Oral Administration of Proteoglycan Isolated from *Phellinus linteus* in the Prevention and Treatment of Collagen-Induced Arthritis in Mice

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To examine whether oral administration of proteoglycan derived from *Phellinus linteus*, which is known as the medicinal mushroom, can prevent or treat collagen-induced arthritis (CIA) in mice as experimental model of autoimmune disease. CIA was induced by intradermal injection of type II collagen (CII) emulsified with complete freund's adjuvant (CFA) into the base of the tail (on day 7) followed by a booster injection on day 21 into the footfad. To examine the ability of proteoglycan to effect the inhibition of CIA, doses of proteoglycan were orally administered on day 0 (pre-administration) or day 28 (post-administration) at two groups. The inhibition of CIA by oral administration of proteoglycan was associated with decrease in anti-CII IgG and IgG2a antibodies (Abs) as well as varying kinds of cytokines including IL-12, TNF-α, and IFN-γ. The results showed that administration of proteoglycan was followed by decrease of CIA of the mice in pre- and post-administration groups. Our findings suggest that immunomodulating proteoglycan isolated from *P. linteus* may be crucially involved in the prevention and treatment of autoimmune joint inflammation such as rheumatoid arthritis, although no definite role of anti-CII Abs in the human disease has been established.

Key words RA; CIA; *Phellinus linteus*; proteoglycan; Th cell

The differentiation of naive $CD4^+$ T cells into mature effector cells is conducted by secretion of an array of cytokines that dictate the nature of the immune response against infectious organisms and self-Antigens (Ags). Activation of the $CD4^+$ Th1 cell that produces interleukin-2 (IL-2) and interferon-gamma (IFN-γ) is necessary in order to eliminate intracellular pathogens[1,2]; however, these cells elicit the inflammation and tissue destruction observed in autoimmune diseases.[3] Also, dendritic cells (DCs) and macrophages secrete IL-12 and tumor necrosis factor-alpa (TNF-α) which those activate Th1 cell and have effect on pro-inflammatory cytokines. The Th2 cell, which produces IL-4, IL-5, IL-10, IL-13, and TGF-β, controls humoral immunity to extracellular organisms. That inhibits cell-mediated inflammatory responses, and can protect against arthritis.[4] The development of Th1 or Th2 types from naive cells to effector cells is regulated by the presence of specific cytokines in the microenvironment at the time of T cell priming. For the Th1 type, IL-12 is a necessary cytokine of differentiation,[5] whereas for Th2 type, IL-4 and IL-10 are critical.[6]

Cytokines are major mediators of inflammation in autoimmune arthritis such as rheumatoid arthritis (RA).[7] A myriad of cytokines are found within the rheumatoid joint. Some appear to exacerbate disease, whereas others may decrease inflammation. In many cases, their precise roles are, as yet, incompletely defined. The balance of Th1 and Th2 cytokines is thought to influence autoimmune arthritis. Several human autoimmune diseases and their corresponding animal models are characterized by the dominance of Th1 cells.[8,9] Analysis of T cell subsets in patients with RA and in mice with collagen-induced arthritis (CIA) reveals that a higher ratio of IFN-γ to IL-4 is produced from lymph node cultures of collagen-immunized mice, which peaks at the onset of disease symptoms.[3]

At present, a great deal of attention is being focused on nonsteroidal anti-inflammatory drugs based on inhibiting cyclooxygenase (COX) enzymes. The two isoforms, COX-1 and COX-2, are central to the production of prostaglandins (PGEs), produced in excess at sites of inflammation. COX-1 synthesizes PGEs that are involved in the regulation of normal cell activity, whereas COX-2 produces PGEs mainly where inflammation occurs.[10,11] Thus, selective inhibition of COX-2 in particular is sufficient to significantly limit inflammation, and COX-2 inhibitors are heavily prescribed.[12] There are still some concerns about side effects arising from the inhibition of a key enzyme in tissues and organs other than those affected by the disease, such as the kidney and the brain.[13]

Finally, other types of trials are aimed at determining the effects of anti-arthritis drugs on various molecular responses, such as the production and subsequent effect of cytokines. For example, a recent trial linked the immune suppressive effect of dexamethasone on IL-10 production and on the Th1/Th2 cell balance in RA.[14] Dexamethasone therapy in RA patients leads to a rapid, clinically beneficial effect, and the up-regulation of IL-10 production observed after administration may be involved in the prolonged clinical benefit. At the same time, there is an immunosuppressive effect accompanied by a relative shift toward Th2 cell activity. Such results offer significant insights into the individual mechanisms that affect the progression and outcome of the disease.

Many biologically active glucans have been isolated from various mushrooms, and some of these glucans, such as lentinan and schizophyllan are used clinically for immune therapy.[15—17] However, no report related to RA has been found. Therefore, we tested the ability of proteoglycan iso-

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lated from *Phellinus linteus* to control Th1/Th2 cytokines balance in CIA mice.

MATERIALS AND METHODS

Characterization of Proteoglycan Purified from *P. linteus* Origin of *P. linteus* used in this study is cultivated-fruiting body (Nam-San No. 1). In our unpublished data, we investigated the sequences of internal transcribed regions containing 5.8S rDNA and identified *P. linteus* (GenBank accession NO. AF080457). The proteoglycan derived from *P. linteus* (PL) was extracted 4 times at 100 °C in distilled water for 30 min, concentrated under reduced pressure, and precipitated with 3 volumes of ethanol. The precipitates were dissolved in distilled water, dialyzed against distilled water, and a gradient NaCl solution (0 to 2 M). The fraction eluted to 0.43 M NaCl gradient. The fraction was not a pure glucan, but rather it was a proteoglycan. Its protein portion consisted of predominantly acidic amino acids, such as aspartic and glutamic acids. The molecular mass was about 150000 by gel-chromatography on Sepharose CL-4B. Amino acids in protein-bounded polysaccharide were determined with a Biochrom 20 amino acid analyzer (Pharmacia, CA, U.S.A.) using an acid hydrolysis and ninhydrin. The 1 mg of protein-bound polysaccharide was hydrolysated with 2 M trifluoroacetic acid (TFA) and evaporated. In order to detect monosaccharide, a column was used with a high-performance liquid chromatography (Waters, Milford, MA, U.S.A.) with a Sugar-Pak column (7.8×300 Milipore, Tokyo, Japan) and detected with a differential refractive index (RI) detector (RID-6A) at 80 °C. Two eluants (0.15 M NaOH and 0.1 M NaOH) were used at a flow rate of 1.0 ml min^{-1}. FT-IR (Magna-IR 560) was analyzed using the KBr disc method for detecting functional groups. The FT-IR spectrum of β-glucan derived from *L. edodes* was conducted and compared to the standard. ^{1}H- and ^{13}C-NMR (Varian, Unity-inova 500) were performed with D_2O as a solvent to determine structure analysis of protein-bound polysaccharide.

Animals Pathogen-free male DBA/1 inbred mice, aged 6 to 8 weeks, were purchased from the Catholic Medical Institute. All the mice were housed in accordance with approved guidelines and were provided with food and water as libitum. The Catholic Medical Institute Animal Care and Use Committee approved all animal use.

Type II Collagen (CII) Immunization Bovine CII (Elastin Products, Owensville, MO) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml and stored at 70 °C until use. For immunization, 100 μg of CII was emulsified with an equal volume of CFA and administered intradermally at the base of the tail of the mice (on day 7). A booster, administered as above, was given days 14 after the primary injection (on day 21). CII for footpad injections was prepared as above and consisted of 100 μl injection into each footpad. One group was immunized intradermally in the tail with 100 μg of CII in CFA (on days 7) after a dose of 200 mg/kg of proteoglycan through oral administration 3 times every 2 d (pre-administration; on day 0). Another group was the administration of same of proteoglycan (post-administration; on day 28) after the injection in footpad as booster. The other group was CIA as control.

Clinical Assessment of Arthritis Mouse paws were scored for arthritis, as previously described,[18] using a macroscopic scoring system ranging from 0 to 4 (0, no swelling or redness; 1, swelling/redness of paw or one joint; 2, two joints involved; 3, more than two joints involved; and 4, severs arthritis the entire paw and joints). The arthritic score for each mouse is the sum of the scores of all four paws.

CII Ab Titration Seven days after the booster, titers of anti-CII Abs in the serum samples were determined by ELISA, as previously described.[19] All samples were measured in triplicate. Serum was isolated from the mouse at days 35 after the booster. Sample sera were diluted 1/100000 and incubated in murine collagen type II-coated wells. Biotin-labeled goat anti-mouse IgG (R&D Systems, Minneapolis, MN, U.S.A.) was used to measure CII-specific total IgG. IgG1 and IgG2a were measured using biotinylated rat anti-mouse IgG1 or IgG2a (R&D Systems, Minneapolis, MN, U.S.A.) and then streptavidin-peroxidase. Plates were developed with Peroxidase Substrate System 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The plate wells were averaged. A serum sample consisting of pooled serum from control mice was tested at various dilutions and used as a standard to generate a curve from which relative titers of the other serum samples were calculated.

Leukocyte Isolation The leukocytes of Peyer's patch (PP), inguinal lymph nodes (iLN), and mesenteric lymph nodes (mLN) from both experimental groups were isolated aseptically on days 35. To isolate their leukocytes, the tissue was stripped of fat and cut into small pieces. Single-cell suspensions were made by pressing it through 70-μm-mesh cell strainers (Becton Dickinson) into RPMI 1640. Purified cell suspensions were washed three times in complete RPMI 1640 supplemented with 10% fetal calf serum and used for *in vivo* cytokines after stimulation with 10 μg/ml of CII for 72 h and the rest was used to survey the RNA expression level.

Detection of RNA Expression Level Total cellular RNA was isolated using TRIZOL (Life Technologies, Poole, U.K.). RNA was reverse transcribed into cDNA as follows: an aliquot of RNA was mixed with a master mix containing a buffer for Maloney murine leukemia virus-reverse transcriptase (MMLV RTase), 2′-deoxynucleoside-5′-triphosphates (dNTPs), ribonuclease (RNase) inhibitor (8 units), reverse transcriptase from MMLV (40 units), and random hexamers (final 2.5 mM), and incubated at 37 °C for 60 min, at 99 °C form 5 min, and then at 4 °C until use. An aliquot of cDNA was mixed with $MgCl_2$ (final 2 mM), a PCR buffer Taq DNA polymerase (0.5 units), a set of primers (Table 1), and 33 cycles of PCR reaction. (step 1, 45 s at 94 °C; step 2, 42 s at 56 °C; step 3, 30 s at 72 °C) were performed in a Perkin-Elmer Cetus DNA thermal cycler to amplify DNA. The resulting DNA fragment was detected by agarose gel electrophoresis after staining with ethidium bromide. β-actin mRNA was used as a control.

***Ex vivo* Cytokine Production** Cytokine levels *ex vivo* culture supernatants were determined by a standard sandwich enzyme-linked immunosolvent assay (ELISA). Briefly, Nunc MaxiSorp (Nalge Nunc International, Roskilde, Denmark) plates were coated for 24 h at 4 °C with rat anti-murine IL-4, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β monoclonal antibodies (R&D Systems, Minneapolis, MN, U.S.A.). The plates were blocked, supernatant samples were added, and the

Table 1. Sequences of the Primers Used in RT-PCR

Cytokines		Sequences (5′ to 3′)	Size (bp)
TNF-α	Sense	TCT CAT CAG TTC TAT GGC CC	213
	Antisense	GGG AGT AGA CAA GGT ACA AC	
IFN-γ	Sense	GCT CTG AGA CAA TGA ACG CT	227
	Antisense	AAA GAG ATA ATC TGG CTC TGC	
IL-12	Sense	ATGAACTCCTTCTCCACAAG	289
	Antisense	CTACATTTGCCGAAGAGCCC	
IL-4	Sense	TCG GCA TTT TGA ACG AGG TC	217
	Antisense	GAA AAG CCC GAA AGA GTC TC	
IL-10	Sense	ATG CAG GAC TTT AAG GGT TAC TTG	254
	Antisense	TAG ACA CCT TGG TCT TGG AGC TTA	
TFG-β	Sense	ACC GCA ACA ACG CCA TCT AT	201
	Antisense	GTA ACG CCA GGA ATT GTT GC	
β-actin	Sense	TGA CCG GCT TGT ATG CTA TC	223
	Antisense	CAG TGT GAG CCAGGA TAT AG	

plates were incubated for 1 h at 4 °C. The plates were then washed and incubated for 60 min at room temperature with biotin-conjugated rat anti-murine IL-4, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β monoclonal antibodies followed by an alkaline phosphatase-conjugated goat anti-biotin monoclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.). The fluorescent substrate for alkaline phosphatase (R&D Systems, Minneapolis, MN, U.S.A.) was used to develop the assay, and fluorescence was measured with microtiter plate reader (Dynex, Chantilly, VA, U.S.A.), using excitation and emission wavelengths 450 nm. To quantify the amount of cytokine present in test samples, values were extrapolated from standard curves established by analyzing different dilutions of recombinant murine IL-4, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β.

Statistical Analysis Unless otherwise indicated, the results were expressed as the mean ± S.D. of data obtained from triplicate experiments. Statistical analysis was performed by a paired Student *t*-test. Differences at $*p<0.05$ were considered statistically significant.

RESULTS

Characterization of Proteoglycan Isolated from *P. linteus* The anion exchange chromatography profile is shown in Fig. 2A. The sugar fractions were Nos. 21—27. A fraction (0.43 M) of NaCl gradient elution containing the protein-bound polysaccharide has an obvious peak when using the DNS method.[12] The concentrated polysaccharide solution was then applied to the Sepharose CL-4B column (1.5×105 cm) and eluted with the same buffer solution at the flow rate of 1 ml min^{-1}. The results in Fig. 2B presumed that the molecular weight of protein-bound polysaccharide was about 150000 by gel-chromatography on Sepharose CL-4B. For the sake of comparison, the spectrum of β-glucan from *L. edodes* is shown in Fig. 1C (upper panel). The spectrum of purified protein-bound polysaccharide is shown in Fig. 1C (lower panel). These two spectra are very much the same. In the FT-IR spectrum of the two polysaccharides, the band corresponding to the ν(C=O) vibration in the carboxyl group at 1650 cm^{-1} indicates that this carbonyl group was hydrogen-bonded. Furthermore the banding-like-structure in the region of 2870—2930 cm^{-1} together with the ν(C–H) vibrations, as well as a continuous absorption beginning at about the region of 3400 cm^{-1} are characteristic of a carbohydrate ring. The spectrum of a β-glycosidic bond appeared at approximately 915 cm^{-1}, compared with (1,6) branched (1,3)-β-D-linked glucan from *L. edodes*. In addition, the purified protein-bound polysaccharide had an α-glycosidic bond at 860 cm^{-1}, but did not found the β-glucan from *L. edodes*. It was suggested that the purified protein-bound polysaccharide had both α- and β-glycosidic bonds and was a proteo-heteroglycan. The ^{13}C-NMR spectrum of the purified protein-bound polysaccharide was shown in Fig. 1D (upper panel). It showed multiple resonances that revealed the structural complexity of the glucan. The β configuration of the polysaccharide was confirmed by peaks in the region of δ 104.86, and the α conformation at 100.51. The branching points at C-6 were shown by the signal of the *O*-substituted carbon atoms at δ 62.9. The broad C-3 signal in the region at δ 81.4 could be ascribed to the presence in the polysaccharide of linear D-(1,3), branched D-(1,6), and terminal D-residues. The presence of the D-(1,6) linked residues was evidenced by the low-intensity signal at δ 69.5. The peaks of C-2, C-4, and C-5 were δ 72.6, δ 70.2, and δ 75.8, respectively. The signals of ^{1}H-NMR were 5.05 (α-C-1), 4.73 ppm (β-C-1), 3.64 ppm (C-5), 3.52 ppm (C-4), 3.56 ppm (C-3), and 3.43 ppm (C-2) and are shown in Fig. 1D (lower panel). On the basis of these results, the proteo-heteroglycan has been determined to be a noble biomolecule combined α- and β-linkages. The monosaccharide composition of the purified protein-bound polysaccharide for the fruiting body of *P. linteus* is shown in Table 2. Thus the polysaccharide was not a glucan, but rather found to be proteo-heteroglycan with a small amount of arabinose and xylose. The main sugars were glucose (41.6%), galactose (23.4%), and mannose (23.8%). It was thought that hexose sugar was the main composition of the backbone and pentose sugar constituted a small part of the side chain. The composition of hydrolysated amino acids is shown in Table 3. Abundance of hydrolysated amino acids was produced, but few physiological amino acids were in the purified protein-bound polysaccharide. Its protein portion predominantly consisted of aspartic acid, glutamic acid, serine, glycine, and alanine. The molar ratios of aspartic acid and glutamic acid having acidic side chains were 9.0% and 11.1%, respectively.

Role of Oral Administration of Proteoglycan Isolated from *P. linteus* in Development of CIA Signs of arthritis are observed on day 35 (Fig. 2). All mice had developed joint inflammation by day 30, and reached a peak on day 35. Thereafter, the arthritis had rapidly subsided by day 56, although complete remission had not been seen at least by day 70. The oral administration of 200 mg/kg of proteoglycan on days 0 (pre-administration) slightly decreased the joint inflammation from the primary injection on day 7 to the booster on day 21. After the booster, joint inflammation increased by degrees and the arthritis reached a peak on day 35 and then declined dramatically. Also, the oral administration of 200 mg/kg of proteoglycan on day 28 (post-administration) markedly reduces the joint inflammation. Comparing

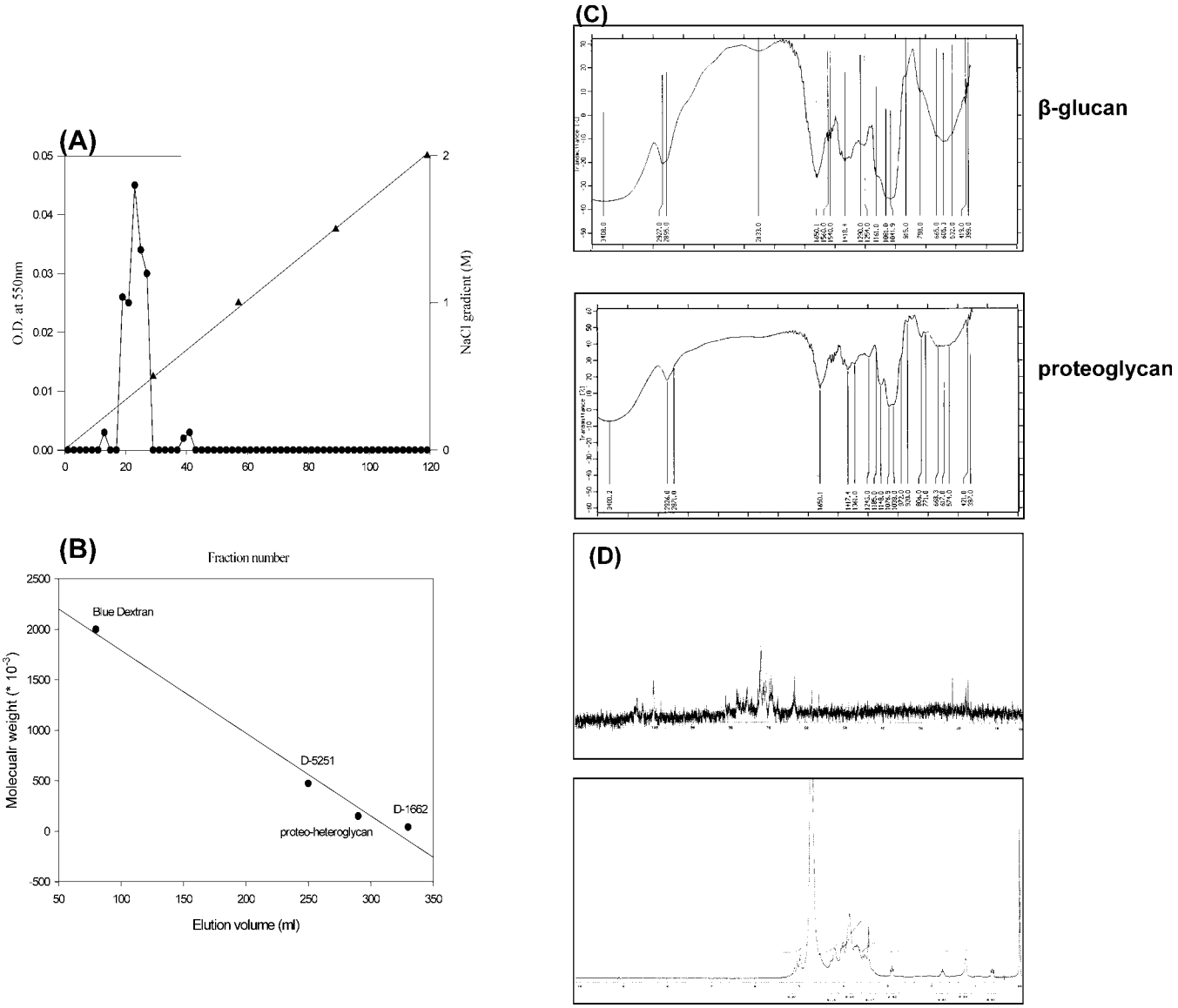

Fig. 1. Protein and Carbohydrate Patterns of Proteoglycan Isolated from *P. linteus*

(A) Anion Exchange Chromatography of Crude Proteo-Heteroglycan Derived from Fruiting Body of *P. linteus* on DEAE-Cellulose Column (3×45 cm)

The column was eluted with distilled water. Absorbance was measured by the DNS method. ●, content of sugar; ▲, NaCl gradient.

(B) Gel Filtration of Proteo-Heteroglycan on Sepharose CL-4B Column (1.5×105 cm)

The molecular weight markers used were: (1) Blue dextran (M.W. 2000000), dextran-5251 (M.W. 473000), and dextran-1662 (M.W. 41272) were used as standard dextran.

(C) FT-IR Spectrum Comparison of (Upper Panel) β-Glucan (Standard) from *Lentinus edodes* and (Lower Panel) Acidic Proteo-Heteroglycan from *P. linteus*

(D) NMR Spectrum of Acidic Proteo-Heteroglycan in D_2O ^{13}C-NMR (Upper Panel) and ^{1}H-NMR (Lower Panel)

the arthritic index of pre- and post-administration groups, the proteoglycan isolated from *P. linteus* is good for the preventing as well as remedying on a medical cure in CIA.

Decreased Ag-Specific IgG2a Ab Level in Pre- and Post-administration of Proteoglycan Because development of CII-specific Abs requires T cell help, we measured the effect of the pre- and post-administration of proteoglycan on Ab isotypes against murine CII *in vivo* by measuring CII-specific IgG, IgG1, and IgG2a production. From the mice that were immunized with CII in CFA, CII-specific Ab levels were determined in immune sera, and samples were collected at 14 d (day 35) after immunization (booster, days 21). CII-specific IgG, IgG1, and IgG2a were undetectable before immunization and there were no significant difference in the IgG1 level in between proteoglycan-induced and CIA mice after immunization (Fig. 3). In contrast, the CII-specific IgG2a and IgG levels were significantly lower in pre- and post-administration groups of proteoglycan compared with CIA controls. The lower amount of IgG2a suggests that T-cell specific expression of proteoglycan treatment reduced inflammatory Th1-type cytokines *in vivo* in CIA. Taken together, we conclude from these studies that the deceleration of arthritis by the oral administration of proteoglycan is attributable to the control of Th1-type cytokines. Instead, Th1-type cytokines are thought to influence the autoimmune arthritis.

Cytokine Gene Expression in CIA after Oral Administration of Proteoglycan We next examined the cytokine

Table 2. Component of Monosaccharide Contents in Acidic Proteo-Heteroglycan from *P. linteus*

Contents	Glucose	Galactose	Mannose	Arabinose	Xylose
PL[a)] (M%)	41.6	23.4	23.8	5.8	4.2

a) PL; acidic proteo-heteroglycan purified from *P. linteus.*

Table 3. Hydrolysated Amino Acid Composition of Acidic Proteo-Heteroglycan from *P. linteus*

Amino acid	M%
Aspartic acid	9.0
Threonine	6.9
Serine	11.8
Glutamic acid	11.1
Glycine	12.0
Alanine	12.7
Cystine	0.7
Valine	5.1
Methionine	1.0
Isoleucine	2.9
Leucine	5.6
Tyrosine	1.6
Phenylalanine	8.4
Histidine	1.2
Lysine	4.2
Arginine	2.8
Proline	3.0

gene expression in iLN, mLN, and PP induced by proteoglycan-treatment and CIA mice. To detect levels of mouse cytokine mRNA, RT-PCR was performed. RT-PCR allows comparison of mRNA levels among samples by using a housekeeping gene in the mRNA as a standard, such as β-actin, which is assumed to be present at similar levels in each sample.

Messenger RNA of Th1-type cytokines, such as TNF-α and IFN-γ that are known as pro- and inflammatory cytokines, is the obvious difference in iLN, as compared to oral administration and CIA mice (Fig. 4). The amount of TNF-α and IFN-β were stable in all isolated leukocytes of CIA mice. However, oral administration of proteoglycan caused a great reduction of TNF-α and IFN-γ in iLN and mLN. The post-administration led to an especially level of TNF-α and IFN-γ mRNA in dLN. The pre-administration has some decreased in and IFN-γ in iLN and mLN. In the post-administration, the level of TNF-α and IFN-γ is completely absent regardless of the tissues isolated in this study. The above two cytokines are thought to the exacerbate CIA. The oral administration of proteoglycan isolated from *P. linteus* mainly decreases these Th1-type cytokines in iLN of CIA mice. Also, IL-12, which is secreted from macrophages and DCs, and induces the production of Th1-type cells, is found in smaller amount iLN in the proteoglycan treated CIA mice. The above results suggest that IL-12 is directly related to suppressing CIA. Messenger RNA of Th2-type cytokines, such as IL-4 and IL-10, which are known as anti-inflammatory cytokines, experienced very slight changes. The mRNA level of IL-4 is stable in all isolated tissues. IL-10 had a small increase in iLN and PP in the pre-administration group and in mLN in the post-administration group. IL-10 partially inhibits of

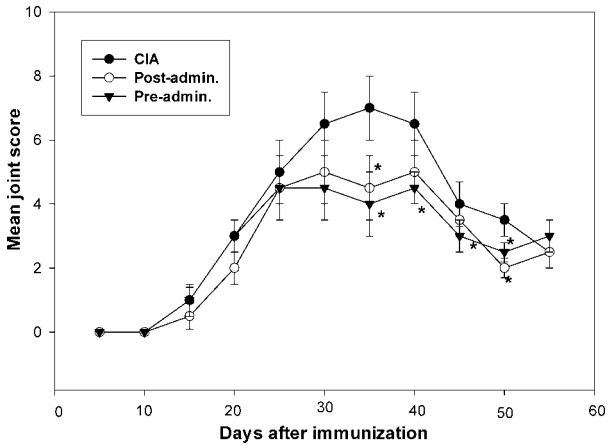

Fig. 2. Arthritic Index of Pre- (Day 0) and Post-administration (Day 28) through Oral Administration (200 mg/kg) of Proteoglycan Isolated from *P. linteus*

One group was immunized intradermally in the tail with 100 μg of CII in CFA (day 7) after a dose of 5 mg of proteoglycan through oral administration (pre-admin.; O). After another group was injected in footpading (booster; day 21), the same of proteoglycan was treatment at days 28 (post-admin.; ▼). The other group was CIA as control (CIA; △). (n=5)

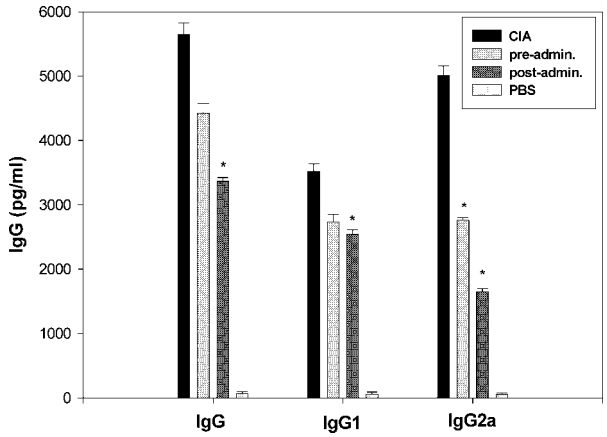

Fig. 3. Titer of Ab Isotypes against Murine Collagen Type II

Serum was isolated from the mouse at days 35 after the booster. Sample sera were diluted 1/100000 and incubated in murine collagen type II-coated wells. After binding mouse Ab isotopes, the titers of each Ab isotype were measured with biotin-conjugated anti-rat whole IgG Ab. n=5 in each groups. *, $p<0.05$ compared with CIA-induced group by t-test. PBS presented negative control in ELISA.

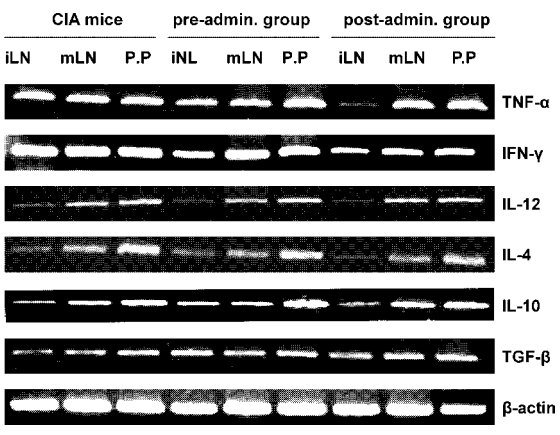

Fig. 4. Expression of mRNA Level in CIA Induced Mouse Administrated with Proteoglycan

RNA was extracted, and PCR was performed as described in Materials and Methods. (iLN, inguinal lymph node; mLN, mesenteric lymph nodes; PP, Peyer's patch).

Th1-type cytokines. Additionally, TGF-β was somewhat augmented in iLN and mLN after the pre-and post-administration of proteoglycan. This result, too, suggests that this cytokine lessens inflammation in CIA mice by inhibiting the expression of B7 in DCs and macrophages because it limits the interaction of CTLA4 and B7. However, unlike other cytokines was abundantly expressed in all leucocytes of proteoglycan administration and CIA mice. The amount of TGF-β cytokine was stable in CIA mice and acts as an anti-inflammatory.

Measurement of Cytokines Using Sandwich ELISA It suggests that the proteoglycan plays an important role in autoimmune arthritis by controlling DCs and macrophages, resulting in the selective release of inflammatory cytokines. Therefore, we also analyzed the effects of *in vivo* proteoglycan treatments on lymphocyte cytokine profiles in response to autoimmune arthritis. As shown in Fig. 5, lymphocytes isolated from proteoglycan-treated CIA mice also exhibited some important differences in cytokine production, and the most significant changes were associated with Th1-type cy-

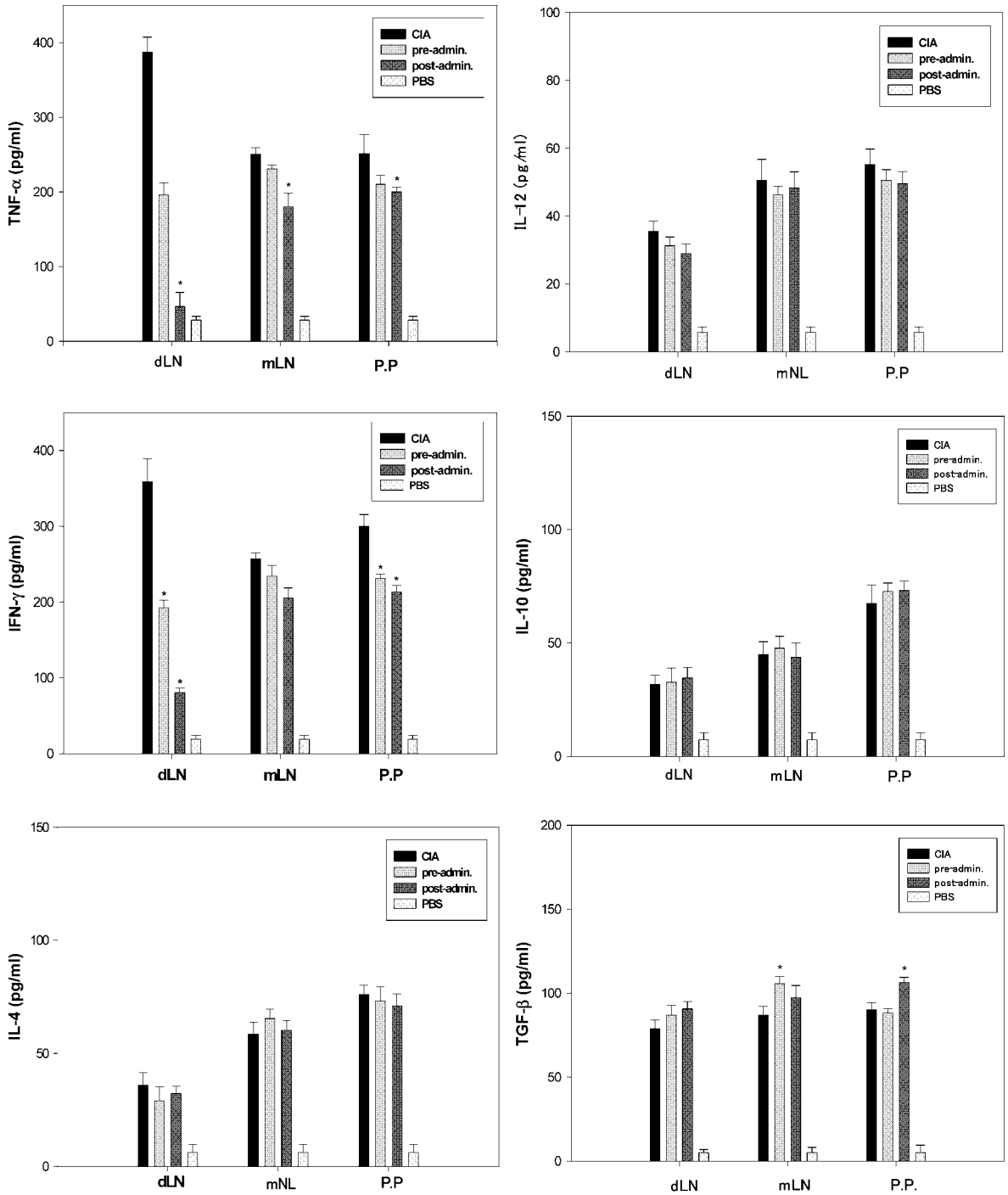

Fig. 5. Measurement of Cytokines Using Sandwich ELISA against Murine Collagen Type II

Mice were immunized with CII in CFA at days 7 and 21. Proteoglycan was administered oral feeding from at days 0 (pre-admin.) and 28 (post-admin.). Mice were scarified at days 35 and leukocytes were isolated from inguinal lymph nodes (iLN), mesenteric lymph nodes (mLN), and Peyer's patch (PP). Leukocytes were treated with 10 μg/ml of CII *in vitro* and ELISA was performed using supernatant after 72 h. The data represent the mean±S.D. at least 5 mice per group. *, $p<0.05$ compared with CIA-induced group by *t*-test. PBS presented negative control in ELISA.

tokines, TNF-α and IFN-γ. Lymphocytes isolated from post-administrated mice released significant amounts of TNF-α and IFN-γ in iLN after the induction of CIA. Similarly, the post-administration group showed a significantly lower level of production of TNF-α and IFN-γ in iLN but not as lower of a level in the pre-administration. Also, pre- and post-administration groups showed some reductions in these two cytokines in mLN and PP. Lymphocytes also produced insignificant levels of IL-10 and TGF-β when stimulated with the proteoglycan. In contrast to the IL-10 and TGF-β, cells from pre- and post-administration groups exhibited a lower level of IL-4 and IL-12 production in iLN. These results demonstrate that IL-10 and TGF-β can be independently regulated regardless of IL-4 production. Therefore, it is thought that this proteoglycan strongly regulates typical Th1 response.

DISCUSSION

Polysaccharides, that is particularly abundant in *P. linteus*, which is a known bioactive material, especially an anticancer agent.[20] The process of isolating and purifying a water-soluble glycan from *P. linteus* was achieved by hot water extraction, filtration, solvent precipitation, dialysis, and freeze-drying. Acidic fractions of the polysaccharide were separated from crude polysaccharides by DEAE-cellulose anion exchange chromatography at 0.43 M NaCl. The molecular weight of the proteo-heteroglycan after Sepharose CL-4B gel filtration chromatography was about 150000. The total carbohydrate consisted of 78.8% Crude polysaccharide and 72.2% purified glycan, respectively. The sugar of the proteo-heteroglycan was composed of mannose, galactose, glucose, arabinose, and xylose. The amino acid pattern showed that the fractions contained a large mount of aspartic acid, glutamic acid, alanine, glycine, and serine. The fractions for both α-glycan at 860 cm^{-1} and β-glycan at 910 cm^{-1} had the characteristics of IR spectrum absorption as compared to those for β-glucan derived from *L. edodes*. A ^{13}C- and ^{1}H-NMR spectroscopy showed that the acidic proteo-heteroglycan was a noble biomolecule mixed both α- and β-linkages, and a (1,6) branched type (1,3) glycan.

After a decade of few notable advances in RA therapy, several biological response modifiers (BRMs) that target the pathophysiological processes of RA are on the horizon. The United States Food and Drug Administration (FDA) have approved two such agents that inhibit TNF-α activity, ethanercept and infliximab, for the treatment of RA. Other agents are in earlier stages of evaluation; some are no longer being studied owing to a lack of efficacy or unexpected toxicities. Although much is still to be learned about these BRMs, they hold the promise of fundamentally changing treatment options for RA. Some of these agents prove rapid symptomatic relief in the absence of significant adverse effects, of even more importance, some BRMs slow disease progression and allow patients to enjoy a healthier an more fulfilling life. These representative BRMs are the polysaccharides of mushrooms. Higher basidiomycetes mushrooms are used in folk medicine throughout the world, as they have been since ancient times. Mushrooms are a nutritionally functional food and a source of physiologically beneficial medicines. Mushrooms influence various physiological properties, such as immunological enhancement, homeostasis maintenance, and biorhythm regulation. They have also been used in the prevention and treatment of cancer, cerebral stroke, and heart disease.[21] The extract of *P. linteus* is considered to be the most potent and has been shown to inhibit Sarcoma 180 growth in 96.7% of the time in immunocompetent mice.[22] However, no report was found concerning whether the proteoglycan isolated from *P. linteus* effects autoimmune disease. The present study implies that proteoglycan derived from *P. linteus* may play a role in the decrease of autoimmune arthritis of the mice in the pre- and post-administration groups. Also, this study focuses on these agents aimed at modulating the activity of pro-inflammatory cytokines, particularly TNF-α.

The balance of Th1 and Th2 cytokines is thought to influence the autoimmune arthritis process in that Th1-type cytokines are associated with disease.[23] Our finding was expected whether it was the Th1-type cytokines that decreased or the Th2-type cytokines that increased due to the proteoglycan treatment. The observed suppressive effects that proteoglycan has on T cell responses in CIA may be more critical in diseases that Th1-type cells are dominant. The success of proteoglycan in suppressing non- and established diseases suggests that T cell response is critical in two conditions of CIA. Once the T cell response has been suppressed or after the second CII injection (booster), the reduction of TNF-α and IFN-γ may limit the production of inflammatory cells in the synovium, such as macrophages and DCs, that may selectively control the inflammatory response to CII and result in the observed inhibition of disease.

The precise mechanism that proteoglycan used to restore CIA is unclear at present. However, decreases in anti-CII and the isotype IgG2a Abs, which followed the administration of proteoglycan, appear to have, at least in part, contributed to the recovery of autoimmune disease because these Abs, especially the IgG2a, played a critical role in CIA.[24,25] Marked reduction of anti-CII IgG2a Abs may be due to the decreased secretion of TNF-α and IFN-γ observed in mice given the proteoglycan because this Th1 cell-producing cytokine was involved in the isotype IgG2a Abs production.[26,27]

Clinical symptoms of disease in patients with rheumatoid arthritis cycle between relapses and flares. Therefore, it was important to determine whether Th2 cytokines can suppress severe inflammation or Th1 cytokines can be suppressed. In proteoglycan-treated group of arthritis, the reduction of TNF-α and IFN-γ, and some augmenting of IL-10 and TGF-β significantly suppress joint inflammation. Arthritis and certain infectious diseases can induce exaggerated production of the inflammatory cytokine, TNF-α. A midline survey of the published literature concluded that TNF-α plays a major role in the pathogenesis of septic shock and related syndromes.[28] Elevated plasma concentrations of TNF-α have been observed in malarial patients.[29] The evidence supporting TNF-α involvement in diverse conditions, such as immune deficiency[30,31] and inflammatory joint disease,[32] has been steadily accumulating during the last decade. These observations provide the rationale for the development of potential therapeutic strategies based on the down-regulation of TNF-α and other inflammatory cytokines. Also, the down-regulation of CIA occurs by shifting the CII-specific T cell response toward a Th2-type profile. IL-10 and TGF-β effectively down-regulate inflammatory responses in mice. Re-

cently, Seder *et al.*[33] have completed a study of factors involved in the differentiation of TGF-β producing cells from naive $CD4^+$ T cells and also found that IL-4 producing T cells enhance TGF-β production in an *in vivo* priming culture using TCR transgenic mice and that IFN-γ inhibits TGF-β production. However, although IL-4 appears to be an important factor associated with the differentiation of TGF-β secreting cells, IL-4 is not essential, as these authors and others[34] have found that T cells from IL-4-deficient animals could be induced to produce TGF-β, albeit at lower levels. Seder *et al.*[33] also found that TGF-β positively regulated its own production and that the presence of IL-10 in their culture system was associated with an increase in TGF-β production, presumably by its down-regulatory, findings that were confirmed in this laboratory. Marth *et al.*[35] have demonstrated that anti-IL-12 given *in vivo* enhanced oral tolerance and induced the production of TGF-β. Thus, it appears that the induction of TGF-β is favored by IL-4 and conditions that decrease IFN-γ and that once TGF-β secretion occurs, TGF-β itself further enhances its own production. What role IL-13 may play in the absence of IL-4 in the differentiation of TGF-β-secreting cells needs to be determined, although Seder *et al.*[33] found no role for IL-13. In addition to regulatory cells that primarily secrete TGF-β, cultures of cells with IL-10 have been reported to lead to the generation of regulatory cells, which primarily secrete IL-10 in addition to TGF-β.[36] Finally, the inhibition of Th1 responses and the TGF-β production induced by anti-IL-12 were not associated with increasing IL-4. This finding argues that typical Th2 responses, *i.e.*, IL-4 production, and TGF-β can be independently regulated at the T cell level despite the fact that they may appear together, presumably because of a common negative regulatory function of IL-12. While IL-10 production was increased in mice treated with anti-IL-12, this appeared to be a non-T cell response. While we cannot exclude the possibility that IL-10 may have some role in *in vivo* suppression induced by oral administration, a large amount of prior data on oral tolerance has demonstrated that the transfer of T cells from orally tolerized animals to naive animals is often sufficient for the transfer of tolerance.

We may thought that proteoglycan (PL) extracted from medicinal mushroom, *Phellinus linteus*, inhibit the production of anti-collagen type II antibody and improve inflammatory arthritis in mouse model. The exact mechanism of PL could not know against inflammatory response. However, Soltys & Quinn[37] suggested that a possible role of soluble β-glucan in enhancing the removal of inflammatory cells from the sites of inflammation. The balance between phagocyte apoptosis and necrosis in inflamed tissues seems to play an important role in the resolution and/or control of inflammation. We may think that PL has a similar effect for anti-CII induced arthritis in mice. Thus, part of the anti-CII effects of soluble PL treatment *in vivo* may results from the enhanced apoptosis of a portion of the activated lymphocyte population. Clearly, further studies are necessary to determine if PL interacts with specific lymphocyte subpopulations and if PL induced apoptosis plays a physiologically important role in resolving inflammatory disease.

In summary, oral administration of proteoglycan isolated from *P. linteus* results in the reduction of CIA in mice and is associated with decreased production of anti-CII IgG2a Abs, especially such as TNF-α and IFN-γ, as well as some enhanced secretion of cytokines including IL-10 and TGF-β in protein level. The decreased joint inflammation appeared to be due to the absorption of proteoglycan in the gut. These results suggest that macrophages or DCs are stimulated by oral administration of proteoglycan at PP and control the activation of T and B-lymphocytes at iLN and mLN. It is possible that the use of BRM, such as the proteoglycan derived from *P. linteus*, to manipulate the production of pro-inflammatory and anti-inflammatory cytokines by activated lymphocytes. Thus, proteoglycan isolated from *P. linteus* may be crucially involved in the prevention as well as remedy of autoimmune joint inflammation such as RA, although no definite role of anti-CII Abs in the human disease has been established.

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