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Immunoregulatory Effects of *Phellinus linteus* (Berk. et Curt) Teng Extract on the Cytokine Production, T Cell Population and Immunoglobulin E Level in Murine Mesenteric Lymph Node Lymphocytes

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ABSTRACT : *Phellinus linteus* (PL), one of the immune-regulatory substances, is recognized to play the role in the metabolic process on inflammation and immunity. It has been traditionally used in the oriental medicine to treat inflammatory related disease. The purpose of this study was to evaluate the effects of water extracts of PL on the mesenteric lymph node lymphocytes immune function in the ICR male mice. Control mice received vehicle only. The PL treated mice were administered the respective extract by oral gavages for 4 weeks. IgE concentrations in serum and MLN lymphocytes were significantly lower in PL treated mice than in control mice. PL increased the proportion of $CD4^+$ and $CD8^+$ T cells in MLN lymphocytes. PL significantly decreased Th2 cytokine concentrations and mRNA expression levels in cytokine secretions. Therefore, water extracts of PL modulate inflammatory parameters through regulation of immunoglobulin production resulting from decreased Th2 cytokine secretion and mRNA expression levels and reduce pro-inflammatory cytokine secretion and mRNA expression in MLN lymphocytes.

Key word : *Phellinus linteus*, mesenteric lymph node, cytokine, IgE, Immune-regulation

INTRODUCTION

The edible basidiomycete fungus *Phellinus linteus* (PL) has been used for the immunochemotherapy of various cancers in oriental countries. The fungus has antitumor and immunostimulatory properties (Han *et al.*, 1999; Kim *et al.*, 1996). The antitumor activity results from activation of the host's immune response. Polysaccharide from PL is well known to act as a biological response modifier (BRM), due to its modulation of host biological responses against tumors (Wasser and Weis, 1999). The immunological activities of PL have been investigated extensively (Kim *et al.*, 1996; Oh *et al.*, 1992; Song *et al.*, 1995; Lee *et al.*, 1996); PL positively stimulates the immune function of T-lymphocytes, natural killer (NK) cells, macrophages, and B-lymphocytes.

The water soluble polysaccharides of PL reportedly stimulate systemic immunity (i.e., the spleen). However, the immunomodulatory properties of this fungus on the intestinal immune system have not previously been investigated. Thus, we evaluated the immune modulating effects of PL extract on mesenteric lymph node (MLN) lymphocytes in this report. It was examined the modulating effects of PL extracts on inflammatory parameters such as immunoglobulin (Ig) production, T-cell populations and cytokine secretions in murine MLN lymphocytes.

MATERIALS AND METHODS

Reagents

Propylene glycol and cocanavalin A (Con A) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS) and Trizol reagent for RNA preparation were purchased from Invitrogen Corporation (Carlsbad, CA, USA). CD4-FITC and CD8-PE monoclonal antibodies were purchased from Santa Cruz Biote chnology, Inc. (Santa Cruz, CA, USA). Antibodies used to detect IgE were purchased from Zymed Laboratories (San Francisco, CA, USA) and Biosource International (Comarillo, CA, USA). Monoclonal antibodies used to measure cytokines

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were purchased from ID Labs (Ontario, Canada). PCR reagents were purchased from Promega (Madison, WI, USA).

Preparation of PL extract

PL was ground to a fine powder with a grinder. Powder (3 kg) was extracted with water (36 L) for 1 day at room temperature. After filtration, the residue was extracted at room temperature and then filtered again. The water was removed with a rotary evaporator under vacuum at 40 ℃. The extract (60 g) was stored at −20 ℃ until used. PL extracts were dissolved in propylene glycol and used for the animal experiments.

Animals

Four-week-old male ICR mice were obtained from Samtako Bio Korea (Osan, Korea). All animal care was performed within the guidelines approved by the institutional animal care and use committee. All mice were kept in stainless steel bottom cages in a room controlled at 23 ± 1 ℃ and 55 ± 5% humidity under a 12-h dark/light cycle. Mice were fed a commercial diet (Purina chow) and given free access to water *ad libitum*. After a weeklong adaptation period, mice were randomized into two groups of ten mice each. Control group mice received the vehicle (propylene glycol) only. The PL group mice were administered the respective extract (2 ㎎/㎏ B.W/day) by oral gavage. Food intake was measured by subtracting the remaining feed from the amount offered every other day and body weights were recorded every week. After 4 weeks of experimental feeding and oral gavage of extract or propylene glycol, mice were anesthetized, and peripheral blood was collected by retroorbital bleeding for the analysis of IgE production.

Lymphocyte culture

MLNs were removed from mice under sterile conditions. MLN lymphocytes were squeezed out into RPMI 1640 medium. After incubating the cells at 37 ℃ for 30 min to remove fibroblasts, 5 ㎖ of the cell suspension was layered on 4 ㎖ of Lympholyte-mice (Hornby, Canada) before centrifugation at 1500 xg for 30 min. The lymphocyte band at the interface of what was recovered and the cells were rinsed three times with RPMI 1640 medium. Cell viability, measured by trypan blue staining, was greater than 95%. The MLN lymphocytes isolated from each group of mice were cultured in 10% FBS/RPMI 1640 medium. The lymphocytes were adjusted to 2×10^6 cells/㎖ in 24-well microtiter plates in a final volume of 1 ㎖. Cells were incubated at 37℃ for 48 h in the absence or presence of Con A (25 ㎍/㎖). The concentrations of IgE were measured by sandwich ELISA methods in supernatant, as described previously (Lim *et al.*, 1995, 1997). The concentrations of cytokines (IFN-γ, interleukin (IL)-2, IL-4, and IL-10) were also measured by ELISA using cytokine-specific capture and detection monoclonal antibodies as described previously (Lim *et al.*, 2000, 2002).

T-cell population analysis

Either CD4-FITC or CD8-PE monoclonal antibodies (5 ㎕) were added to MLN lymphocytes, suspended at 1×10^6 cells/㎖ and incubated at 4 ℃ for 30 min. The lymphocytes were rinsed three times with PBS containing 10% FBS and centrifuged at 300 xg for 5 min. The stained lymphocytes were fixed with 2% paraformaldehyde and counted by EPICS Altra™ flow cytometry (Beckman Coulter, Cupertino, CA) (Lim *et al.*, 2000). Each analysis, including the negative control samples, was based on at least 10^4 events after the dead cells and residual erythrocytes were eliminated by gating on the basis of the forward angle light scatter.

RT-PCR analysis of cytokines

Total RNA was isolated from MLNs using Trizol reagent. One ㎍ of total RNA was used for single strand cDNA synthesis. Reverse transcription was performed at 30 ℃ for 10 min, 42 ℃ for 30 min and then at 99 ℃ for 5 min to inactivate avian myeloblastosis virus (AMV) RT XL. The following primers were used: IFN-γ, (GenBank™ accession number M28621), forward 5'-TGCATCTTG-GCTTTGCAGCTCTTC-CTCATGGC-3' and reverse 5'-TGCACCTGTGGGTTGTTG-ACCTCAAACTTGGC-3'; IL-2, (GenBank™ accession number NM008366), forward 5'-AACAGCGCACCCACTTCA-3' and reverse 5'-TTGAGATGATGCTTTGACA-3'; IL-4, (GenBank™ accession number BC027514), forward 5'-ACGGCA-CAGAGCTATTGAT-G-3' and reverse 5'-ATGGTGGCTC AG TACTACGA-3'; IL-10, (GenBank™ accession number M37897), forward 5'-CAAACAAAGGACCAGCTGGAC-3' and reverse 5'-GTCCTGCATTAAG-GAGTCG-3'; β-actin, (GenBank™ accession number BC013380), forward 5'-GTGGG-GCGCCCCAG-GCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGAT-3'. The IFN-γ and IL-2 amplifications were performed after an initial denaturation at 94 ℃ for 5 min, by denaturing at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, extension at 72 ℃ for 1 min for 30 cycles and finally 72 ℃ for 10 min (MJ Research, MA, USA). The IL-2, -4, and -10 amplifications were performed using similar conditions with 33 cycles, while β-actin was amplified in 25 cycles. The PCR products were loaded onto 1.2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Statistical analysis

The data are presented as mean ± SEM. Differences between

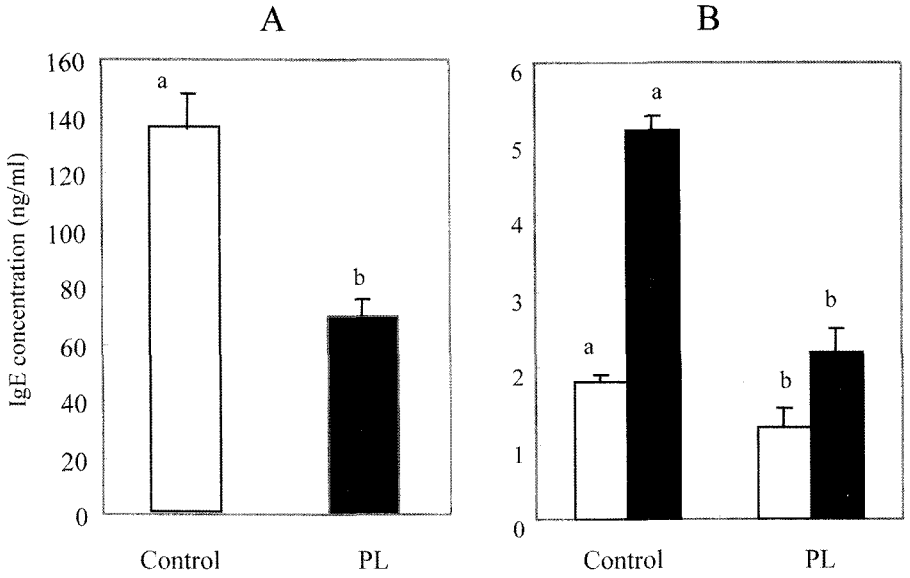

Fig. 1. IgE concentrations in serum and mesenteric lymph node (MLN) lymphocytes. Control group mice received vehicle (propylene glycol) only. *Phellinus linteus* extract (PL) group mice were administered the respective extract by oral gavage for 4 weeks A; IgE concentrations in the serum. Each bar represents mean ± SEM of ten mice. Mean values with different superscripts are significantly different ($p < 0.05$) B; IgE concentrations in the MLN lymphocytes. The MLN lymphocytes were adjusted to 2×10^6 cells/ml in 24-well microtiter plates and incubated at 37℃ for 48 h in the absence or presence of Con A (25 ㎍/㎖) The concentrations of IgE were measured by sandwich ELISA methods in supernatant, as described in the *materials and methods*. Each bar represents mean ± SEM of ten mice. Mean values with different superscripts are significantly different ($p < 0.05$).

the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package SPSS 10.0 (SPSS institute, Chicago, IL) was used for these analyses.

RESULTS

IgE concentrations in serum and MLN lymphocytes

Body weight gain and food intake did not differ significantly among the two groups (data not shown). Serum IgE concentrations were significantly decreased in mice administered PL compared to levels in mice in the control group (Fig. 1A).

After 48 h of MLN lymphocytes incubation in the absence or presence of Con A, IgE concentrations in the PL groups were significantly higher in MLN lymphocytes not stimulated with Con A. The IgE concentration pattern in MLN lymphocytes treated with Con A was highly similar to that in non-stimulated cells (Fig. 1B). PL significantly decreased IgE concentrations below the concentration in the control group regardless of Con A treatment.

T-cell populations of MLN lymphocytes

The proportions of $CD4^+$ and $CD8^+$ T cells are shown in Table 1 and Fig. 2. The proportion of $CD4^+$ T cells was significantly higher in the PL groups than in the control groups,

Table 1. T-cell population of MLN lymphocytes in mice given extracts (Unit: %)

Groups	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Control	55.12 ± 1.0^a	15.21 ± 0.4^b	3.63 ± 0.10^a
PL	61.08 ± 0.3^b	14.38 ± 0.1^a	4.26 ± 0.05^b

Values represent the means ± SEM of ten mice. Values with different superscripts are significantly different ($P < 0.05$). Either CD4-FITC or CD8-PE monoclonal antibodies (5 ㎕) were added to MLN lymphocytes suspended at 1×106 cells/㎖ and incubated at 4℃ for 30 min. The stained lymphocytes were fixed with 2% paraformaldehyde and counted

while the proportion of $CD8^+$ T cells was lower in the PL group. Interestingly, the $CD8^+$ cell population was not decreased in mice administered PL but remained at the control level. As a result, the ratio of $CD4^+/CD8^+$ T cells in MLN lymphocytes was higher in the PL groups than in the control group.

Th1 and Th2 cytokine secretions in MLN lymphocytes

Various types of lymphokines specifically regulate Ig production by class (Pene *et al.* 1988). The Th1 and Th2 cytokine secretions in MLN lymphocytes after administration of extract in the treated groups are shown in Fig. 3A. After 48 h of cultivation of lymphocytes with Con A, IFN-γ concentrations were significantly increased and IL-4 and IL-10 concentrations were

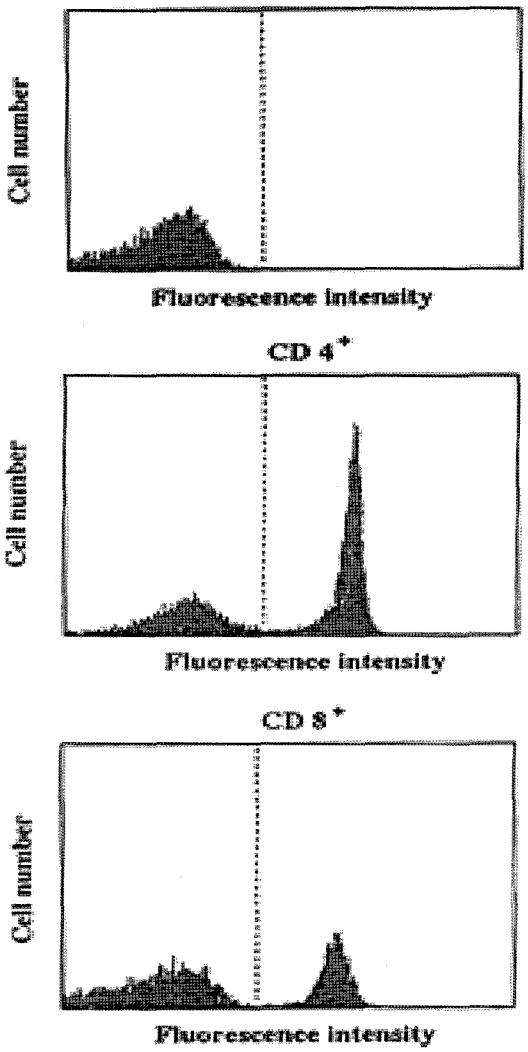

Fig. 2. Flow cytometer histograms illustrating expression of $CD4^+$ and $CD8^+$ by mesenteric lymph node MLN lymphocytes Cells were stained with fluorececein-labeled anti-CD4 and with phycoerythrin labeled anti-CD8. Negative control histogram is that observed with cells not stained for the CD surface markers. Histograms were obtained from the population of the red fluorescence of PE-stained cells such that all events represent either $CD4^+$ and $CD8^+$ Tcells. Each histogram was obtained from analyses of at least 10^4 cells after removing dead cells and erythrocytes according to the sizing with forward angle scatter. Each panel depicts results from on rat fed the control diet.

markedly decreased in mice in the treated groups compared to levels of the control group. RT-PCR analysis for Th1 and Th2 cytokines was conducted to determine if the extracts influence expression of IFN-γ, IL-2, IL-4 and IL-10 in MLN lymphocytes. mRNA expression levels of IL-4 and IL-10 were significantly decreased in the PL group as compared to the control groups (Fig. 3B).

DISCUSSION

In the current study, we demonstrate that water extracts of PL modulate inflammatory parameters in murine MLN lymphocytes. IgE plays a major role in the development of food allergies, while IgA is thought to suppress food allergies by preventing antigen binding to the intestinal mucosa (Metcalfe, 1991). Lymphocytes in the intestinal mucosa first interact with antigens in the organized lymphoid tissues (Peyer's patches and lymphoid follicles in the colon) and further differentiate and mature in the germinal centers of the lymphoid follicles. Thereafter, they rapidly leave the mucosa and migrate through the MLN and the thoracic duct to reach the systemic circulation (James & Kiyono, 1999). In the present study, we show that PL significantly decreased IgE concentrations in serum and MLN lymphocytes (Fig. 1). These results suggest that decreased IgE concentrations may prevent the development of food allergies by activating the intestinal immune response due to the modulation of Ig production in MLN lymphocytes.

PL increased the proportions of $CD4^+$ and $CD8^+$ T cells in MLN lymphocytes (Table 1). $CD8^+$ T cells may regulate the development of $CD4^+$ helper T cells by producing IFN-γ or other regulatory cytokines which suppress the development of Th2 cells and favor Th1 cell growth (Cher & Mosmann, 1987). Th1 cells produce IFN-γ, IL-2 and TNF-α, which influence cell-mediated immunity, while Th2 cells produce IL-4, and IL-10, which regulate humoral or antibody-mediated immunity, particularly IgE synthesis (Fiorentino *et al.*, 1989). PL significantly increased IFN-γ concentrations in MLN lymphocytes (Fig. 3). A greater proportion of $CD4^+$ T cells and greater IFN-γ concentrations were observed in the PL groups compared to the control group. Therefore, T cell differentiation to Th1 cells may be increased by the increased concentration of Th1 cytokines induced by PL administration.

The balance between pro-inflammatory and anti-inflammatory cytokines in the gastrointestinal immune response produced by $CD4^+$ T-helper subset 1 and 2 (Th1 and Th2 cells) of T lymphocytes is an important part of the pathophysiological mechanism involved in inflammation (Mosmann TR & Moore KW., 1991; Powrie *et al.*, 1994; Berg *et al.*, 1996; Simpson *et al.*, 1997). In this study, PL significantly increased IFN-γ secretions and mRNA expression levels (Fig. 3). Reduction of Th2 cytokine secretion and mRNA expression of IL-4 and -10 in MLN lymphocytes by PL may result in decreased autoantibody production (IgE), which may prevent the development of autoimmune disease or IBD (Lim *et al.*, 2000). Also, PL significantly decreased the secretion and mRNA expression of IL-4 and IL-10, pro-inflammatory cytokines. These observations further suggest that they may inhibit food allergies and

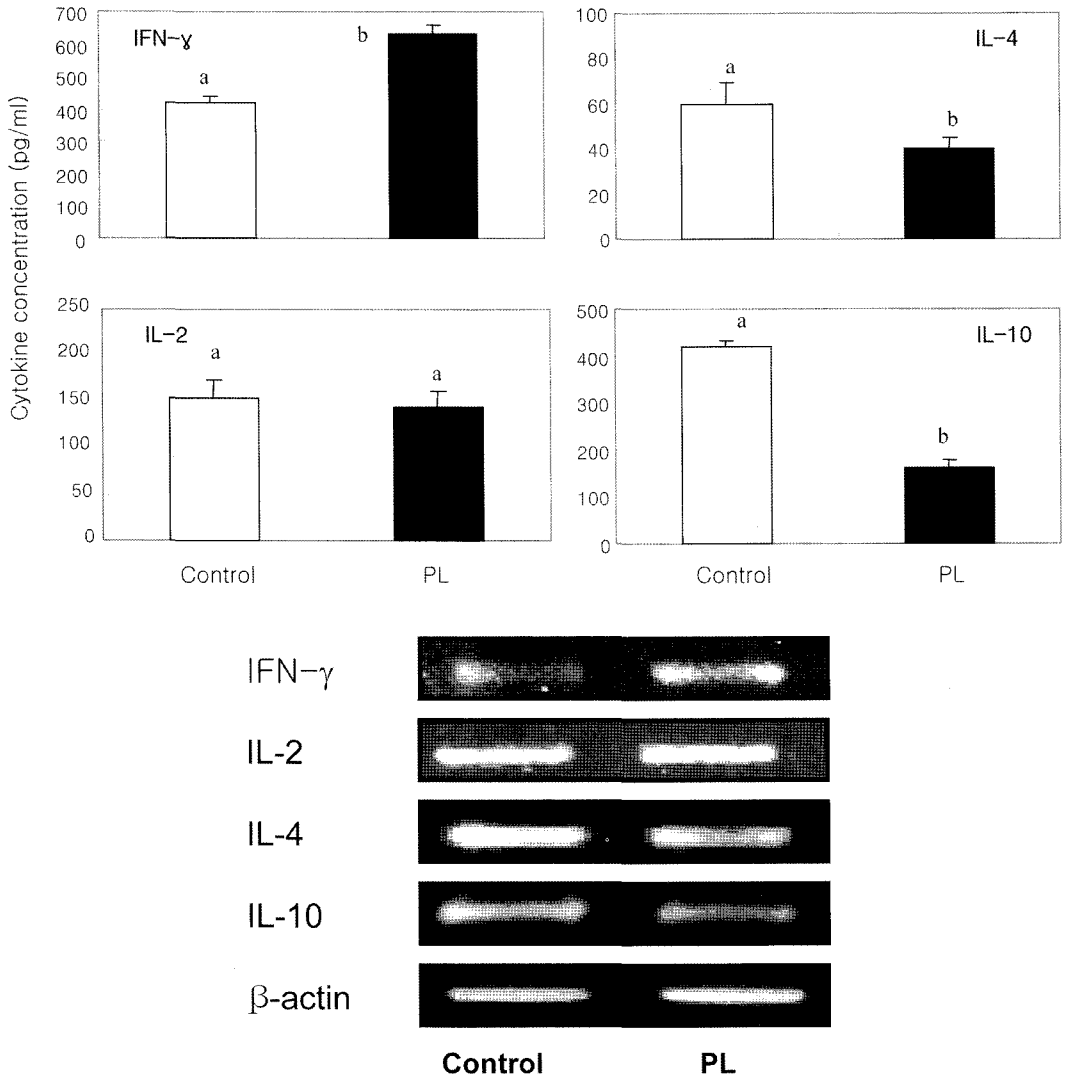

Fig. 3. Th1 and Th2 cytokine secretion and mRNA expression in MLN lymphocytes A; ConA-induced Th1 and Th2 cytokine secretion in MLN lymphocytes. MLN lymphocytes were adjusted to 2×10^6 cells/ml in 24-well microtiter plates and incubated at 37℃ for 48 h in the absence or presence of Con A(25 ㎍/㎖). The concentrations of all cytokines were measured by sandwich ELISA methods in supernatant. Each bar represents mean ± SEM of ten mice. Mean values with different superscripts are significantly different ($P < 0.05$) B; Th1 and Th2 cytokine mRNA expression in MLN lymphocytes measured by RT-PCR analysis One g of total RNA was used for single strand cDNA synthesis. Reverse transcription and PCR conditions are described in the *materials and methods*. The relative levels of all cytokines vs β-actin were determined by densitometry. Values are means ± SEM of ten mice. * $p < 0.05$ relative to control cells. Densitometric quantification of the bands was measured with Bio-1D software (Bio-Rad Laboratories, Inc., Hercules, CA). The data represent typical results obtained in at least three independent experiments. PL, *Phellinus linteus* extract.

IBD by suppressing IL-4 and IL-10 production in areas of inflammation.

We conclude that water extracts of PL modulate inflammatory parameters through regulation of immunoglobulin production resulting from decreased Th2 cytokine secretion and mRNA expression and reduce pro-inflammatory cytokine secretion and mRNA expression in MLN lymphocytes.

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