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Full Length Research Paper

Effect of *ginseng* polysaccharide on TNF-α and IFN-γ produced by enteric mucosal lymphocytes in collagen induced arthritic rats

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To investigate the effects of *ginseng* polysaccharide on T-lymphocytes of enteric mucosal lymphocytes in rats with collagen induced arthritis (CIA). The peyer's patche lymphocyte (PPL), intraepithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) of SD rats were isolated. The PPL, IEL and LPL were checked with flow cytometry and these lymphocytes were co-cultured with ginseng polysaccharide separately at different dosages. ELISA measured the expression levels of TNF-α and IFN-γ in supernatants. The results showed that *ginseng* polysaccharide could increased the protein expression of TNF-α and IFN-γ in the supernatant of PPL on normal rats, and it could lower the protein expression of TNF-α while increase the IFN-γ protein level in the supernatant of PPL on CIA rats. *Ginseng* polysaccharide could lower the protein levels both TNF-α and IFN-γ in the supernatant of LPL on normal rats. And it could lower the protein levels of TNF-α and IFN-γ in the supernatant of both IEL and LPL on CIA rats. *Ginseng* polysaccharide could decrease the expression of TNF-α and IFN-γ, regulate the function of Lymphocytes in enteric mucosal immune system in CIA rats, which indicate that G*inseng* polysaccharide could be used in the treatment of autoimmune disease.

Key words: *Ginseng* polysaccharide, collagen induced arthritis, lymphocyte, enteric mucosal immunity, TNF-α, IFN-γ.

INTRODUCTION

Polysaccharide is one of the complicated ingredients of herbal medicine, which play an important role in traditional Chinese medical treatment. Polysaccharides and polysaccharide-protein complex participate in mediating functions of many kinds of cells, which can regulate immune system through stimulating various immunocytes, promoting the synthesis of cytokines such as interleukin (IL), tumor necrosis factor (TNF), interferon (IFN) etc., and regulating antibodies and complements (Ooi and Liu, 2000). Yet, it is still unclear about the pathway and mechanism of the regulation on intestinal mucosal immunity by polysaccharides.

Nowadays, many researchers are keen on the lymphocytes in intestinal mucosal immune system that refers to the gut-associated lymphoid tissue (GALT) distributed over the internal surface of gastrointestinal tract, including Peyer's Patches (PP), mesenteric lymph nodes and quantities of lymphocyte dispersaling over

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Abbreviations: IL, Interleukin; **TNF,** tumor necrosis factor; **IFN,** interferon; **CIA,** induced arthritis; **PPL,** peyer's patche lymphocyte; **IEL,** intraepithelial lymphocyte; **LPL,** lamina propria lymphocyte; **GALT,** gut-associated lymphoid tissue; **PP,** peyer's patches; **LP,** lamina propria.

Lamina propria (LP) and enteric epithelium (Sato and Iwasaki, 2005). We supposed that the polysaccharides which had not been absorbed might stimulate the lymphocytes of the intestinal mucosal system, thus to take a part in immune regulation. In our research, we selected g*inseng* polysaccharide, popular in research as the representatives of polysaccharides, and hoped to explore their effects on the correlated intestinal mucosal immunocytes.

MATERIALS AND METHODS

Animals and polysaccharides

Sprague Dawley (SD) rats, male, 8 to 10 weeks old with a mean weight of 180 to 200 g were purchased from the Center of Experimental Animals, the Guangzhou University of Traditional Chinese Medicine. Rats were housed in a temperature-, humidity- and light-controlled environment with free access to rodent chow and water.

The light–dark cycle was 12:12 h with the light phase from 06:00 to 18:00. The rodent license of the laboratory (NO.SCXK 2003-0001) was issued by National Science and Technology Ministry of China. Ginseng polysaccharide, purchased from Shenyang Shuangding Pharmaceuticals Company Limited (National license number for drugs: Z20025235, Batch No：05030102), was extracted from ginseng with 75% ethanol, and purified with 0.3% charcoal, resin column filtration, washing and precipitation with acetone. It contains monosaccharides, oligosaccharides and polysaccharides. The quality is controlled with containing not less than 30.0 mg/10 ml galacturonic acid.

Induction of CIA and evaluation of arthritis

Soluble pure rat type II collagen (CII) was purchased from Rikard Holmdahl (Lund University, Sweden) and complete Freund's adjuvant was from Sigma Corporation, USA. CII was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml. CIA was induced by immunization once with 100 µg type II collagen emulsified with equal complete Freund's adjuvant under fur skin at the hind paws (Holmdahl et al., 1986). The degree of arthritis was examined every 3 days. The severity of arthritis was expressed as mean arthritic index on a 0 to 4 scale according to the following criteria (Holmdahl et al., 1988): 0 = no edema or swelling, 1 = slight edema and erythema limited to the foot and/or ankle, 2 = slight edema and erythema from the ankle to the tarsal bone, 3 = moderate edema and erythema from the ankle to the tarsal bone, and 4 = severe edema and erythema from the ankle to the entire leg. Each limb was graded, and thus the maximum possible score was 16 for each animal.

Preparation of peyer's patch lymphocytes (PPL) suspension

Four weeks after the immunization, the lymphocytes of Peyer's patches were harvested referring to Lyscom's method (Lyscom and Brueton, 1982). Briefly, Peyer's patches were removed, and the tissues were mechanically dissociated into phosphate buffered saline (PBS).

Lymphocytes were isolated by density centrifugation with Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Amersham Biosciences, Sweden). The lymphocytes were collected by centrifugation at 1500 rpm for 5 min after being washed twice with PBS, and collected by repeated centrifugation and then suspended in PBS at a density of 1×10^6 cells/ml for flow cytometry.

Intraepithelial lymphocyte (IEL) isolation

According to the findings reported by Wyatt (Wyatt et al., 1996), the entire intestine was flushed three times with D-Hank's wash containing antibiotic, then turned over and placed in the centrifuge tube with pre-heated 15 ml spin medium containing 1 mM EDTA and 1 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO). Sections were stirred vigorously on a hot plate/stirrer at 600 to 800 rpm for 30 min at 37°C. The tissue was strained from the supernatant using a #300 basket style sieve after the stirring. The supernatant was centrifuged at 1000 × g for 1 min at 20°C and the supernatant was carefully removed from the pellet by pipetting. The pellet was resuspended by medium consisted of RPMI 1640 solution supplemented with 20% fetal bovine serum in 5.4 ml, and 3.6 ml 100% Percoll was added into, the total volume of 40% Percoll was increased to 9 ml and then vortexed vigorously to mix. Using a 14 gauge, 10 cm teat cannula, 1 ml of 70% Percoll was underlaid carefully and then centrifuged for 30 min at 600 ×g at 25°C. Iso-osmotic Percoll is defined as 90.8% Percoll (GE Healthcare Bio-Sciences, Amersham Biosciences, Sweden) and 9.2% 15 M sodium chloride solution. Following centrifugation, the 40/70-interface layer was collected, washed twice in wash medium and cells were counted.

Lamina propria lymphocyte (LPL) isolation

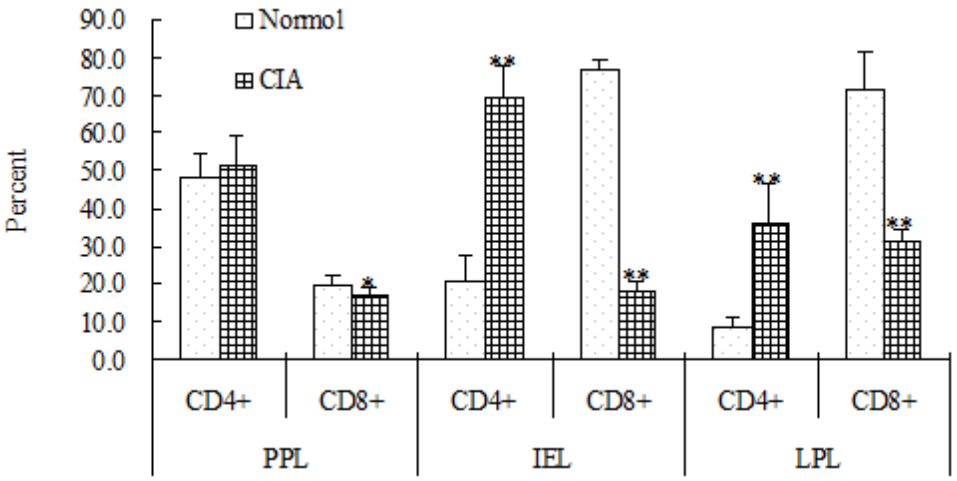
LPL was isolated by following process of intestine described for IEL mentioned above. Tissues were resuspended in spin medium containing 0.1% collagenase II and stirred for 30 min at 37°C on a Thermolyne Nuova II hot plate/stirrer at 600 to 800 rpm. The following steps were the same as the intraepithelial lymphocyte isolation.

Flow cytometry

Flow cytometry was performed on freshly isolated cell suspensions. Immunofluorescence staining of cell surface markers was performed using the immunofluorescence antibody: fluorescein isothiocyannate (FITC)-anti rat CD4, R-Phycoerythrin (PE)-anti rat CD3e, PE-cy5-anti rat CD8a (eBioscience; San Diego, CA, USA). Each sample of 0.1 ml cell suspension was incubated for 30 min at 4°C in the dark, with a solution consisting of 6 µL 0.01 M PBS and an appropriate concentration of antibodies. After washing in saline, the cells were resuspended and fixed. Flow cytometric analysis was performed using a FACS Caliburflow cytometer utilizing CELL Quest software (Becton Dickinson; San Jose, CA, USA). Analysis was carried out on 1×10^4 cells for each sample.

Lymphocyte culture

These lymphocytes were maintained in RPMI-1640 at the density of 1×10^9/L, supplemented with 10% fetal bovine serum, and add 200 µL/well cell suspension to 96-well flat-bottomed microtiter plates. Then 50 µL/well *ginseng* polysaccharide at different concentrations was added according to our previous tests. The *ginseng* Polysaccharide concentrations used were 0.15, 0.3 and 0.6 mg/mL. The RPMI-1640 at 50 µL/well was as the control well. The cells were incubated at 37°C under 5% CO2 and 95% air for 48 h, the supernatant were collected for standby.



F$igure 1. Percentages of CD4+ and CD8+ T cells in PPL, IEL and LPL. Data are expressed as mean ± SD. The CD4+ and CD8+ T cells were determined by flow cytometry. More CD4+ T cells were detected in IEL and LPL in CIA models whereas less CD8+ T cells were detected (*p < 0.05, **p < 0.01 vs. normal).

ELISA assay

TNF-α and IFN-γ levels in supernatant were determined by ELISA test kits (BMS Inc) with reference to the manual. Briefly, the ELISA plates were added with samples at 100 µl/well and diluted HRP-conjugate at 50 µl/well incubating for 4 h, washing with washing buffer. Adding with TMB for half hour, and 1 M H_2SO_4 was used to end the reaction and the plates were read at 450 nm on a multiscan (Labsystems, Multiskan Ascent Finland).

Statistics

Using SPSS 11.5 software, ANOVA was used to determine the significance in the data set. Student-Newman-Keuls test was employed for variable between both groups when equal variances assumed and Dunnetts's t test for equal variances not assumed. Chi square test was employed for the incidence data analysis.

RESULTS

Result one: more CD4+ T cells were detected in IEL and LPL in CIA models whereas less CD8+ T cells were detected ($p < 0.01$, Figure 1). Result two: compared to normal rats, the level of TNF-α in supernatant of PPL on CIA rats were increased ($p < 0.05$). Ginseng polysaccharide could significantly increased the level of TNF-α in supernatant of PPL on normal rats ($p < 0.01$), while significantly decreased the level of TNF-α in PPL of CIA rats ($p < 0.05$, 0.01; Figure 2). Result three: the level of TNF-α in supernatant of IEL on CIA rats was similar to normal rats. Ginseng polysaccharide (0.15, 0.3 mg/ml) could significantly lower the level of TNF-α in supernatant of IEL on CIA rats ($p < 0.05$, Figure 3). Result four: the level of TNF-α in supernatant of LPL on CIA rats was similar to normal rats. *Ginseng* polysaccharide could significantly lower the level of TNF-α in supernatant of LPL on normal rats ($p < 0.05$, 0.01).

Ginseng polysaccharide (0.15 mg/ml) could significantly lower the level of TNF-α in supernatant of LPL on CIA rats ($p < 0.05$, Figure 4). Result five: the level of IFN-γ in supernatant of PPL on CIA rats was significantly lower than normal rats ($p < 0.05$). *Ginseng* polysaccharide could significantly increase the level of IFN-γ in supernatant of IEL both on normal and CIA rats ($p < 0.05$, 0.01, Figure 5).

Result six: the level of IFN-γ in supernatant of IEL on CIA rats was similar to normal rats. *Ginseng* polysaccharide could significantly lower the level of IFN-γ in supernatant of PPL on CIA rats ($p < 0.01$, Figure 6). Result seven: the level of IFN-γ in supernatant of LPL on CIA rats was similar to normal rats. *Ginseng* polysaccharide could significantly lower the level of IFN-γ in supernatant of LPL both on normal and CIA rats ($p < 0.05$, 0.01, Figure 7).

DISCUSSION

Recently, there has been an increasing interest in systematic screening of biological activity of polysaccharides, which play a powerful immune energizing role in particular and have been studied extensively (Liu et al., 1999; Kim et al., 1998). Polysaccharides have the function of activating the maturity, differentiation and proliferation of various immunocytes, increasing non-specific cytotoxic of macrophagocytes, inducing the production and the cytokine receptors expression of cytokines such as IL-1, IL-2, TNF, IFN, etc., enhancing antibody-forming, activating the classical and alternative pathway of complement system and so on (Mose et al., 1998;

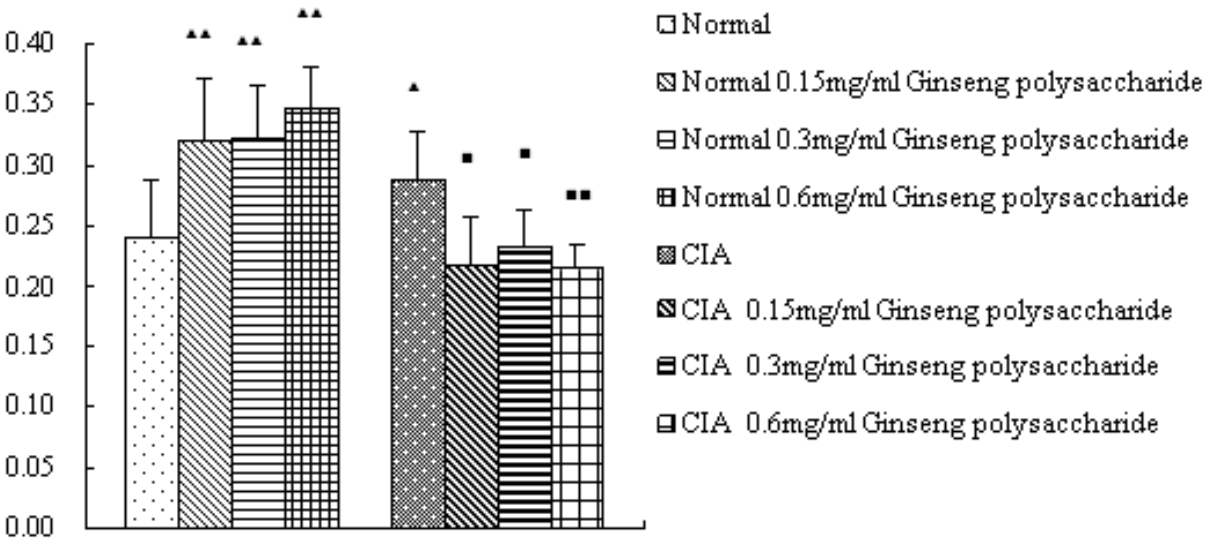


Figure 2. Effects of *ginseng* polysaccharide on TNF-α in PPL supernatants. Data are expressed as mean ± SD. The TNF-α level was determined with ELISA kit. Compared to normal rats, the level of TNF-α in supernatant of PPL on CIA rats was increased. *Ginseng* polysaccharide could significantly increase the level of TNF-α in supernatant of PPL on normal rats, while significantly decrease the level of TNF-α in PPL of CIA rats (▲ $p < 0.05$, ▲▲ $p < 0.01$ vs. normal. ■ $p < 0.05$, ■■ $p < 0.01$ vs. CIA).

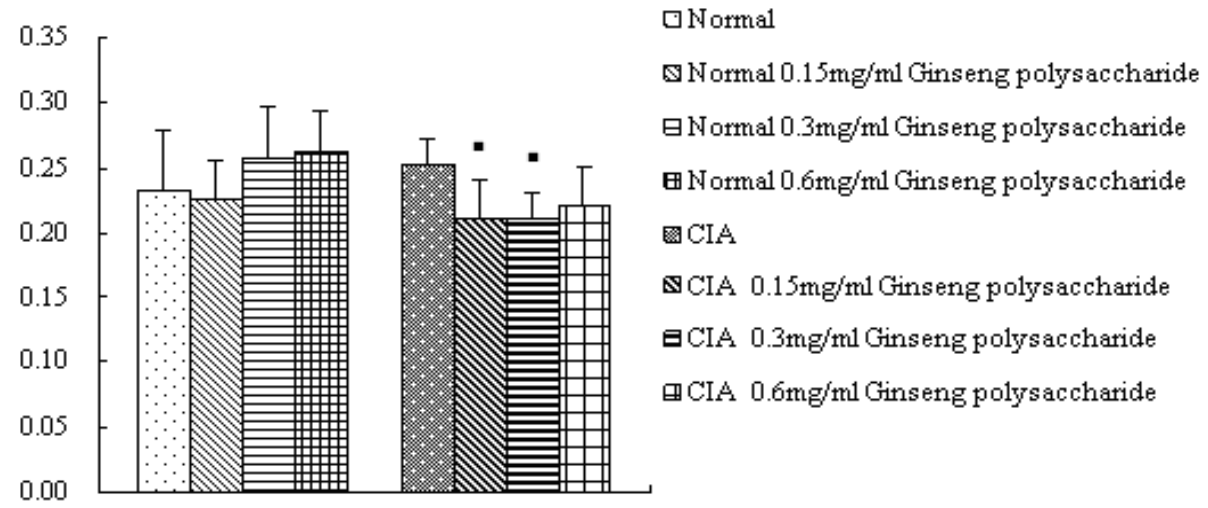


Figure 3. Effects of *ginseng* polysaccharide on TNF-α in IEL supernatants. Data are expressed as mean ± SD. The TNF-α level was determined with ELISA kit. *Ginseng* polysaccharide in 0.15 and 0.3 mg/ml could significantly decrease the level of TNF-α in supernatant of IEL on CIA rats (■ $p < 0.05$, ■■ $p < 0.01$ vs. CIA).

Shimizu et al., 1991; Yamaoka et al., 1995; Liu et al., 1989).

Though, polysaccharides are enhancing the immune response usually, but the use of herbs containing Polysaccharides for treating arthritis are reported (Li, 1991; Liu et al., 2006). Mucosal immune system is an integrated network of tissues, lymphoid and constitutive cells and effector molecules characterized by production of secretory IgA (sIgA), Th1 and Th2 type CD4+ T lymphocytes responses and CD8+ CTL responses. It functions through protecting the host from infection of the mucous membrane surfaces; on the other hand, it can induce tolerance to the antigen (Didierlaurent et al., 2002; Tlaskalova-Hogenova et al., 2002). Collagen-induced arthritis (CIA) as a common animal model for auto-immune disease shows immunologic abnormality (Svendsen et al., 2004).

It has been reported that oral administration of CII can

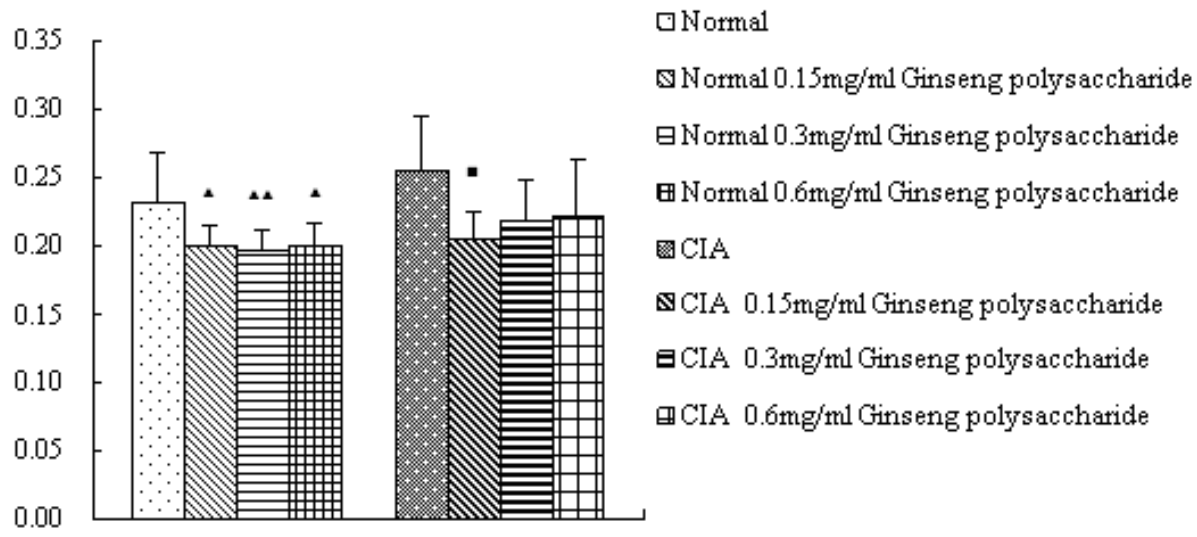


Figure 4. Effects of *ginseng* polysaccharide on TNF-α in LPL supernatants. Data are expressed as mean ± SD. The TNF-α level was determined with ELISA kit. *Ginseng* polysaccharide could significantly decrease the level of TNF-α in supernatant of LPL on normal rats, while in 0.15 mg/ml could significantly decrease the level of TNF-α in PPL of CIA rats (▲ $p < 0.05$, ▲▲ $p < 0.01$ vs. normal. ■ $p < 0.05$, ■■ $p < 0.01$ vs. CIA).

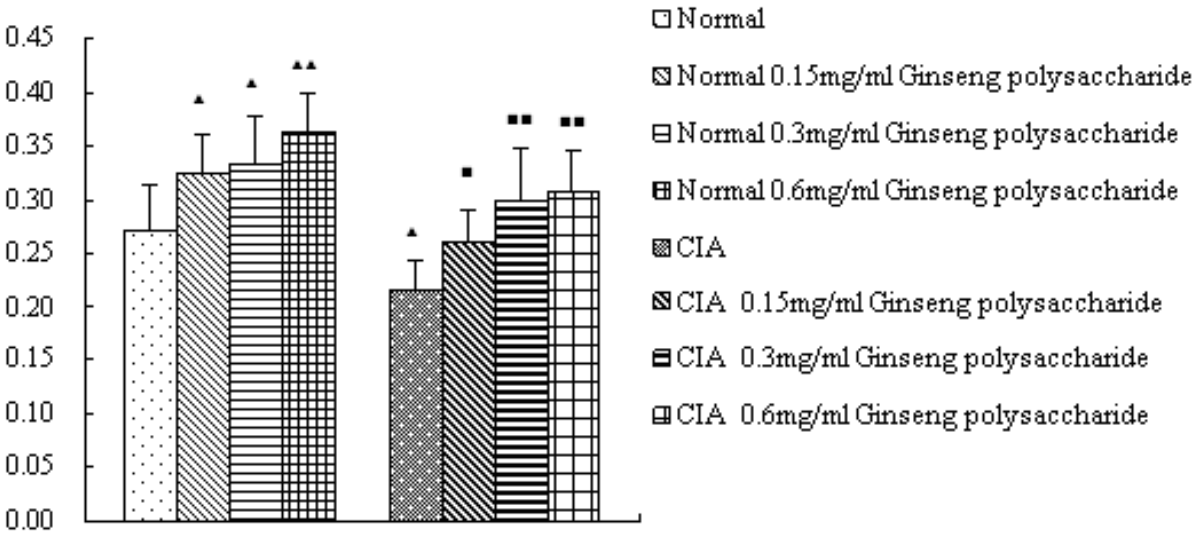


Figure 5. Effects of *ginseng* polysaccharide on IFN-γ in PPL supernatants. Data are expressed as mean ± SD. The IFN-γ level was determined with ELISA kit. Compared to normal rats, the level of IFN-γ in supernatant of PPL on CIA rats was decreased. *Ginseng* polysaccharide could significantly increase the level of IFN-γ in supernatant of PPL on normal and CIA rats (▲ $p < 0.05$, ▲▲ $p < 0.01$ vs. normal. ■ $p < 0.05$, ■■ $p < 0.01$ vs. CIA).

reduce synovial hyperplasia, mononuclear infiltration, pannus formation, and cartilage erosions on CIA models (Thompson et al., 1993). Polysaccharides, as large molecules, are not easily assimilated in the alimentary canal. Our results show that the ratio of CD4+ T cell to CD8+ T cell of CIA model is ascending, which means the immune response is positive. And this indicates the hyperfunction of mucosal immune system on CIA model, correlate with the results of earlier researches. So, intestinal mucus maybe serves as the means of immune regulation of polysaccharides. The mucosal surfaces of the intestine are major sites of antigen exposure (Yuan and Walker, 2004; Kiyono and Fukuyama, 2004), lymphocytes within gut-associated lymphoid tissue are mainly activated T cells (Erickson and Hubbard, 2000). In the immunoreactions, both TNF-α and IFN-γ are the

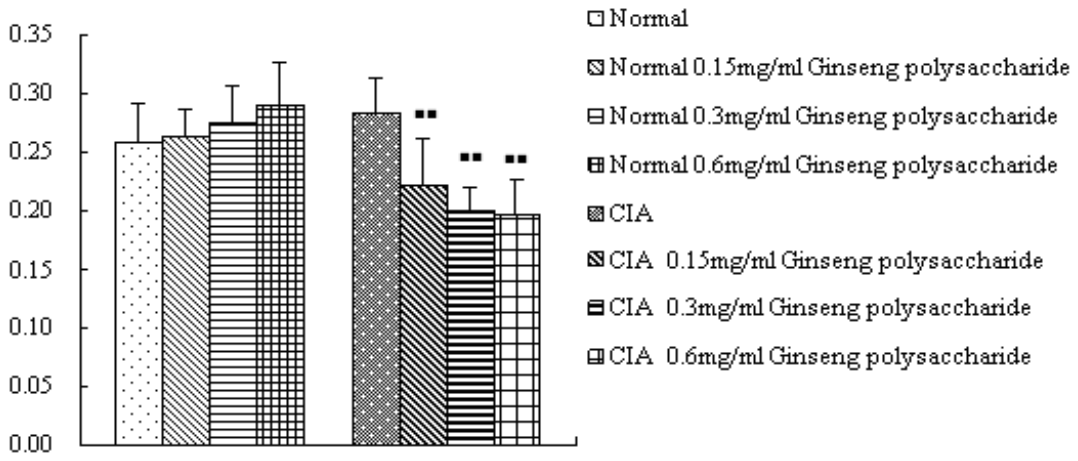


Figure 6. Effects of *ginseng* polysaccharide on IFN-γ in IEL supernatants. Data are expressed as mean ± SD. The IFN-γ level was determined with ELISA kit. *Ginseng* polysacchoride could significantly decrease the level of IFN-γ in supernatant of IEL on CIA rats (▪ $P<0.05$, ▪▪ $P<0.01$ vs. CIA).

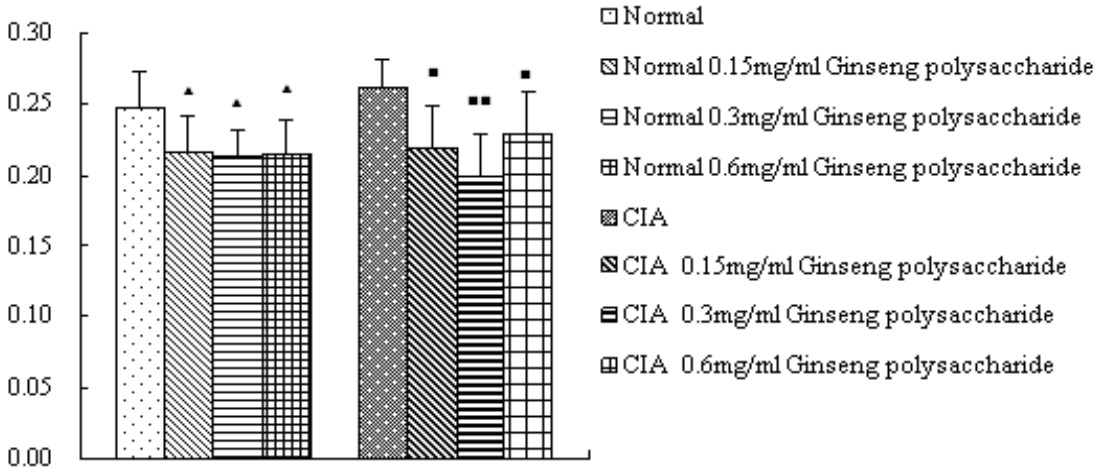


Figure 7. Effects of *ginseng* polysaccharide on IFN-γ in LPL supernatants. Data are expressed as mean ± SD. The IFN-γ level was determined with ELISA kit. *Ginseng* polysaccharide could significantly decrease the level of IFN-γ in supernatant of LPL on normal and CIA rats (▲ $p < 0.05$, ▲▲ $p < 0.01$ vs. normal. ▪ $p < 0.05$, ▪▪ $p < 0.01$ vs. CIA).

important cytokines. TNF-α is produced by mononuclear macrophage chiefly as well as by NK cell and T cell; while IFN-γ comes from activated Th1 lymph cell, also from NK cell. The level of TNF-α and IFN-γ means the degree of immunoreactions.

The results showed that the secretion of TNF-α and IFN-γ could both be increased by co-cultured with *ginseng* polysaccharide and lymphocyte from peyer's patch. So, *ginseng* polysaccharide could activate T-lymph cell, reducing the immunological enhancement in peyer's patch. Co-culture the *ginseng* polysaccharide with intraepithelial lymphocyte, and then the level of TNF-α and IFN-γ showed no significant change, the probable reason was that the dose of polysaccharide was too low to activate Th1 cell-Th2 cell.

Some research (Xiao and Link, 1997) indicated that low dose of antigen could lead to the inhibition of the active induced by T cell, the antigen was uptaken by antigen presenting cells after entering the mucosa, and then it went through the complex procedure of processing, submitting, inducing Th2 cell to secret suppressor cytokines as IL-4, IL-5 and TGF-β, etc., which caused the inhibition of activating immunologic response and the decreasing of TNF-α and IFN-γ secretion under the effect of these cytokines. The ability of lymph cell secreting TNF-α and IFN-γ was inhibited in different levels during the co-culture of g*inseng* polysaccharide by lamina propria lymphocytes. The reason maybe was: (1) the

activation of Th2 cells and the secretion of the inhibitors like IL-4, IL-10, TGF-β inhibited the organic immunologic response of foreign antigens; (2) because the $CD4^+$ cell content in lamina propria is only 8.06%, the relative concentration of antigen increased, which caused $CD4^+$ T cell apoptosis.

This research showed that g*inseng* polysaccharide could activate Peyer's patch lymphocytes, and cause the immune hypofunction of the lymphocytes in the enteric epithelium and lamina propria. Yet, we are still not clear about if the lowered function could decrease the immunologic inhibition reflected by intestinal mucosal immunity usually and the mechanism of the formation of the immunologic influence needs further study.

ACKNOWLEDGEMENTS

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