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Synergistic Immunostimulating Activity of Pidotimod and Red Ginseng Acidic Polysaccharide against Cyclophosphamide-induced Immunosuppression

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We investigated the synergistic effect of combined treatment with red ginseng acidic polysaccharide (RGAP) from *Panax ginseng* C.A. Meyer and pidotimod in cyclophosphamide-treated mice. The combination of pidotimod and RGAP restored concanavalin A-induced splenic T cell proliferation and LPS-stimulated B cell proliferation significantly. The production of nitric oxide from peritoneal macrophages was increased by the combinations. NK cell activity was increased by RGAP alone or in combination with pidotimod. A synergistic increase in the level of serum IL-12 and interferon-gamm was observed when the combination of the two was used. RGAP alone or in combination with pidotimod modulated the level of serum C-reactive protein to a near-normal level. These results indicate that combinations of pidotimod and RGAP are synergistic and suggest that combination therapy using pidotimod and RGAP for improving immune activity may provide an additional benefit over the use of the two drugs by themselves.

Key words: *Panax ginseng* C.A. Meyer, Red ginseng acidic polysaccharide, Pidotimod, Cyclophosphamide, Immunostimulation, Combination

INTRODUCTION

Panax ginseng C.A. Meyer (Korean ginseng, mainly produced in Korea and northeast China), is a herbal root and has been extensively used as a traditional medicine and functional food in the Orient for more than 3,000 years. The known biochemical and pharmacological activities of ginseng include antiaging, antidiabetic, anticarcinogenic, analgesic, antipyretic, antistress, antifatigue, and tranquilizing activities, as well as the promotion of DNA, RNA, and protein synthesis activities (Abe et al., 1979; Huang, 1989; Sun et al., 1991). We reviewed the biochemical and pharmacological studies of Korean ginseng and found them to be mainly concentrated on ginseng saponins (ginsenosides) as active components. However, non-saponin components have also received a great deal of attention for their mitogenic activities (Eun et al., 1989), hypoglycemic activities (Konno et al., 1985; Konno et al., 1983), and antitumor activities (Lee et al., 1997; Moon et al., 1983). Li et al. reported that coarse polysaccharide from *Panax quinquefolium* could improve lymphocyte transformation in immunosuppressed mice (Li, 1996). Among the polysaccharides which are isolated from ginseng root, it is considered that acidic polysaccharides were more effective than neutral polysaccharides in their immunostimulating activities. They were found to activate macrophage and NK cells, induce the production of nitric oxide synthase (NOS), and increase the number of macrophages (Kim et al., 1990; Lim et al., 2004). In addition, polysaccharides isolated from *Panax ginseng* showed strong stimulation of inducible NOS (iNOS) in RAW 264.7 cells (Friedl et al., 2001).

Pidotimod ((*R*)-3-[(*S*)-(5-oxo-2-pyrrolidinyl)carbonyl] thiazolidine-4-carboxylic acid) is a biological immunoregulatory modifier synthesized by Poli Industria Chimica, Italy (Magni et al., 1994). It is the first compound of a new class of immunity modifiers with peptide-like structures, and has been well characterized and is known to be chemically pure (Crimella et al., 1994). Treatment with

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pidotimod increased macrophage activity and humoral immune functions (Coppi and Manzardo, 1994). Furthermore, pidotimod is able to activate NK cells, a front line of defense that is capable of quickly expressing itself towards aggressors (Migliorati et al., 1994).

An essential facet of traditional oriental medicine is "combination". The modern concept of "combination" relates to molecules possessing the same or similar functions, which are combined as a group and which show particular activities (Wu and Wu, 2002). The advantage to the use of the combination would be to reduce drug dosages, thereby lowering the potential risk of toxicity, and also to reduce the development of drug resistance. Many chemicals including many clinically used drugs when exposed under certain doses and durations suppress the immune response and may cause increased incidences of infectious diseases and cancers (Barron et al., 2003). Therefore, cyclophosphamide was used as an immunosuppressant in this study and the synergistic effect of the combined treatment of red ginseng acidic polysaccharide (RGAP) from *Panax ginseng* C.A. Meyer and pidotimod was investigated in BALB/c mice. The purpose of this study has been to confirm the synergistic effect of the combination to provide a reference for the combinational application of western and oriental medicines.

MATERIALS AND METHODS

Materials

RGAP was provided by Korean Tobacco and Ginseng company (Korea). It was isolated from *Panax ginseng* C.A. Meyer as described previously (Kyeong, 2000). RGAP was confirmed to be the acidic polysaccharide fraction composed of about 56.9% uronic acid, 28.3% neutral sugar and less than 1% protein. The analysis of component sugars in RGAP by GC revealed that the polysaccharides in RGAP contained about 51.8 mole % glucuronic acid, 26.1 mole % glucose, and 5.1 mole % galacturonic acid as major components, and arabinose, rhamnose, and galactose as minor components. One milligram of RGAP contained less than 0.006 EU (endotoxin units) of endotoxin, which did not affect the experimental result obtained by RGAP.

All the reagents were purchased from Sigma-Aldrich (USA) except for those as indicated.

Animals and treatments

Specific pathogen-free male BALB/c mice (six-week-old) were purchased from Koatech Company, Korea. They were acclimatized for at least three days before the experiment. Mice were housed in an air-conditioned SPF room with a 12-h light/dark cycle, and provided with Certified Rodent Diet (Koatech Company, Korea) and tap water *ad libitum*. The experimental protocol was approved by our institutional animal care and use committee.

The mice were divided into seven groups of twelve. All animals received cyclophosphamide (Choongwre Pharma Company, Korea) intraperitoneally once per three days to establish the immunosuppressive animal model, except for control group which received normal saline. The different groups were treated with the following: pidotimod (Yungjin Pharm. Company, Korea) at a dose of 200 mg/kg given orally; RGAP at a dose of 300 mg/kg given orally; and the combinations of pidotimod (200 mg/kg) and RGAP at different doses of 33, 100, and 300 mg/kg. Mice were sacrificed after three weeks of administration. Bodyweight gain (percentage) and relative weight of the spleen (organ weight/100 g of bodyweight) were determined for each animal.

Peritoneal macrophage culture

Murine peritoneal cells were harvested by peritoneal lavage with 5 mL of PBS, according to a procedure reported previously (Komutarin et al., 2004). The cells were kept cold on ice until transferred and plated in a complete medium consisting of RPMI-1640 with 10% FBS, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 mg/ml streptomycin. After incubating at 37ºC in 5% CO_2 for 2 h, the non-adherent cells were removed by washing twice with cold PBS. The adherent cells were used to perform the following experiment.

Assay of NO production and cell viability

Peritoneal macrophages (2×10^5 cells per well) from BALB/c mice were cultured in the absence or presence of LPS (20 μg/mL). NO synthesis was examined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using the Griess reagent (An et al., 2006). Briefly, 50 μL of supernatant was removed from the conditioned medium and incubated with the same volume of modified Griess reagent at room temperature for 15 min. The absorbance at 540 nm was measured by means of an automatic microplate reader (JASCO, V-530).

Cell viability was evaluated by an MTT reduction assay (Byun et al., 2006). After various treatments, the cells were co-incubated with MTT (0.25 mg/mL) for 4 h at 37ºC. The formazan crystals in the cells were solubilized with DMSO. The level of MTT formazan was determined by measuring the optical density at a wavelength of 540 nm.

Assay of splenocyte proliferation

BALB/c mice were sacrificed and their spleens were removed aseptically. A single cell suspension was prepared after cell debris and clumps had been removed.

Splenocytes were washed three times with PBS and then resuspended in RPMI-1640 medium containing 5% FBS. They were seeded in 96-well plates at 2×10^5 cells per well and incubated in the absence or presence of either LPS (1 μg/mL) or Concanavalin A (Con A, 0.25 μg/mL). After the cells had been cultured for 72 h at 37ºC in a humidified 5% CO_2 incubator, the number of proliferating cells was determined by the MTT assay at a wavelength of 540 nm.

Assay of splenic NK cell activity

After the preparation of a single splenocyte suspension, equal volume of Histopaque® was added and centrifuged at 400 *g* for 30 min at room temperature to separate mononuclear cells. The upper layer to within 0.5 cm of the opaque interface was transferred and seeded at 2×10^5 cells/mL followed by washing with PBS. YAC-1 cells were used as target cells. The effecter-to-target cell ratio was 50:1. After the cells had been co-cultured for 72 h at 37ºC in humidified 5% CO_2, the number of proliferating cells was determined by the MTT assay. Splenic NK cell activity was calculated as follows:

$$\text{NK cell activity} = [(OD_Y + OD_{NK} - OD_{(Y+NK)})/(OD_Y + OD_{NK})] \times 100\%$$

where OD_Y = optical density of wells containing YAC-1 cells only; OD_{NK} = optical density of wells containing NK cells only; and $OD_{(Y+NK)}$ = optical density of wells containing both YAC-1 cells and NK cells.

Assay of cytokines and CRP in serum

Blood was obtained from the posterior vena cava and centrifuged at 3,000 rpm and 4ºC for 15 min. The amounts of IL-12, IFN-γ, and CRP in the supernatant were analyzed by an enzyme-linked immunosorbent assay using commercial ELISA system assay kits (Pierce Biotechnology Inc., USA).

Statistical analysis

The data were expressed as mean±S.E.M. The groups were analyzed using an analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons (SPSS version 13.0). A *p* value of less than 0.05 was considered significant.

RESULTS

Effect of pidotimod and RGAP on bodyweights and spleen weights of mice

The effects of pidotimod and RGAP on the bodyweights and spleen weights of mice are presented in Table I. Cyclophosphamide elicited a significant reduction in the rate of increase of bodyweight to just 7.51% compared to the control value of 17.31%. Bodyweight was significantly restored when mice were treated with pidotimod alone or in combination with RGAP.

Table I. Effects of pidotimod and RGAP alone or by combination against cyclophosphamide-induced immunosuppression on bodyweight gain (percentage) and relative organ weight (organ weight/100 g of bodyweight) of spleen

Group	Δbodyweight/ bodyweight (%)	Spleen/bodyweight (%)
control	17.31±1.19 [a]	4.07±0.14 [a]
Cy	7.51±0.51 [b]	2.89±0.04 [b]
Pi+Cy	15.63±2.47 [a]	3.32±0.08 [c]
R(300) +Cy	10.92±1.12 [b]	3.42±0.19 [c]
Pi+R(33)+Cy	14.26±1.91 [a]	2.98±0.08 [b]
Pi+R(100)+Cy	17.05±2.40 [a]	3.38±0.15 [c]
Pi+R(300)+Cy	14.42±1.25 [a]	3.40±0.14 [c]

Cy: cyclophosphamide; Pi: pidotimod; R: RGAP. The results are presented as the mean±S.E.M. of 12 animals per group. Values with different superscripts are significantly different at $P < 0.05$.

Relative spleen weight was extremely reduced to 2.89% by cyclophosphamide. Treatments with pidotimod and RGAP individually increased the relative spleen weight to 3.32% and 3.42%, respectively. When pidotimod was combined with RGAP, the spleen index was also significantly restored.

Effect of pidotimod and RGAP on NO production by LPS-stimulated mouse peritoneal macrophages

To determine the effect of pidotimod and RGAP on the production of NO of mouse peritoneal macrophages, the cells were stimulated by LPS (20 μg/mL) for 24 h. The results indicated that LPS and/or samples did not affect the viability of macrophages in the present study (data not shown). Fig. 1 shows that the stimulation of peritoneal macrophages with LPS resulted in a manifold increase in NO production compared to the control group in the absence of LPS. Cyclophosphamide significantly inhibited the LPS-stimulated NO production by 35.9%, compared to the LPS-stimulated control group. The mean values for the pidotimod- or RGAP-treated groups were not significantly different from the mean value of the cyclophosphamide-treated group. However, NO production was significantly increased by combinations of pidotimod (200 mg/kg) and RGAP at doses of 100 and 300 mg/kg to 86.1% and 87.1%, respectively, compared to the single treatment.

Effect of pidotimod and RGAP on splenocyte proliferation

The effects of pidotimod and RGAP on mitogen-stimulated murine spleen lymphocyte proliferation are pre-

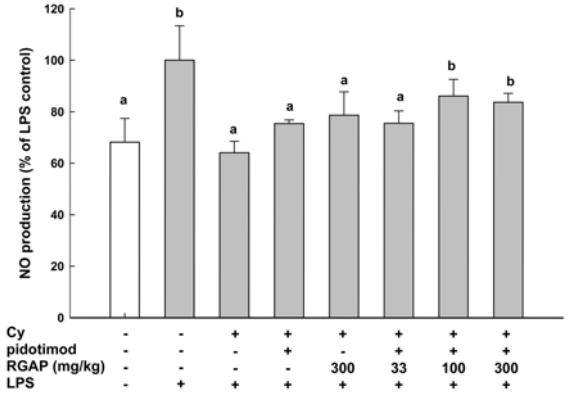

Fig. 1. Effects of pidotimod and RGAP alone or by combination against cyclophosphamide-induced immunosuppression on NO production by LPS-stimulated mouse peritoneal macrophage. The results are presented as the mean ± S.E.M. of 12 animals per group and expressed as percentage values, taking the LPS-treated control group as 100%. Values with different superscripts are significantly different at $P < 0.05$.

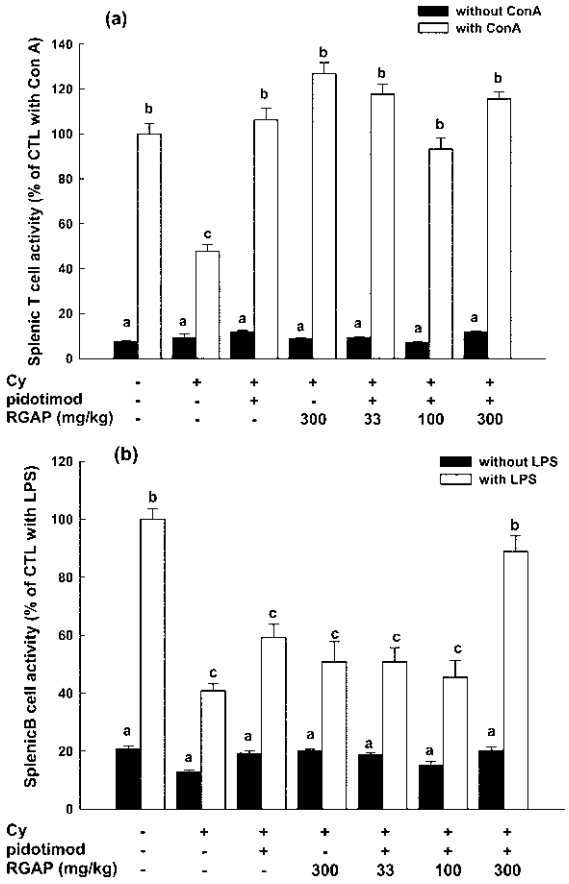

Fig. 2. Effects of pidotimod and RGAP alone or by combination against cyclophosphamide-induced immunosuppression on mice splenic T cell (a) and B cell (b) proliferations. The results are presented as the mean ± S.E.M. of 12 animals per group and expressed as percentage values taking control group with Con A or LPS as 100%. Values with different superscripts are significantly different at $P < 0.05$.

sented in Fig. 2(a) and (b). It is apparent that treatment with cyclophosphamide significantly reduced Con A- or LPS-stimulated cell proliferation to 47.7% and 40.8% compared to the control group in the absence of Con A or LPS, respectively. The results also show that the strongly diminished Con A-induced proliferative response in cyclophosphamide-treated mice was greatly recovered by pidotimod, RGAP, and the combinations of the two. However, the decrease in B cell proliferation was only significantly restored by the combination of pidotimod and RGAP (300 mg/kg).

Effect of pidotimod and RGAP on splenic NK cell activity

The splenic NK cell activity was determined by examining cytotoxicity towards YAC-1 cells by means of an MTT assay. As shown in Fig. 3, cyclophosphamide significantly inhibited the NK cell activity to 59.4%. When the immunosuppressed mice were treated with pidotimod and RGAP individually or simultaneously, the decline in the NK activity was significantly reversed compared to the cyclophosphamide-treated mice.

Effect of pidotimod and RGAP on cytokines and CRP

Mouse serum IL-12, IFN-γ, and CPR were evaluated. As shown in Table II, cyclophosphamide injection caused significant reductions in the levels of IL-12, IFN-γ, and CRP to 67.0%, 82.8%, and 50.0%, respectively. In contrast to the individual treatments, combined treatment of

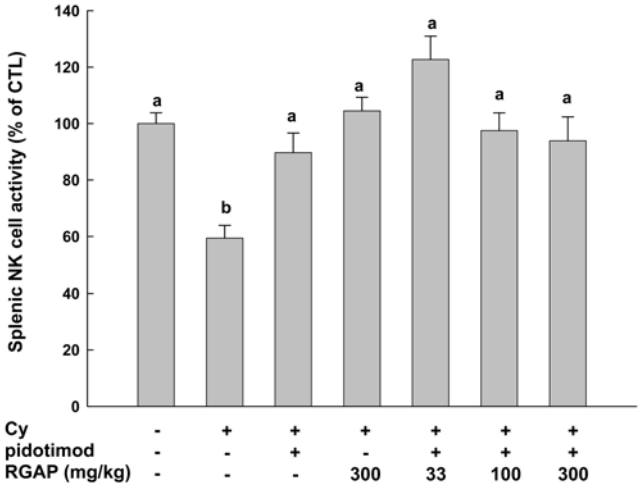

Fig. 3. Effects of pidotimod and RGAP alone or by combination against cyclophosphamide-induced immunosuppression on mice splenic NK cell activity. The results are presented as the mean±S.E.M. of 12 animals per group and expressed as percentage values taking control group as 100%. Values with different superscripts are significantly different at $P < 0.05$.

Table II. Effects of pidotimod and RGAP alone or by combination against cyclophosphamide-induced immunosuppression on mice serum IL-12, IFN-γ, and CRP levels

Group	IL-12	IFN-γ	CRP
control	100.0±12.6 [a]	100.0±5.3 [a]	100.0±14.5 [a]
Cy	67.0± 5.5 [b]	82.8±1.9 [b]	50.0±10.6 [b]
Pi+Cy	61.0± 4.0 [b]	88.4±1.9 [b]	52.4±10.9 [b]
R(300) +Cy	82.3± 7.1 [ab]	96.9±3.5 [a]	83.4± 9.8 [ac]
Pi+R(33)+Cy	73.7± 7.3 [b]	99.8±3.8 [a]	42.5± 6.7 [b]
Pi+R(100)+Cy	127.9± 8.3 [c]	100.5±4.7 [a]	59.4±12.1 [b]
Pi+R(300)+Cy	79.8± 4.7 [ab]	102.1±4.9 [a]	86.2±11.5 [ac]

Cy: cyclophosphamide; Pi: pidotimod; R: RGAP. The results are presented as the mean±S.E.M. of 12 animals per group. Values with different superscripts are significantly different at $P < 0.05$.

pidotimod and RGAP (100 mg/kg) acted synergistically in enhancing the IL-12 level that had been decreased by cyclophosphamide. A significant increase in levels of serum IFN-γ was observed when mice were treated with RGAP. Pidotimod showed no effect like this until it was combined with RGAP. Similarly, RGAP alone or in combination with pidotimod induced elevations in serum CRP against cyclophosphamide and restored CRP to near-normal levels.

DISCUSSION

Integrative medicine, combining the theories and treatments of both western medicine and traditional medicine, has become the developing trend of medicine along with the social development since 1970's (Feng, 1977; Liu, 2003). Previous reports have indicated some progress in pre-clinical and clinical research (Chen et al., 2003; Wang and Zhou, 2003). In this study, we have demonstrated the synergistic effect of a combination of RGAP and pidotimod in immunosuppressant models induced by cyclophosphamide. The action of cyclophosphamide is primarily directed towards the depletion of B lymphocytes. Cyclophosphamide has also been reported to induce the depletion of T cells (Mahiou et al., 2001; Rollinghoff et al., 1977) and the deficiency of macrophages (Santosuosso et al., 2002). Accordingly, cyclophosphamide impaired all of the immune parameters examined in this study.

Lymphocyte proliferation induced by Con A is commonly used as a method of detection for T lymphocyte immunity in vitro, while lymphocyte proliferation induced by LPS is often used to detect B lymphocyte immunity (Cerqueira et al., 2004). Cyclophosphamide-induced suppression of ConA-induced T-lymphocyte proliferation and LPS-induced B-lymphocyte proliferation was identified in this study. Combined treatment with pidotimod and RGAP significantly enhanced the proliferation of splenocytes against cyclophosphamide. In addition, the combinations are suggested to have greater sensitivity for the T cells of splenocytes than the B cells, which was manifested in a better T-cell-mediated immunostimulating effect. NK cell activity of splenocytes was determined to assess the effects of samples on innate immune responses. Shin reported that RGAP restored NK cell activity suppressed by paclitaxel (Shin et al., 2004). Our results have further shown that treatments with pidotimod and RGAP, both individually and in combination, can enhance NK cell activity.

Macrophages are an integral part of the immune system, playing an important role in the initiation and regulation of immune response (Gordon, 1998). Macrophage activation by LPS, the major component of the cell walls of gram-negative bacteria, results in the release of several inflammatory mediators such as NO and the pro-inflammatory cytokines (An et al., 2006). NO is a highly reactive molecule produced from a guanidine nitrogen of NOS., The iNOS, the high-output isoform, is highly expressed in LPS-activated macrophage of the three NOSs (Petros et al., 1994), which is mediated by cytokines, INF-γ, and tumor necrosis factor alpha (TNF-α) (Kroncke et al., 1998). Low concentrations of NO from activated macrophages are beneficial, and along with other reactive nitrogen intermediates, are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in the function of natural killer cells and in the expression of cytokines such as IFN-γ and transforming growth factor-β (Bogdan et al., 2000). A combination of pidotimod and RGAP has been shown to significantly restore NO production suppressed by cyclophosphamide, which supports the results that they also increased INF-γ production in a dose-dependent manner in this study.

IL-12 is known as a T cell stimulating factor, which can stimulate the growth and function of T cells. It is involved in the differentiation of naïve T cells into Th1 cells and stimulates the production of IFN-γ and TNF-α from T and NK cells. Pidotimod and RGAP acted synergistically in enhancing the IL-12 level, which was decreased by cyclophosphamide, whereas individual treatments showed no enhancing effect. Moreover, when combined with RGAP, pidotimod was able to fully restore the decrease of IFN-γ induced by cyclophosphamide. IFN-γ is involved in the regulation of immune and inflammatory responses. It is produced in activated T cells and NK cells. Thus, our findings suggest that RGAP may include some factors capable of inducing T helper activity and up-regulating NK cell activity. CRP is a plasma protein, an acute phase protein produced by the liver (Lind, 2003). It is also believed to play an important role in innate immunity as an early defense system against infec-

tions. It was found that RGAP is capable of preventing the decrease of CRP caused by cyclophosphamide and modulating it to a near-normal level, which suggests that it does not cause any inflammatory reaction.

In summary, the present study has demonstrated that the combination of pidotimod and RGAP acts synergistically in enhancing the immunostimulating effect in cyclophosphamide-induced immunosuppressed mice. Pidotimod and RGAP used in combination significantly restored the spleen index decreased by cyclophosphamide, and also increased Con A- and LPS-induced splenocyte proliferation. The cotreatments also enhanced NK cell activity and NO production by peritoneal macrophages. IL-12 and IFN-γ in serum were restored by combined treatments. In view of these data the finding that combined therapy with pidotimod and RGAP in our immunosuppressed model is of particular importance. The effect of combination of pidotimod and RGAP on the humoral immune system is already being addressed and our findings will follow in future communications.

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