Shinji Murosaki · Koutarou Muroyama
Yoshihiro Yamamoto · Yasunobu Yoshikai

Antitumor effect of heat-killed *Lactobacillus plantarum L-137*
through restoration of impaired interleukin-12 production
in tumor-bearing mice

Received: 15 July 1999 / Accepted: 28 January 2000

Abstract We have previously reported that heat-killed *Lactobacillus plantarum* L-137 is a potent inducer of interleukin-12 (IL-12) in vivo as well as in vitro in mice. In order to develop effective usage of *L. plantarum* L-137 for tumor immunotherapy, we examined its antitumor effect against DBA/2 mice inoculated with syngenic P388D1 tumor cells in different treatment schedules. Daily injection of *L. plantarum* L-137 from the day of tumor inoculation induced a steep increase in plasma IL-12 only after the first injection but not after subsequent injections, and had no effect on tumor growth and survival time. In contrast, daily injection of *L. plantarum* L-137 from the 7th day after tumor inoculation exerted a marked antitumor effect but such an effect was not evident in mice treated with *L. plantarum* L-137 twice a week from the 7th day. IL-12 production was considerably impaired at the first injection but steeply increased after the third injection in the mice injected daily with *L. plantarum* L-137 from the 7th day. Our results suggest that daily administration of *L. plantarum* L-137 is required to exert an antitumor effect at the late stages of tumor development when IL-12 production is considerably impaired.

Key words *Lactobacillus plantarum* · IL-12 · Antitumor immunity

Introduction

Interleukin-12 (IL-12) was formally known as natural killer (NK) cell stimulatory factor [17] and cytotoxic lymphocyte maturation factor [26]. It is a heterodimeric cytokine produced primarily by antigen-presenting cells. IL-12 has abilities to induce naive CD4⁺ T cells to differentiate into Th1 cells [14], to enhance the cytotoxicity of NK and CD8⁺ T cells [3, 9], and to stimulate NK, NKT and Th1 cells to produce interferon γ (IFNγ) [17, 18, 20]. In addition to enhancing cell-mediated immunity, IFNγ can suppress tumor growth through up-regulation of MHC expression on tumor cells [19] and inhibition of tumor angiogenesis [1]. On the basis of these effects, IL-12 has been shown to exert potent antitumor activities against many murine tumor [27].

OK-432, a preparation of penicillin-inactivated low-virulence *Streptococcus pyogenes* that is extensively used in Japan for cancer immunotherapy, has recently been shown to exert its antitumor effect by promoting a Th1-dominant response via IL-12 induction [8]. Other bacterial preparations, such as *Mycobacterium bovis* BCG and *Lactobacillus casei*, are well known to exert antitumor effects [11, 16, 29]. Among these, heat-killed *L. casei* has been shown to induce IL-12 secretion by macrophages, thereby inhibiting IgE production [25]. We have also developed a potent IL-12 inducer, heat-killed *Lactobacillus plantarum* L-137, which suppresses naturally fed antigen-specific IgE production by induction of IL-12 production in a murine model of IgE production associated with predominant Th2-like responses [22]. However, the precise interaction between endogenous IL-12 induced by these immunopotentiators and the antitumor effect has not been established.
In this study, to develop effective usage of IL-12 inducers for tumor therapy, we examined the antitumor effect of *L. plantarum* L-137 on tumor-bearing mice in the different treatment schedules.

**Materials and methods**

**Mice**

Female 7-week-old DBA/2 mice were obtained from Charle River Japan (Atsugi, Japan). The mice were maintained in our facility and fed a commercial nonpurified diet (CE-2; CLEA Japan, Tokyo, Japan) and tap water for 1 or 2 weeks before experiments were begun. A notice from the Prime Minister’s Office of Japan (no. 6, 27 March 1980) for the care and use of laboratory animals was followed.

**Preparation of *L. plantarum* L-137**

Heat-killed *Lactobacillus plantarum* L-137 was prepared according to the method previously described [22]. The preparation was suspended in saline and sonicated before use in experiments.

**Tumor**

P388D1, a mouse macrophage cell line derived from DBA/2, was obtained from the Institute for Fermentation (Osaka, Japan). This cell line was maintained in vitro in RPMI-1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with HEPES, l-glutamine, penicillin, streptomycin, 2-mercaptoethanol and 10% fetal bovine serum (BioWhittaker, Walkersville, Md.).

IL-12 induction by *L. plantarum* L-137

Normal or tumor-bearing mice were given intraperitoneal injections of 500 µg *L. plantarum* L-137 singly or repeatedly at 1- to 4-day intervals. Plasma from these mice 4 h after the injection was collected and analyzed for IL-10, IL-12 or tumor necrosis factor α (TNFα) levels with an enzyme-linked immunosorbent assay (ELISA).

**Determination of cytokines**

The concentrations of IL-10, TNFα, and IL-12 in plasma were examined by sandwich ELISA. The capture antibodies used were rat anti-(mouse IL-10) monoclonal antibodies (mAb) (Clone 16E3, Endogen, Cambridge, Mass.), rat anti-(mouse IL-12) mAb (clone C17.8, Genzyme, Cambridge, Mass.), and hamster anti-(mouse TNFα) mAb (Genzyme). Detection antibodies used were rat anti-(mouse IL-10) mAb (clone 2A5, Endogen), rat anti-(mouse IL-12) mAb (Clone C15.6, Genzyme), and polyclonal rabbit anti-(mouse TNFα) (Genzyme). Standard cytokines used were recombinant mouse (rm) IL-10 (Genzyme), rmIL-12 (Genzyme), and rmTNFα (Genzyme). Each well of the 96-well flat-bottom plates was coated with 100 µl capture antibody (6 µg/ml) diluted with borate buffer (pH 8.5). After overnight incubation at room temperature, the wells were blocked with 1% bovine serum albumin in borate buffer for 30 min at room temperature. The wells were washed three times with the same buffer containing 0.05% Tween 20 (borate/Tween), and then plasma or the serially diluted standard cytokine was incubated for 90 min at room temperature. The wells were washed with borate/Tween before diluted detection antibody was added. Following incubation and a wash with borate/Tween before diluted detection antibody was added. After overnight incubation at room temperature, the wells were washed and then plasma or the serially diluted standard cytokine was incubated for 90 min at room temperature. The wells were washed three times with the same buffer containing 0.05% Tween 20 (borate/Tween), and then plasma or the serially diluted standard cytokine was incubated for 90 min at room temperature. The wells were washed with borate/Tween before diluted detection antibody was added. Following incubation and a wash with borate/Tween, diluted peroxidase-conjugated mouse anti-(rat IgG1) mAb (Experimental Immunology, Belgium) or diluted peroxidase-conjugated rat anti-(rabbit IgG) mAb (Zymed Laboratories, San Francisco, Calif.) was added to the wells for detection of IL-10 and IL-12 or TNFα. After incubation for 90 min, the wells were washed with borate/Tween and o-phenylenediamine (1 mg/ml) in phosphate buffer (pH 7.0, 0.2 µl/ml H2O2) was applied. Reactions were stopped with 0.75 M H2SO4 after incubation for 40 min. The absorbance at 492 nm minus that at 630 nm was measured with a microplate reader MTP-32 (Corona Electric, Ibaragi, Japan).

In vivo treatment of tumor-bearing mice with *L. plantarum* L-137

P388D1 cells (1 × 10⁶ cells) were injected intraperitoneally into DBA/2 mice on day 0. The mice were then given repeated intraperitoneal injections of 500 µg *L. plantarum* L-137 on days 0, 3, 6 and 10, or on days 7, 10, 13 and 17, or for 5 consecutive days from day 0 or from day 7. The body weights of the tumor-inoculated mice were measured every 2-4 days for evaluation of tumor growth.

**Statistical analysis**

The resulting survivals were analyzed by generalized Wilcoxon test [10] to assess the significance over time. Comparisons between two groups were analyzed by Student’s *t*-test. Significance was assessed at *P* < 0.05.

**Results**

Induction of IL-12 by repeated administration of *L. plantarum* L-137 to normal mice

We have previously reported that a single administration of *L. plantarum* L-137 to normal mice increased plasma IL-12 levels in a dose-dependent manner, with a steep increase at a dose of 500 µg [22]. However, the effects of repeated administration of *L. plantarum* L-137 on the induction of IL-12 are not known. To evaluate the effect of daily administration of *L. plantarum* L-137 on IL-12 production, we administered 500 µg to normal mice for 3 consecutive days and measured plasma IL-12, TNFα and IL-10 levels 4 h after the injection. Although the first injection of 500 µg *L. plantarum* L-137 steeply increased the plasma IL-12 level, the following injections failed to produce any further increase (Fig. 1A). Similarly, the increase in plasma TNFα level was significant at the first injection of *L. plantarum* L-137 but marginal at the following injections (Fig. 1B). In contrast, a significant increase in plasma IL-10 level was induced by the first injection of *L. plantarum* L-137 and this was enhanced by the following injections (Fig. 1C). Thus, the diminished production of IL-12 in response to *L. plantarum* L-137 after the second injection may be closely correlated with the enhanced increase in plasma IL-10 level. We next administered 500 µg *L. plantarum* L-137 to normal mice at 1- to 4-day intervals and measured plasma IL-12 levels 4 h after the injections. Although the second injection of *L. plantarum* L-137, 1 or 2 days after the first injection, did not increase the plasma IL-12 level, significant increases in plasma IL-12 levels were observed when the second injection was 3 or 4 days after the first injection (Fig. 2). These results