Effect of Orally Administered Heat-Killed *Enterococcus faecalis* FK-23 Preparation on Leukopenia in Mice Treated with Cyclophosphamide

Takashi HASEGAWA, Yukari OGURA, Takateru INOMATA, Yuko MURAKUMO, Tetsuro YAMAMOTO, Shigeru ABE, and Hideyo YAMAGUCHI

Veterinary Teaching Hospital, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, \(^1\)Nichi-Nichi Pharmaceutical Company, Mie 518-14, and \(^2\)Department of Medical Microbiology, School of Medicine, Toyko University, Tokyo 173, Japan

(Received 1 June 1994/Accepted 11 August 1994)

**ABSTRACT**. Cyclophosphamide (CY) treated mice were orally administered heat-killed *Enterococcus faecalis* FK-23 preparation (FK-23) to define the effect of FK-23 on leukopenia associated with chemotherapy. FK-23 had no inhibiting effect on CY induced leukopenia, whereas it augmented leukocyte reconstitution in CY-treated mice. The percentage of neutrophils increased in the mice orally given FK-23. Furthermore, an increase in a myeloid/erythroid ratio and neutrophilic lineages was found in bone marrow of FK-23 treated mice. These findings suggest that FK-23 may have a potent effect on the augmentation of leukocyte reconstituting capacity in patients receiving chemotherapy.—**KEY WORDS**: heat-killed *Enterococcus faecalis*, leukopenia, reconstitution.

---


Myelosuppression and its related leukopenia are major problems of patients receiving chemotherapy. Recombinant human granulocyte colony stimulating factor (rhG-CSF) have been used in small animals to prevent the leukopenia caused by chemotherapy [10]. Neutralizing antibodies to rhG-CSF, however, were found in the cases of canine cyclic hemopoiesis with G-CSF therapy [2]. This evidence indicates that heterogeneous CSFs may not be suitable for cytokine therapy in veterinary medicine. Attention has been recently focused on the usefulness of biological response modifiers (BRMs) to prevent the leukopenia in patients with chemotherapy, since the exogenous administration of BRMs generally induces the endogenous production of cytokines [3, 7]. Heat-killed *Enterococcus faecalis* FK-23 preparation (FK-23), one of the exogenous BRMs, has been reported to induce tumor necrosis factor (TNF) in mice [8]. It has been demonstrated that TNF stimulates hematopoietic capacity in γ-ray irradiated mice [11]. FK-23, therefore, may protect leukopenia associated with the administration of chemotherapeutic agents in vivo. The present study was designed to investigate the effects of orally administered FK-23 on leukopenia in mice injected intraperitoneally (i.p.) with cyclophosphamide (CY).

Six-week-old female ICR mice, obtained from Japan SLC (Shizuoka, Japan), were used in the present study. Each experimental group consisted of five mice. FK-23 was kindly provided by Nichi-Nichi Pharmaceutical Company (Mie, Japan). This FK-23 is freeze-dried whole bacteria. Mice were injected i.p. with 250 mg/kg of CY (Shionogi, Osaka, Japan). FK-23 preparation in saline was given orally to the mice by a feeding needle. Blood samples were obtained by retroorbital bleeding every day. The number of leukocytes in the peripheral blood was determined by a hemocytometer with Turk’s solution. Differential count was carried out microscopically on blood smears stained with Giemsa solution. In some experiments, bone marrow examination was carried out on day 4. Bone marrow smears were prepared from the femur of anesthetized mice, fixed with methanol, stained with Giemsa solution, and then observed microscopically to evaluate the myeloid/erythroid (M/E) ratio. The statistical significance of the data was determined by Student’s t-test. A p-value less than 0.05 was considered significant.

The effect of FK-23 on the kinetics of the number of peripheral blood leukocytes in CY-induced leukopenic mice was examined. CY-treated mice were orally administered FK-23 at daily dosages of 50, 100, 200, and 400 mg/kg every day. Leukocyte reconstitution was enhanced in the mice treated with 400 mg/kg of FK-23 at 7 and 9 days and the other groups of mice treated with FK-23 at only 9 days after CY treatment, although any dosages of FK-23 tested did not inhibit leukopenia induced by CY (Fig. 1).

Mice received 400 mg/kg of FK-23 at three different times to explore the effects of beginning of FK-23 administration. As shown in Fig. 2, the number of circulating leukocytes in CY-treated control mice decreased from 6,000 ± 543 / μl on day 0 to 1,279±80 / μl on day 4, and then gradually increased to 3,675±195 / μl on day 7. Any dosages of FK-23 used did not show effects

---

**Fig. 1.** Kinetics of the number of circulating leukocytes in mice treated with various doses of FK-23 following CY injection. Mice were injected i.p. with CY at the dose of 250 mg/kg, and then orally administered different doses of FK-23 (O; 0 mg/kg, ●; 50 mg/kg, ▲; 100 mg/kg, ■; 200 mg/kg, □; 400 mg/kg) for 9 consecutive days. The number of leukocytes was determined by a hemocytometer. The mean of five mice ± standard error is given.
Fig. 2. Effect of treatment with FK-23 at three different starting times on the number of peripheral blood leukocytes in CY-treated mice. Mice were injected i.p. with CY at the dose of 250 mg/kg, and the administration of FK-23 was started at 2 days before (○; pre-treatment), the same time (■; simultaneous treatment), and 2 days after (▲; post-treatment) CY injection. These groups of mice were orally administered 400 mg/kg of FK-23 for 5 to 9 days. Open circle (○) represents a control group without FK-23 administration. The number of leukocytes was determined by a hemocytometer. The mean of five mice ± standard error is given. * and ** indicate p<0.05 and p<0.01, respectively, when compared to the control group.

Fig. 3. Effect of treatment with FK-23 at three different starting times on the differential count of circulating leukocytes in mice treated with CY. Mice were injected i.p. with CY at the dose of 250 mg/kg, and the administration of FK-23 was started at 2 days before (pre-treatment), the same time (simultaneous treatment), and 2 days after (post-treatment) CY injection. The number of leukocyte differential in each group was determined microscopically. The mean of five mice is given.
inhibiting a decrease in the number of circulating leukocytes in mice injected with CY. On day 4, the number of peripheral leukocytes in pre-, simultaneous-, and post-treatment with FK-23 was 1,495 ± 184 /μl, 1,100 ± 122/μl, and 1,044 ± 185/μl, respectively. Leukocyte reconstituting capacity, however, was augmented by the administration of FK-23. The number of circulating leukocytes of groups of the mice given pre-, simultaneous-, and post-treatment with FK-23 was 9,983 ± 2,132 /μl (p <0.05), 11,988 ± 2,573 /μl (p<0.01), and 10,360 ± 2,411 /μl (p<0.05), respectively on day 7 (Fig. 2). There was no significant difference among three groups of the mice given FK-23 at different times in their levels of the stimulation of the leukocyte reconstitution. Blood smear examination revealed that the increased number of leukocytes mainly consisted of neutrophils in every group treated with FK-23 (Fig. 3).

The treatment of mice with FK-23 also resulted in a significant increase of M/E ratio. In CY-treated control mice, the ratio was 0.94 ± 0.03, which was almost same to 0.97 ± 0.08 in normal mice. In contrast, the values are 1.61 ± 0.11 (p<0.001), 1.92 ± 0.11 (p<0.001), and 1.78 ± 0.01 (p<0.001) in the groups of pre-, simultaneous-, and post-treatment, respectively (Fig. 4). In addition, increased neutrophilic lineages were found in bone marrow of mice in simultaneous-treatment of FK-23 (Fig. 5).

Fig. 4. The myeloid/erythroid (M/E) ratio of bone marrow cells in mice treated with FK-23. Mice were injected i.p. with CY at the dose of 250 mg/kg, and the administration of FK-23 was started at 2 days before (bar 2), the same time (bar 3), and 2 days after (bar 4) CY injection. Bar 1 and 5 represent control and healthy mice, respectively. M/E ratio was determined by the microscopical examination of bone marrow smears. The mean of three to five mice ± standard error is given. **represents p<0.01, when compared to the control group.

Fig. 5. Photomicrographs of smears of bone marrow cells on day 4 in non-treatment (A) and simultaneous-treatment of FK-23 (B). An increase in the number of neutrophilic lineages was observed in mice treated with FK-23. × 100.
Similar results were obtained on the bone marrow smear in pre- and post-treatment of FK-23 (data not shown).

Augmented leukocyte reconstitution was demonstrated in each group of the pre-, simultaneous-, and post-treatment of FK-23. Blood smear examination revealed that increased leukocytes were mainly neutrophils in FK-23 treated mice irrespective of administration timing (Fig. 3). FK-23 treatment also gave an increase in M/E ratio and neutrophilic lineages in bone marrow of mice injected with CY in all groups tested. These findings suggest that orally administered FK-23 augments leukocyte reconstituting capacity, perhaps through activation of bone marrow. FK-23 directly or indirectly may stimulate not only neutrophil proliferation and differentiation in bone marrow but also release of neutrophils from bone marrow storage pool in CY-treated mice.

Neutrophilia can be induced by the administration of some hematopoietic growth factors [4, 6, 9]. FK-23 has been reported to activate TNF synthesis in vivo [8]. TNF is found to stimulate production of GM-CSF in fibroblasts and vascular endothelial cells [1, 9]. An α-glucan, SPR-901 (RBS), significantly stimulated the proliferative response of bone marrow cells to GM-CSF in mice treated with 5-fluorouracil [3]. GM-CSF, therefore, may play an important role as a factor in the stimulation of leukocyte reconstituting capacity in mice treated with FK-23. The contribution of G-CSF and macrophage CSF (M-CSF) can not be ruled out in the stimulation of leukocyte reconstitution in mice treated with FK-23. Since FK-23 has been reported to activate macrophage functions [8], macrophages activated by FK-23 treatment may produce M-CSF in mice. In addition, M-CSF acted on macrophages to stimulate production of GM- and G-CSF [5]. It is possible that G- and M-CSF may be involved in the stimulation of leukocyte reconstitution in mice treated with FK-23.

CY induced decrease in the number of circulating leukocytes was observed in both pre- and simultaneous-treatment of FK-23, indicating that FK-23 had no inhibiting effect of myelosuppression associated with the administration of CY. This result is not surprising, because enhanced leukocyte reconstitution may be attributed to the endogenous CSFs induced by FK-23. Welte et al. [12] reported that circulating leukocytes were restored 7 to 8 days after CY treatment in monkeys given pre- and post-treatment with G-CSF. Therefore, the restoration of the number of circulating leukocytes may be assumed to be dependent on the timing of CY administration rather than on the timing of the induction of CSFs by FK-23 treatment.

Orally administered FK-23 accelerated leukocyte reconstitution in mice injected with CY, suggesting that FK-23 may be a useful supportive agents on the augmentation of leukocyte reconstituting capacity in patients receiving chemotherapy. The mechanisms of the induction of CSFs are still obscure. The more detailed information about the biological and therapeutical properties of FK-23 are needed before therapeutic application of this agent in clinical areas.

REFERENCES