), 30% (3 of 10 [$P < 0.05$]), 55% (11 of 20 [$P < 0.05$]), and 70% (7 of 10 [$P < 0.05$]), respectively (Fig. 4A). In the *S. aureus* infection experiments, the survival rate of the control group was 0% (0 of 10) and the survival rates of the groups treated with vitamin B2 at 20, 40, and 80 mg/kg/12 h were 20% (2 of 10), 50% (5 of 10 [$P < 0.05$]), and 70% (7 of 10 [$P < 0.05$]), respectively (Fig. 4B).

Infusion of vitamin B2 with or without APC for 6 h was commenced 6 h after LPS or SEB injection. The survival rate of the control group was 0% (0 of 10) for LPS-treated mice. The survival rates of the groups treated with vitamin B2 (10 mg/kg/6 h), APC (75 units/kg/6 h), and vitamin B2 plus APC were 50% (5 of 10 [$P < 0.05$]), 30% (3 of 10), and 90% (9 of 10 [$P < 0.05$]), respectively (Fig. 4C). In SEB-treated mice, the survival rates of the control group and the groups treated with vitamin B2 (20 mg/kg/6 h), APC (300 units/kg/6 h), and vitamin B2 plus APC were 0% (0 of 10), 60% (6 of 10 [$P < 0.05$]), 40% (4 of 10), and 90% (9 of 10 [$P < 0.05$]), respectively (Fig. 4D).

Treatment with vitamin B2 lowered the levels of cytokines (4, 10, 11) and NO (12) that are implicated in the pathogenesis of sepsis and likely enhanced the eradication of bacteria. Thus, vitamin B2 may provide an approach for immunotherapy in the treatment of many inflammatory diseases (including systemic inflammatory response syndrome, cachexia, chronic rheumatism, and nephritis) without excessive suppression of the host defense system. Simultaneous administration of vitamin B2 with APC reduced the mortality of endotoxin- and exotoxin-induced shock, suggesting that therapy with vitamin B2 combined with agents having other properties could be an effective approach to treat sepsis.

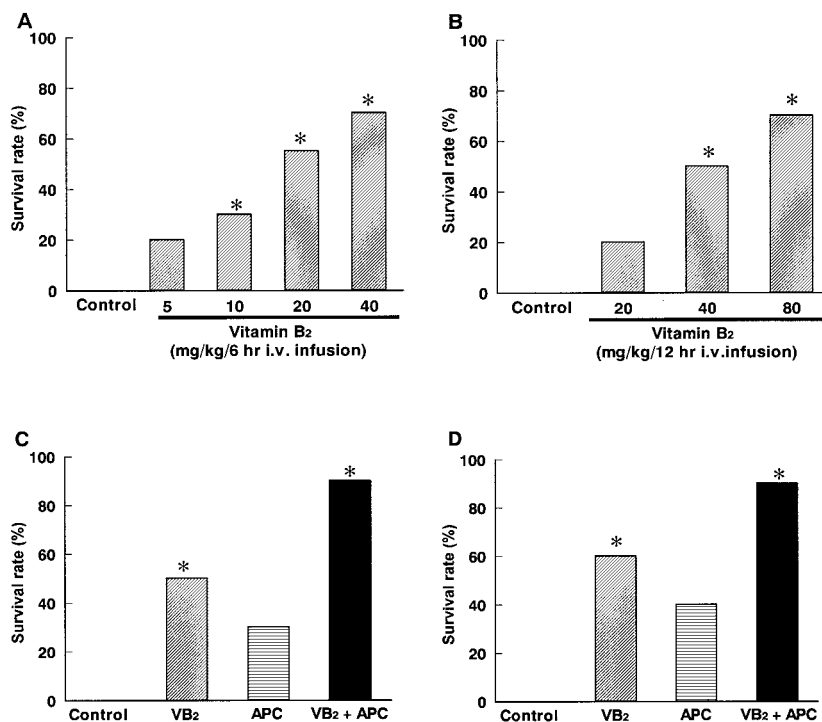


FIG. 4. Therapeutic effect of intravenous (i.v.) infusion of highly purified vitamin B₂ (VB₂) on levels of mouse mortality caused by bacterial infection and toxin-induced shock. (A) *E. coli* infection; (B) *S. aureus* infection; (C) LPS-induced shock; (D) SEB-induced shock. *, $P < 0.05$ versus the results for the control group (Steel test).

In conclusion, treatment with highly purified vitamin B₂ (riboflavin 5'-sodium phosphate; purity > 97%) reduced levels of mortality in mice with septic shock and may be useful for the treatment of patients with sepsis.

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