Effects of Oral Administration of *Phellinus linteus* on the Productions of the Th1- and Th2-type Cytokines in Mice

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ABSTRACT

Background: The mushroom *Phellinus linteus* (PL) has been shown to have the anti-tumor and immunostimulatory effects. We hypothesized that the hot water extract of PL (WEPL) exerts its significant immunostimulatory effect by inducing production of the Th1-derived cytokine interferon-γ (IFN-γ) by T lymphocytes. **Methods:** T lymphocytes were isolated from the mice fed with 200 mg/kg of WEPL once a day for 4 weeks, and then stimulated with the mitogen concanavaline A (Con A). IFN-γ gene and intracellular protein expressions were analyzed by RT-PCR and flow cytometry, respectively. The production of IFN-γ was measured by enzyme-linked immunosorbent assay. **Results:** WEPL significantly enhanced the transcription of IFN-γ mRNA. The effect of WEPL on IFN-γ expression was further supported by a concomitant increase in the number of cells with intracellular IFN-γ protein as well as the secretion of IFN-γ. However, WEPL did not modulate either gene expression or protein secretion of interleukin-4, a Th2-associated cytokine, by Con A-stimulated T lymphocytes. **Conclusion:** Our results demonstrate that one of the potentially beneficial anti-tumor and immunostimulatory effects of WEPL may be mediated through the enhancement of IFN-γ secretion by T lymphocytes. **(Immune Network 2003;3(3):182-187)**

Key Words: *Phellinus linteus*, interferon-γ, immunity, T lymphocyte

Introduction

Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Korea, China and Japan continues to rely on mushroom-derived preparations. *Phellinus linteus* (PL), commonly referred to as 'Sangwhang' in Korea, is a fungus belonging to the *Hymenochaetaceae basidiomycetes* and is found mainly in tropical America and Africa (1). PL has been used as a traditional medicine in Korea, China, Japan and other Asia countries for the treatment of various diseases, including gastroenteric disorder, lymphatic diseases and various cancers (2-5). It was previously reported that PL has the effects of inhibiting tumor growth and metastasis (6). The hot water extract of PL (WEPL) was the most potent, with a growth inhibition rate of about 96.7% to sarcoma 180 transplanted to immune competent ICR mice (7). It has been also reported that the crude polysaccharide purified from WEPL stimulated the proliferation of T lymphocytes (8). However, the molecular mechanism underlying these biological activities of PL remains to be elucidated. Given the potent effects of WEPL on T lymphocyte proliferation, it would be expected that WEPL would alter production of T cell-derived cytokines.

It is currently recognized that helper T (Th) lymphocytes may be divided into two functional subclasses, Th1 and Th2 cells, based upon the cytokines that they produce and their effects on cell-mediated and humoral immunity. Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, and IL-12 and enhance cell-mediated immunity. Th2 cells produce IL-4, IL-5, and IL-6 and exert negative immnuoregulatory effects on cell-mediated immunity, while up-regulating humoral immunity (9). We hypothesize that WEPL exerts its significant immunostimulatory effects by

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differentially regulating the production of Th1- and Th2-derived cytokines. In the present study, we assessed the effects of WEPL on the productions of the Th1-derived cytokine IFN-γ and the Th2-mediated cytokine IL-4 by murine T lymphocytes stimulated with the mitogen concanavaline A (Con A), to test the above hypothesis.

Materials and Methods

WELP preparation. The fruiting body of PL used in this study was kindly provided by Ryu Chunghyun Mushroom (http://mushroom.co.kr). The fruiting body of PL was homogenized and boiled in distilled water for 6 h at 100°C. After filtration to remove debris fragments, the filtrate was concentrated in a rotary evaporator and then precipitated with three volumes of 95% ethanol for 24 h at 4°C. The precipitates were acquired using centrifugation. The precipitates were dissolved in distilled water and dialyzed against distilled water. The precipitates were then freeze-dried.

Animals. Female or male C57BL/6 mice, age 6～7 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained under conventional conditions or in barrier facilities in the Animal Care Unit at the University of Wonkwang (Iksan-Shi, Korea) under an approved protocol. On arrival, mice were acclimated for 1 week on a diet of standard pelleted rodent chow in filter top units in the Animal Care Unit. All mice are tested negative for an extensive battery of viruses and parasites and are maintained in quarters with sentinel mice, which are tested routinely. Animals were separated randomly into two groups: control and WEPL. Mice in the WEPL group were given water containing 200 mg/kg of WELP to drink ad lib once a day for up to 4 weeks. Mice in the matched control group were given PBS from the same source without WEPL. All study groups were fed standard rodent chow ad lib throughout the study. In most studies, initial group size was 4～20 mice each in control and WELP groups, caged in groups of four females or one to three males. Mice were killed in groups of three to five controls or WELP mice, and cells were pooled for the described procedures.

Preparation of T cells from mouse spleen. Spleens and lymph nodes pooled from each group were homogenized in RPMI-1640 (RPMI) cell culture medium (Life Technologies, Grand Island, NY) with supplements of 5% heat-inactivated fetal calf serum (Life Technologies, Rockville, MD), 50μM 2-β mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 50μg/ml gentamicin (Sigma Chemical Co.). The red blood cells were lysed by adding lysing buffer (0.83% NH_4Cl in 1 mM Trizma base, pH 7.3). The cells then were washed and re-suspended in medium. To enrich splenocytes for T cells, single-cell suspensions were incubated with anti-mouse B220 biotinylated monoclonal antibody (mAb) for 10 min at 4°C. After washing with degassed column buffer [phosphate-buffered saline (PBS); 0.5% bovine serum albumin; 2 mM EDTA], cells were subsequently incubated with Streptavidin microbeads (Miltenyi Biotec Inc., Sunnyvale, CA) for 15 min at 4°C. B220-positive B cells were removed by using magnetic columns (Miltenyi Biotec Inc.). As determined by flow cytometric analysis, 90～95% of the B cell-depleted cells were T lymphocytes.

Stimulation fo T lymphocytes. Con A (Sigma Chemical Co.) was used at 0.2μg/mL to induce sub-maximal responses in most experiments. Enriched mouse T cells were placed in 96 wells with complete RPMI in equal numbers for normal and WELP cells in the range of $1～2×10^6$ cells/mL. In parallel cultures, the same number of cells was cultured in the same type of plates without Con A. Plates were incubated in a 37°C, 5% CO_2 humidified incubator for various times as indicated. In another experimental set, we stimulated T lymphocytes with phorbol myristate acetate (PMA; Sigma Chemical Co.) or PMA plus Con A. PMA was served as an immune stimulant control.

T lymphocyte proliferation assay. T cell suspensions were plated into 96-well round-bottom plates at a concentration of $2×10^5$ cells/well and stimulated with or without Con A for 4days. Thereafter the cultures were pulsed with 0.5μCi/well titrated thymidine (Amersham, Buckinghamshire, UK) for 16 h and harvested. The proliferative responses were measured by scintillation counting.

RNA isolation. RNA was isolated using the SNAP total RNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. All steps were performed at room temperature, except if indicated. Centrifugation steps were performed at 13,000 rpm. Briefly, 300μL of lysis buffer (5.3 M guanidine HCl, 1.5% Triton-X100, 2.5 mM Tris HCl, pH 7.5, 0.25 mM EDTA) were added to the cell pellets. The cell pellets were then homogenized by pipetting, and 300μL of isopropanol were added. After mixing, the sample was transferred to a column. The column was centrifuged for 1 min and 300μL of wash solution (100 mM NaCl) was added. The column was centrifuged again for 1 min and again 300μL of wash solution were added. The column was centrifuged for 2 min. After adding 67.5μL of water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. After DNase treatment for 10 min at 37°C, 225μL of binding buffer (7 M guanidine HCl, 2% Triton-X100) were added and mixed. Then 150μL of isopropanol was added. After mixing, the

sample was transferred to a fresh column. The column was washed as described above. After adding 21μL of water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. The RNA concentration was measured spectrophotometrically at 260 nm. RNA was subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. RNA was used only when both 28 and 18 S rRNA bands were intact. Samples were stored at -70°C until use.

cDNA synthesis by reverse transcription (RT). One microgram RNA was reverse transcribed using the Reverse Transcription System (Promega, Madison, WI). Briefly, 10.5μL water containing 1μg RNA was mixed with 0.5μL oligo (dT) primer (500μg/mL) and incubated for 10 min at 70°C. Samples were then incubated at 4°C for at least 5 min. To the mixture, 4μL $MgCl_2$ (25 mM), 2μL 10×RT-buffer, 2μL dNTP (10 mM each), 0.5μL RNasin (50 U/μL) and 0.5μL AMV-RT (30 U/μL) was added and incubated at 42°C for 1 h. The reaction was stopped by heating the samples for 5 min at 72°C. Samples were stored at -20°C until use.

Polymerase chain reaction (PCR). The PCR mixture contained 5μg/mL of 5'-primer, 5μg/mL of 3'-primer, 1.5 mM $MgCl_2$, 200μM of each of the nucleotides dATP, dCTP, dGTP and dTTP, and 2 U AmpliTaq Gold DNA polymerase (PerkinElmer Co., Norwalk, CT) in 1×PCR buffer II (PerkinElmer). The primers used were: IFN-γ; 5'-GCT CTG AGA CAA TGA ACG CT-3' (sense) and 3'-AAA GAG ATA ATC TGG CTC TGC-5' (anti-sense), IL-4; 5'-TCG GCA TTT TGA ACG AGG TC-3' (sense) and 3'-GAA AAG CCC GAA AGA GTC TC-5' (anti-sense), and β-actin; 5'-TGA CCG GCT TGT ATG CTA TC-3' (sense) and 3'-GA TAT AGG ACC GAG TGT GAC-5' (anti-sense). One and 2μL of cDNA were amplified as follows: incubation for 12 min at 94°C, then 24, 27, and 30 cycles (β-actin), or 27, 30, and 33 cycles (IFN-γ and IL-4), each of 45 s at 94°C, 45 s at 55°C, and 2 min at 72°C, and a final incubation for 7 min at 72°C. PCR products were electrophorized on a 2% agarose gel and visualized by ethidium bromide staining.

Cytoplasmic cytokines. Enriched T cells were cultured with or without Con A for the times indicated. The protein secretion inhibitor (10μg/mL) brefeldin A (BFA; Epicentre Technologies, Madison, WI) was added to the culture medium for 4～6 h before cell harvest. Cytoplasmic cytokine-staining was initiated by washing cells with washing buffer [PBS containing 20% heat-inactivated newborn calf serum and 10μg/mL BFA (Sigma Chemical Co.)]. Cells were simultaneously fixed and permeabilized with 2 mL PBS containing 1% formaldehyde, 20μg/mL lysolecithin, and BFA (10μg/mL at 4°C for 30 min. Subsequently, cells were washed with washing buffer and incubated with antibody specific to the mouse cytokine IFN-γ conjugated with FITC (BD Biosciences, Mountain View, CA) at room temperature for 30 min in the dark. Finally, cells were washed twice with washing buffer and re-suspended in PBS containing 1% formaldehyde for flow cytometric analysis. Isotypic antibodies were used as controls and were applied to similarly incubated and permeabilized cells. The mean fluorescence intensity (MIF) of IFN-γ-FITC was measured by using FACSvantage™ flow cytometer (BD Biosciences).

Measurement of secreted cytokines. For determination of IFN-γ and IL-4 productions, spleen T cell suspensions were cultured with or without Con A in 48-well plates (Costar, Cambridge, MA) at a concentration of 5×10^6 cells/well. After 40 h of stimulation with Con A, supernatants were taken and stored at -20°C until analyzed. The levels of IFN-γ, IL-2 and IL-4 were measured using mouse enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Cambridge, MA). The sensitivity of these assays was <5 pg/mL.

Statistical analysis. Statistics were determined by using Mintab software (Cupertino, CA). The analyses were performed using Student's *t* test.

Results

The proliferative response of Con A-stimulated T lymphocytes was measured by a thymidine incorporation assay. The oral administration of WEPL (200 mg/kg) once a day for 4 weeks significantly enhanced

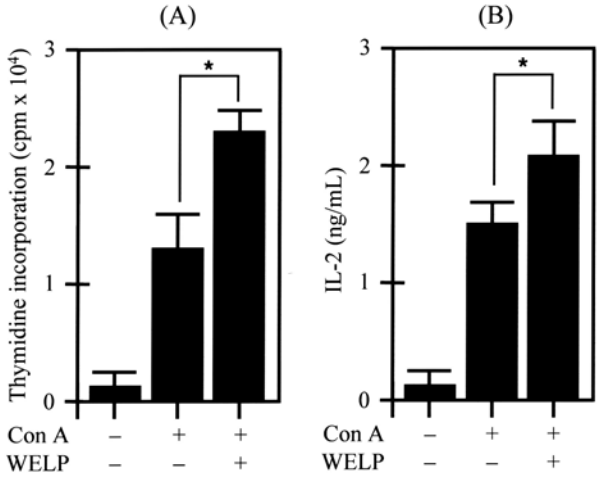

Fig. 1. Proliferation and IL-2 production of Con A-stimulated T lymphocytes from the mice fed either WELP or PBS once a day for 4 weeks. Proliferation (A) was determined by labeling with [^{3}H]thymidine and IL-2 concentration (B) was assayed with ELISA. Data present four independent experiments from WELP- and PBS-treated mice (*$p<0.05$ by ANOVA).

Con A-stimulated T lymphocyte proliferation (Fig. 1A). IL-2 secretion was also enhanced by WEPL administration (Fig. 1B).

The IFN-γ-specific gene expression by Con A-stimulated T lymphocytes was confirmed by RT-PCR. RT-PCR allows comparison of mRNA levels among samples by using a house-keeping gene in the mRNA as a standard, such as β-actin, which is assumed to present at similar levels in each sample. As shown in Fig. 2, WEPL significantly up-regulates the expression of IFN-γ mRNA by Con A-stimulated T lymphocytes (Fig. 2).

In order to examine whether the enhanced gene expression as determined by RT-PCR correlated with the quantitation and identification of cells expressing the intracellular IFN-γ, we performed immunofluorescent staining assay by flow cytometry analysis. Data presented in Fig. 3 show that WEPL significantly increased the number of IFN-γ-positive cells in Con A-stimulated T lymphocytes.

Since WEPL significantly up-regulates the IFN-γ gene expression by Con A-stimulated T lymphocytes,

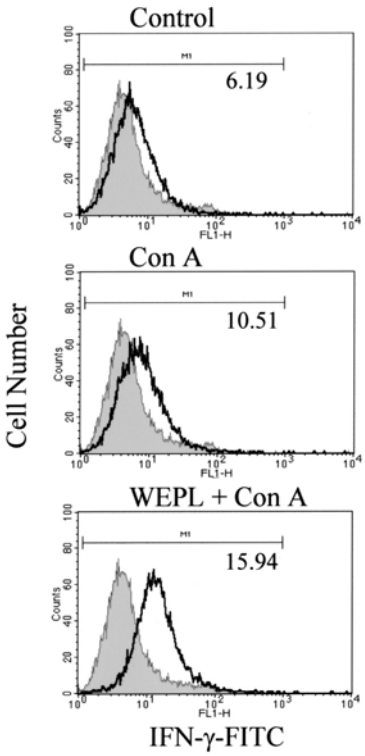

Fig. 3. Intracellular IFN-γ protein levels in Con A-stimulated T lymphocytes from the mice fed either WELP or PBS once a day for 4 weeks. Intracellular IFN-γ expression was determined using flow cytometry. The FL1 axis represents FITC-labeled IFN-γ positive cells and the mean fluorescence intensity is shown inside in each histogram.

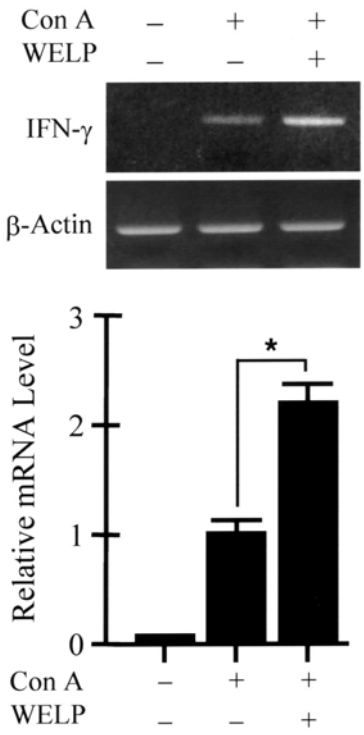

Fig. 2. IFN-γ mRNA levels in Con A-stimulated T lymphocytes from the mice fed either WELP or PBS once a day for 4 weeks. IFN-γ mRNA expression was determined using RT-PCR. Data present four independent experiments from WELP- and PBS-treated mice (*$p < 0.05$ by ANOVA).

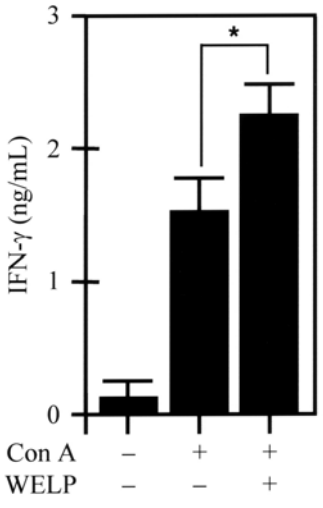

Fig. 4. IFN-γ secretion levels in Con A-stimulated T lymphocytes from the mice fed either WELP or PBS once a day for 4 weeks. IFN-γ secretion was determined using ELISA. Data present four independent experiments from WELP- and PBS-treated mice (*$P < 0.05$ by ANOVA).

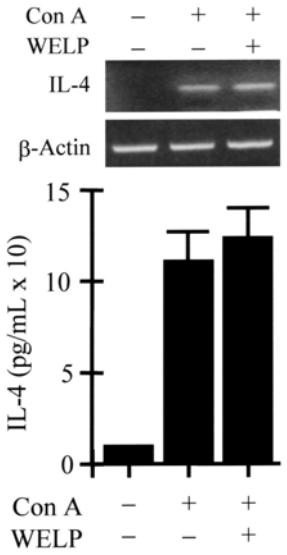

Fig. 5. IL-4 mRNA and secretion levels in Con A-stimulated T lymphocytes from the mice fed either WELP or PBS once a day for 4 weeks. IL-4 mRNA expression and secretion were determined using PT-PCR and ELISA, respectively. Data present four independent experiments from WELP- and PBS-treated mice. There is no significant difference between WELP- and PBS-treated groups.

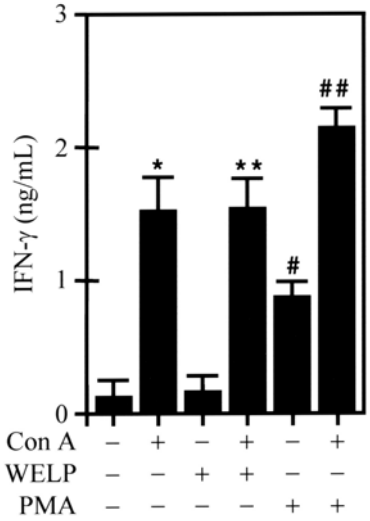

Fig. 6. Effects of WELP on IFN-γ production in T lymphocytes isolated from the mice fed normal diet. T lymphocytes were stimulated for 40 h with 0.2μg/mL of Con A, 200μg/mL of WEPL, Con A plus WEPL, 20 ng/mL of PMA, or Con A plus PMA. IFN-γ secretion was determined using ELISA. Data present four independent experiments (*$P<0.05$ compared with control group, **no significance compared with Con A-treated group, #$P<0.05$ compared with control group, and ##$P<0.05$ compared with PMA-treated group).

we also examined whether WEPL exerted its effect on IFN-γ production. Supernatant from the cultures of Con A-stimulated T lymphocytes was quantitated for IFN-γ protein by ELISA. Supernatant of Con A-stimulated T lymphocytes from the mice fed WEPL had the significantly enhanced level of IFN-γ (Fig. 4).

Since WEPL significantly up-regulates Th1-derived cytokine IFN-γ gene expression and production by Con A-stimulated T lymphocytes, we also examined whether WEPL administration could exert a modulatory effect on the gene expression and production of the Th2-derived cytokine IL-4. Data presented in Fig. 5 show the effects of WEPL on IL-4 gene expression and IL-4 production by Con A-stimulated T lymphocytes. Neither IL-4 gene expression nor IL-4 production was affected by WEPL diet.

Finally, we examined whether the direct exposure of WEPL to T lymphocyte culture could also enhance IFN-γ production. WELP alone had no effect on IFN-γ production, and it did not enhance Con A-induced IFN-γ production (Fig. 6). Unlike WELP, PMA, an immune stimulant, enhanced Con A-induced IFN-γ production (Fig. 6).

Discussion

There is general acceptance that the Th1-derived cytokines, such as IL-2 and IFN-γ, promote cell-mediated immunity. Conversely, the Th2-derived cytokines, such as IL-4 and IL-6, up-regulate humoral immunity. Th2-derived cytokines can also inhibit cell-mediated immunity. Recent study shows that many immune disorders are attributable to the collapse of the system controlling the proportion of Th1 to Th2 cells (10). Restoration of the proper balance between Th1 and Th2 cells is perceived as essential in the treatment of tumors, which are generated when cell-mediated immunity is affected by immuno-suppressing factors. In the present study, we have demonstrated that WEPL can selectively enhance the production of the Th1-derived cytokine IFN-γ, with no effect on the production of the Th2-derived cytokine IL-4, in mitogen-stimulated T lymphocytes.

Our results presented herein show that oral administration of WEPL can enhance proliferative response and IL-2 production in Con A-stimulated T lymphocytes (Fig. 1), suggesting that WEPL would alter production of T cell-derived cytokines. Indeed, WEPL up-regulates the transcription of the Th1-associated IFN-γ gene in Con A-stimulated T lymphocytes (Fig. 2). We also demonstrated that this effect is biologically significant, as WEPL can induce an increase in T lymphocytes with intracellular IFN-γ protein (Fig. 3) as well as the protein secretion of IFN-γ (Fig. 4). However, WEPL did not modulate either gene expression or protein secretion of IL-4,

a Th2- associated cytokine, by Con A-stimulated T lymphocytes (Fig. 5). These effects do not appear to be due to selective cytotoxicity of WEPL, as T lymphocytes isolated from the mice fed with WEPL showed viability comparable to that of lymphocytes isolated from the control mice (not shown). We should note that WEPL becomes effective only when this extract is administrated at least for a week (not shown). The direct exposure of WEPL to T lymphocytes isolated from the mice fed normal diet did not enhance Con A-induced IFN-γ production (Fig. 6), suggesting that WEPL might indirectly affect immune system. Thus, the mechanism of WEPL action is highly different from that of PMA action. The direct immune stimulant PMA stimulated T lymphocytes to produce IFN-γ and enhanced Con A-induced IFN-γ production (Fig. 6).

Many biologically active polysaccharides have been isolated from various mushrooms, and some of these polysaccharides, such as lentinan and schizophyllan, are used clinically for immune therapy (11-15). These polysaccharides enhance and stimulate the immune system of human and mice, and are thus called biological response modifiers. A variety of polysaccharides having different structures have been derived from various sources and by different extraction processes. Polysaccharides derived from microorganisms generally had beta-D-(1,3)-liked anhydro-D-glucose units as a backbone and periodic beta-D-(1,6)-liked side chains. It has been reported that polysaccharides are particularly abundant in WEPL and crude polysaccharides isolated from WEPL stimulated T lymphocyte proliferation (8). Considering that WEPL enhanced T lymphocyte proliferation (Fig. 1) as crude polysaccharides isolated from WEPL did (8), we speculate that polysaccharides contained in WEPL may be responsible for the enhancing effects on IFN-γ production.

In summary, our observations clearly demonstrate that WEPL selectively enhances the Th1-derived cytokine IFN-γ by mitogen-stimulated T lymphocytes. Evaluation of the molecular mechanisms underlying the immunostimulatory activities mediated by WEPL may be useful in the development of new polysaccharide-based pharmaceutical agents.

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