Tryptanthrin Inhibits Interferon-γ Production by Peyer’s Patch Lymphocytes Derived from Mice That Had Been Orally Administered Staphylococcal Enterotoxin

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Tryptanthrin, a biologically active compound found in the medicinal plant Polygonum tinctorium, reportedly has several biological activities. We investigated the effects of tryptanthrin on cytokine production by lymphocytes in response to staphylococcal enterotoxin B (SEB), which causes a variety of disorders in humans based on its induction of large amounts of immunostimulatory cytokines. Tryptanthrin dose-dependently inhibited interferon-γ (IFN-γ) and interleukin-2 production by mouse spleen cells and Peyer’s patch (PP) lymphocytes in vitro. The efficacy of tryptanthrin was further studied in a mouse model in vivo. Tryptanthrin was administered orally 2 h after an oral challenge with SEB. Nineteen hours after SEB administration, PP lymphocytes were prepared, and IFN-γ production by PP lymphocytes was examined. The production of IFN-γ increased after SEB administration, and the elevated IFN-γ production was significantly inhibited by tryptanthrin treatment. These results suggest that tryptanthrin may be effective in the treatment of disorders of the intestines, such as food poisoning, that are associated with activated lymphocytes.

Key words tryptanthrin; superantigen; cytokine

Polygonum tinctorium (P. tinctorium) is a folk medicine used for its anti-inflammatory, anti-pyretic and detoxification effects in China, Korea and Japan. Tryptanthrin, 6,12-dihydro-6,12-dioxoindolo-(2,1-b)-quinazoline is an ingredient of the preparation, and has been reported to have various biological activities, such as anti-microbial,1–3) anti-tumor,4,5) and anti-inflammatory activities.6,7) Recently, tryptanthrin was also found to suppress dextran sodium sulfate-induced colitis in mice, probably based on its inhibition of interleukin-2 (IL-2) production by activated spleen cells.8)

Staphylococcus aureus produces various toxins, including Staphylococcal enterotoxins and toxic shock syndrome toxin-1, which cause a variety of disorders in humans and in other species ranging from food poisoning to toxic shock syndrome.9,10) These toxins are absorbed through the intestinal epithelium in an immunologically intact form,11) and act as superantigens due to their activity to cross-link major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APC) with the Vβ-chains of T cell receptors on T cells. This polyclonal activation of T cells results in the overproduction of cytokines, including interferon-γ (IFN-γ), IL-2 and tumor necrosis factor-α (TNF-α), which are closely associated with the toxin-elicited symptoms.9,10)

In this study, we examined the effect of tryptanthrin in vivo on cytokine production by Staphylococcal enterotoxin B (SEB)-treated mouse lymphocytes as a basis for the possibility that this substance may modify cytokine-related disorders in the intestines by controlling the production of lymphocyte-derived cytokines.

MATERIALS AND METHODS

Mice Six-week old female BALB/c mice were used in this study. They were purchased from Charles River Japan (Kanagawa, Japan) and maintained in a specific pathogen-free environment. The mice were exposed to 12 h light/12 h dark cycles, and provided with standard feed and water.

Preparation of Lymphocyte Suspensions Spleen cells were prepared by passing the tissues through a 100 μm nylon cell strainer (Falcon, NJ, U.S.A.). The cells were washed and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Australia), 50 μg/ml streptomycin and 100 U/ml penicillin G.

Peyer’s patch (PP) lymphocytes were prepared according to the method reported by Wilson and co-workers,12) with slight modification. In brief, all visible PP were collected from the intestines and the PP were then digested with 0.2% collagenase (Amano Enzyme, Nagoya, Japan) dissolved in RPMI 1640 medium. After digestion for 30 min at 37 °C, the PP were forced through a 100 μm nylon cell strainer, and PP lymphocytes were collected from the interface between the two gradients. The collected PP lymphocytes were washed 3 times with medium before use.

Cytokine Production in Vitro and Measurement of Cell Number Mouse lymphocyte suspensions obtained from healthy untreated mice were cultured at a cell density of 4×10⁶ cells/ml with SEB (Toxin Technology, FL, U.S.A.) (10 μg/ml) in the absence or presence of tryptanthrin (Wako Pure Chemicals, Osaka, Japan) at concentrations of 0.1 or 1 μg/ml. In the case of lymphocytes obtained from SEB-treated and tryptanthrin-administered mice, the lymphocytes were cultured at a cell density of 4×10⁶ cells/ml without SEB or tryptanthrin. After incubation for 24 or 48 h, the supernatants were harvested and stored at −80 °C until being assayed for cytokine levels. The amounts of mouse cytokines in the culture supernatants were determined by specific sandwich enzyme-linked immunosorbent assays (ELISA).13,14)

Viable cell number was measured by a fluorometric
method using AlamarBlue, a specific marker for enzyme activity in the mitochondria of viable cells. After removing the supernatants of 48 h cultures to determine the amounts of cytokines, fresh growth medium containing 10% AlamarBlue (Wako Pure Chemicals) was added to the wells, and the cells were incubated for 2 h. Fluorescence intensity was measured using an automated fluorescence microwell plate reader set at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

**SEB and Tryptanthrin Treatment in Vivo** Mice were treated orally by gastric intubation with 50 mg of SEB mixed with 400 µg of soybean trypsin inhibitor (STI) (Sigma, MO, U.S.A.) in 500 µl of PBS. Control mice were given STI alone. Two hours later, the mice were orally treated with tryptanthrin dissolved in a solution of 0.5% hydroxypropyl methylcellulose (Sigma) at doses of 0, 80 or 400 mg/body, respectively. Nineteen hours after SEB administration, the mice were sacrificed and the lymphocyte suspensions were prepared according to the method described above.

**Statistical Analyses** Statistical comparisons between pairs were assessed by Student’s *t*-test. For multiple group comparisons, differences were evaluated by one-way analysis of variance (ANOVA), and each group was compared with the control group by Dunnett’s test.

**RESULTS**

**Tryptanthrin Suppresses Cytokine Production by SEB-Activated Lymphocytes** To investigate whether tryptanthrin suppresses cytokine production by lymphocytes, PP lymphocytes and spleen cells were prepared from healthy mice and stimulated with 10 µg/ml SEB in vitro for 24 and 48 h. The culture supernatants were collected and the levels of the different cytokines were determined. As shown in Fig. 1, PP lymphocytes and spleen cells produced IFN-γ and IL-2 in response to SEB. PP lymphocytes produced IFN-γ spontaneously in the absence of SEB stimulation (Fig. 1A). When the SEB-elicited lymphocytes were simultaneously exposed to tryptanthrin, IFN-γ and IL-2 production was inhibited by tryptanthrin in a manner dependent on the concentration of the drug, both after 24 and 48 h of culture (Figs. 1A, B). Toxicity of tryptanthrin on these lymphocytes was assessed at 48 h by staining with AlamarBlue dye. As a result, there was no difference in the fluorescence intensity (FI) of the treated and control cultured lymphocytes (Fig. 1C), indicating that the concentrations of tryptanthrin used in this study were not toxic to cultured lymphocytes.

**Effect of Orally-Administered Tryptanthrin on Cytokine Production by PP Lymphocytes** To investigate whether the oral administration of SEB can activate lymphocytes in vivo, SEB was administered to mice. Nineteen hours later, spleen cells and PP lymphocytes were prepared, cultured for 24 or 48 h without SEB, and the amounts of cytokines in the culture supernatants were measured. Although IFN-γ was not detected in spleen cell cultures, IFN-γ levels in PP lymphocyte cultures significantly increased after SEB administration (Fig. 2). TNF-α and IL-2 were not detected in the culture supernatants of either PP lymphocytes or spleen cells (data not shown).

The SEB-administered mice were further treated with tryptanthrin to investigate the effect of oral tryptanthrin on IFN-γ production by PP lymphocytes. Nineteen hours after SEB treatment, PP lymphocytes were prepared and cultured. As shown in Fig. 2, the amount of IFN-γ was elevated by the oral administration of SEB. The increased IFN-γ production induced by SEB administration was significantly inhibited by oral treatment with tryptanthrin in a dose-dependent manner. No visible changes were observed in the general conditions of the SEB- and tryptanthrin-administered mice.
administration of SEB alone in our experiments was not sufficient to induce a large amount of the cytokines. Considering that tryptanthrin inhibited not only IFN-γ and IL-2 production in vitro in this study, but also IL-2 production in vivo, it is possible that tryptanthrin inhibits the in vivo production of other inflammatory cytokines, even when these are produced in response to SEB in disease.

The mechanism by which tryptanthrin inhibits IFN-γ production is unclear. The regulation of IFN-γ mRNA transcription is complex and has not been characterized well, but the production of this cytokine induced by LPS plus superantigen is reportedly controlled by NF-κB. Ishihara and coworkers reported that the production of nitric oxide (NO), a known pro-inflammatory molecule, is inhibited by tryptanthrin. They reported that the inhibition of NO production is due to the inhibition of inducible NO synthase protein expression, which is also controlled by NF-κB, suggesting that tryptanthrin may inhibit the expression of IFN-γ through the same pathway. The mechanism by which tryptanthrin affects immune cells must still be defined.

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REFERENCES