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Oral Administration of Highly Oligomeric Procyanidins of Jatoba Reduces the Severity of Collagen-Induced Arthritis

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We have previously reported that highly oligomeric procyanidins (HOPC) purified from Jatoba, a South American herb, ameliorated experimental autoimmune encephalomyelitis (EAE) in mice. In this present study, we report that symptoms of arthritis were also significantly reduced by administering the Jatoba extract, when compared with the vehicle-alone-treated control. Interferon-gamma (IFN-γ) production by the splenocytes from mice injected with procyanidins was also dramatically decreased. The oral administration of purified HOPC was significantly more effective in disease prevention than the ethanol (EtOH) extract of Jatoba. Green tea polyphenol administration, however, surprisingly facilitated disease development. Observation of the joint histopathology on whole paws derived from the HOPC-treated mice showed complete abrogation of collagen induced arthritis (CIA), a characteristic of chronic inflammation in the synovial tissue.

These results demonstrate that HOPC administration had an inhibitory effect on both chronic arthritis and EAE and that the oral administration of HOPC exerted its effect after the induction of secondary immunity.

Key words: rodent; CIA/arthritis; procyanidins

Collagen-induced arthritis (CIA) in mice is a widely studied animal model of inflammatory polyarthritis with similarity to rheumatoid arthritis (RA). CIA is induced after immunizing susceptible strains of mice with articular bovine type II collagen (C II) in complete Freund's adjuvant (CFA), and the resulting disease is primarily mediated by an autoimmune response.[1,2] The significance of the model lies in the fact that CII is a major constituent protein of the cartilage in the diarthrodial joints, the primary site affected in RA.[2] The pathogenic immune response to CII in CIA is rather complex and depends on major histocompatibilty complex (MHC)-restricted CII-specific, Th1-type, IFN-γ-producing T cell and IgG2a-producing B cell responses, in combination with several other cellular and biochemical factors.[2,3] Thus, there is a synergy in the CII specific humoral and cellular immune response that is critical for the pathogenesis of the disease. Treatments designed to interfere with this synergistic response have been shown to prevent the onset of CIA.[4–7] There are many compelling similarities between CIA and RA that make CIA an appropriate model, not only to precisely define the role of T and B cells in the pathogenesis of the disease, but also to develop and test preventive and therapeutic approaches to arthritis in humans. Drug therapy for RA rests mainly on two principals: symptomatic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and treatment with disease-modifying antirheumatic drugs (DMARDs). NSAIDs interfere with only a small segment of the inflammatory cascade, namely prostaglandin production by cyclooxygenases (COXs), but not the underlying immuno-inflammatory events. Therefore, NSAIDs do not slow the progression of the disease. By contrast, DMARDs retard or halt disease progression and, once DMARDs are effective, no further symptomatic therapy is needed. New guidelines for the management of RA by the American College of Rheumatology (ACR) emphasize early diagnosis and implementation of DMARD therapy.[8] Although there are several DMARDs available, all of them show differential responses among patients, and there remains no adequate way to predict which will be the most effective in RA therapy. Clearly, the introduction of biological DMARDs that block tumor necrosis factor alpha (TNF-α) or interleukin-1 (IL-1) has had a major impact on the treatment of RA in the last decade. However, more than half of the patients in clinical trials did not achieve ACR 50 (an improvement of 50% or more in the number of tender and swollen

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Abbreviations: HOPC, highly oligomeric procyanidins; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; C II, type II collagen; CFA, complete Freund's adjuvant; DC, dendritic cells; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin-3-*O*-gallate; EGC, (−)-epigallocatechin

joints), and the enormous costs of biological drugs make them inaccessible to many patients.[9–11] In addition, the toxicity of most DMARDs is a concern. Therefore, the demand for effective, cheap and less toxic DMARDs is still high.

Studies are also in progress to investigate the anti-oxidative actions of polyphenols which are known to be rich in plant extracts. Polyphenols are physiologically active substances found in various foods and plants, including green tea, wine, and cocoa. It has been reported that all the polyphenols have an anti-oxidative action.[12] Anti-arteriosclerotic, anti-hypertensive, and anti-tumor effects are also well known. Although monomer polyphenols also have such effects, highly-polymerized polyphenols have stronger anti-oxidative, anti-mutagenic and anti-tumor activities.[13–15] Polyphenols also affect the immune system;[16,17] *e.g.*, the polyphenols derived from tobacco leaf inhibit IgE-mediated mast cell activation.[18] In our previous study, we showed that the administration of procyanidins that were polymers of flavan-3-OHs (*e.g.*, catechin and epicatechin), ameliorated experimental autoimmune encephalomyelitis (EAE) in a mouse model. The inhibitory activity of polyphenols is dependent on their degree of polymerization, so that monomer catechin-like green tea polyphenols do not prevent EAE.[19] However, there are reports that green tea polyphenols can ameliorate CIA.[20] In this study, we examined whether procyanidins purified from Jatoba could inhibit CIA, and whether the inhibitory activity would be dependent on polymerization. We also investigated the most effective administration mode and administration timing of HOPC.

Materials and Methods

Mice. Male DBA1/J mice were purchased from Charles River Japan. All mice were 8–10 weeks of age when used. The experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Kirin Holdings Co. (Tokyo, Japan).

Materials. A commercial dry powder of Jatoba (*Hymenaea courbaril*) was purchased from Edison SRL. 5 liters of EtOH was added to 500 g of powder, and the mixture stirred overnight. After filtration, 136.1 g of an ethanol extract was obtained by evaporating the solvent. The ethanol extract of Jatoba was found to contain a large amount of procyanidins (≥70% by weight) as estimated by thiolysis (see lates). In addition, the extract was composed solely of (−)-epicatechin (EC) with no trace of gallate esters (unpublished data, manuscript in preparation). The extract was dissolved in 1% or 10% EtOH and used as an inoculum. The green tea polyphenol used was a commercial powder, Polyphenon™ (Tokyo Food Techno). The powder was dissolved in 10% EtOH.

Preparation of HOPC. Fractionation was conducted according to published methods.[21] Briefly, the Jatoba EtOH extract (60 g) was extracted with ethyl acetate, the water and ethyl acetate layers were separated, and the water layer was dissolved in chloroform-methanol (MeOH). The concentration of chloroform was increased stepwise to 50, 60, 70 and 75%, and the precipitates were collected. The precipitated layers obtained at each concentration are designated Fr1, 2, 3 and 4. A 9.7 g amount of Fr3 was obtained, the highest amount of procyanidins being observed in Fr3 (84.3%). The mean degree of polymerization of this fraction was 10.9, as determined by thiolysis. Fr3 was used as HOPC, because this fraction showed the strongest inhibitory activity toward EAE.[19]

Thiolysis analysis. To estimate the degree of polymerization of the procyanidins, thiolysis assays were performed by using scaled down versions of previously published methods.[22,23] Each sample was dissolved in MeOH to a concentration of 4 mg/ml. Twenty-five ml of the solution was mixed with 25 ml of 3.3% (v/v) HCl in MeOH and 50 ml of a 5% (v/v) solution of toluene-α-thiol in MeOH, and the mixture was stirred and heated at 40 °C for 30 min. The resulting solution was analyzed directly by HPLC.

Antigen. Bovine type II collagen (C II; Collagen Technological Workshop, Japan) was dissolved in 0.05 mole/liter acetic acid to a final concentration 3 mg/ml, and the solution stored at −80 °C.

CIA induction. An equal volume of C II was emulsified with Freund's complete adjuvant (4 mg/ml of H37Ra), and the mice were immunized intradermally (i.d.) in the base of the tail (on day 0). The mice were boosted i.d. or intraperitoneally (i.p.) 3 weeks later (on day 21) with the C II solution emulsified in CFA.

Clinical scores expressed the arthritis severity on a scale of 0–4 for each paw, according to changes in erythema and oedema as previously described:[24] score 0, normal; score 1, detectable arthritis with erythema in at least some digits; score 2, significant swelling and redness; score 3, severe swelling and redness from joint to digit; score 4, maximal swelling with ankylosis. The total score was the cumulative value for the four paws, with a maximum of 16 for each mouse.

Jatoba treatment. The EtOH extract of Jatoba at a stock concentration of 5 mg/ml was injected i.p. to give 1 mg per mouse; 200 µl of 1% EtOH was administered i.p. as a control. This treatment was given 3 times a week to each mouse.

The EtOH extract of Jatoba, HOPC or Polyphenon™ at a stock concentration of 62.5 mg/ml was used for oral administration in order to give 12.5 mg per mouse; 200 µl of 10% EtOH was administered orally as a control. This treatment was given 5 times a week to each mouse.

Cytokine measurement. Spleen cells were prepared on day 10, five to 15 animals being used per group. The spleen cells were cultured for 3 days with RPMI 1640 (Sigma) + 10% fetal calf serum (FCS; Roche) either with or without 25 μg/ml of type II collagen. The concentration of IFN-γ was measured in the supernatant from each group. The IFN-γ level was determined by using an OptEIA ELISA set (Becton Dickinson) according to the manufacturer's protocol.

Serum anti-CII Ab level. Serum samples were collected on day 53, and titers of anti-CII IgG Abs were measured by ELISA. Bovine CII (3 μg/ml) was coated on to microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) overnight at 4 °C. After blocking with 10% FCS in phosphate-buffered saline (PBS), a serially diluted serum sample was added, and the plate incubated for 2 h at room temperature. After washing, AP-conjugated rabbit anti-mouse IgG1, IgG2a or IgG Ab (Zymed, CA, USA) was added, and the plate furthers incubated for 2 h at room temperature. After washing, Ab binding was visualized by using the p-nitrophenyl-phosphate phosphatase substrate system (KPL, Maryland, USA). A standard serum sample composed of a mixture of sera from arthritic mice was added to each plate in a serial dilution, and a standard curve was constructed. The standard serum is defined as 100 units/ml, and the titers of the serum samples were determined by using this standard curve.

Histopathology. The mice were sacrificed and fixed for 24 h in 10% phosphate-buffered formalin, before being decalcified for 2 weeks in an EDTA solution. After this decalcification, the paws were embedded in paraffin, before being sectioned and stained with hematoxylin and eosine.

Surface marker. The expression of surface molecules on the spleen cells was analyzed by FACSort (Becton Dickinson Immunocytometry Systems). The cells were preincubated with CD16/CD32 (rat IgG2b, clone 93) to block the Fcγ receptors. After washing, 5×10^5 cells were stained with specific monoclonal Abs (mAbs) for 30 min at 4 °C in 50 μl of PBS containing 5% of FCS and 0.1% of sodium azide. We used fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled mAbs for staining CD3ε (Armenian hamster IgG 145-2C11), CD4 (Rat IgG2b, GK 1.5), CD8α (rat IgG2a, 53–6.7), CD11b (rat IgG2b, M1/70), CD11c (Armenian hamster IgG, N418) and CD45R (B220; rat IgG2a, RA3-6B2). FITC- or PE-labelled mouse, rat or Armenian hamster IgG antibodies were used as isotype controls.

Endotoxin assay. We established that the EtOH extract of Jatoba, HOPC and Polyphenon™ were all endotoxin-free by using a Limlus Color KY Test kit (Wako).

Statistical analysis. Differences between groups were analyzed by the Mann-Whitney *U* test, and significant differences are reported at $p < 0.01$.

Results

Intraperitonial administration of Jatoba ameliorated CIA

The clinical scores and production of Ag-specific cytokines monitored the efficacy of the EtOH extract of Jatoba for modifying CIA. The mice were examined daily for clinical symptoms, and scores were recorded after the 2nd immunization. Strong inhibition of CIA was observed after injecting the Jatoba extract (Fig. 1A). The incidence of disease with the Jatoba extract-injected mice was 50% compared to 100% with the control group.

Some of the mice in the group were sacrificed on day 10, and spleen cells were prepared to examine the Ag-specific production of IFN-γ. The production of 12.5 ng/ml of IFN-γ was observed in the control group after Ag stimulation, whereas this production was reduced to 3.8 ng/ml with the Jatoba extract (Fig. 1B).

The population of spleen cells was analyzed by flow cytometry on day 14 post-induction of CIA. $CD4^+$ T cells (CD3ε, CD4), $CD8^+$ T cells (CD3ε, CD8α), B cells (B220), macrophage (CD11b) and dendritic cells (DC; CD11c) were significantly lower in the mice treated with Jatoba, while the macrophage population increased in mice treated with Jatoba (Fig. 1C).

Oral administration of Jatoba ameliorated CIA

The effect of procyanidins administered by a different route was also investigated. We thought oral administration would result in a mild effect compared with i.p., so in this case, when we induced CIA, we boosted i.p. (not i.d.) with CII-emulsified CFA. As a result, the clinical score for the control was lower than the foregoing data. Mice were orally given the Jatoba extract, HOPC or green tea polyphenols, and the effect monitored by the clinical score and serum antibody level. In our previous study, HOPC was more effective than the EtOH extract, because it contained more highly oligomeric procyanidins.

The mice were examined daily for clinical symptoms, and scores were recorded after the 2nd immunization. Strong inhibition of CIA was observed after the oral administration of Jatoba and HOPC (Fig. 2A). HOPC showed a particularly strong inhibitory effect. The incidence of disease in the HOPC-administered mice was completely inhibited (Table 1). The production of IFN-γ by the spleen cells was inhibited as efficiently as that by the i.p. treatment (Fig. 2B). We next examined the serum antibody levels that are indicative of disease severity. We measured not only total IgG but also the production of the anti-collagen IgG2a subtype, which is produced during a Th1 response, and the IgG1 subtype, indicating a Th2 response. The mean values of IgG1,

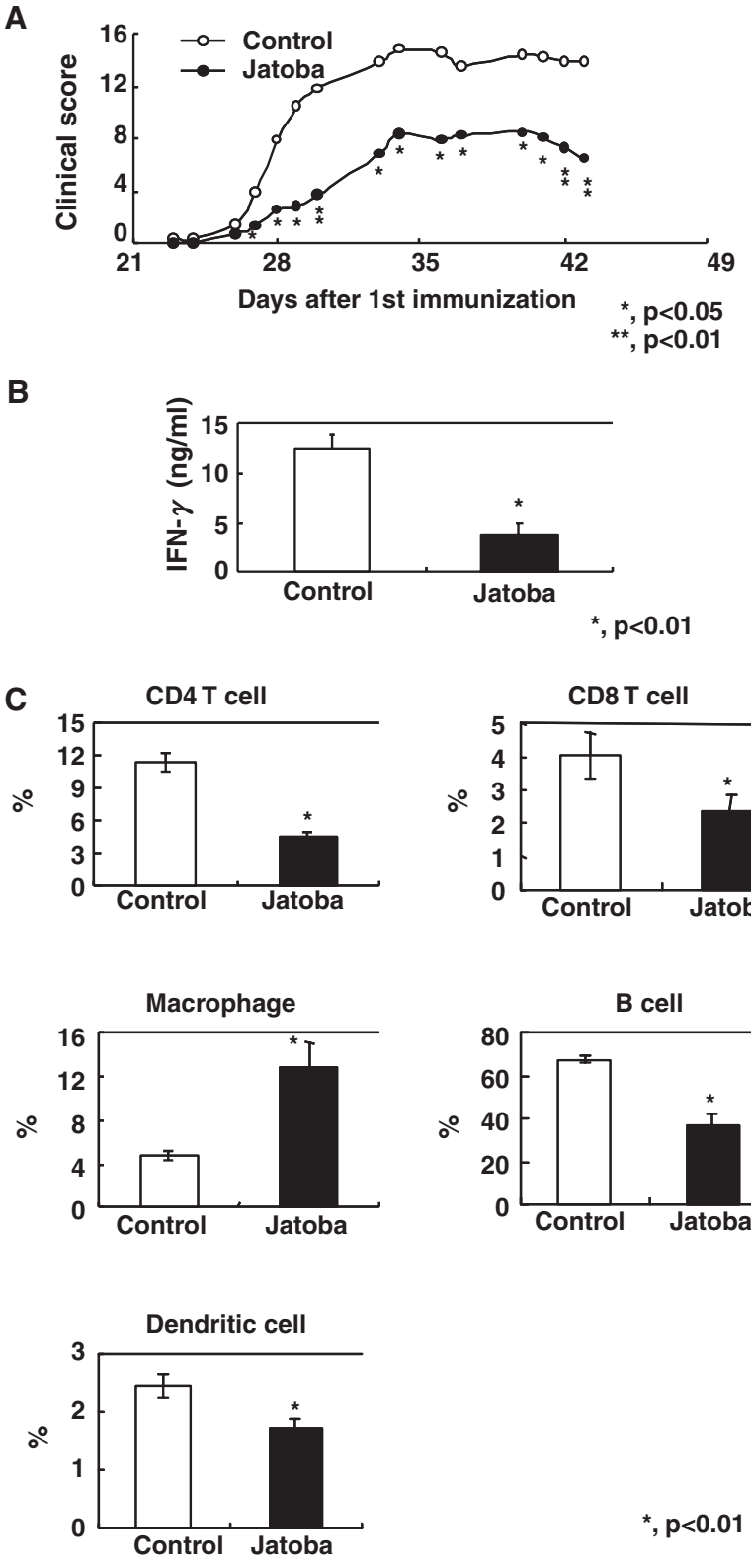

Fig. 1. Improvement of CIA Scores by Jatoba Administration.

A, The EtOH extract of Jatoba was injected i.p. at a dose of 1 mg/mouse 18 times during the period from day 0 to day 42 into mice in which CIA had been induced. Each group consisted of 8 animals. Clinical scores were recorded until day 43. Mean scores are plotted on the graph. B, Simultaneously with the induction of CIA, the EtOH extract of Jatoba was injected i.p. 5 times during the period from day 0 to day 9 into mice at a dose of 1 mg/mouse. Spleen cells were prepared from the mice on day 10, and production by these spleen cells of Ag-specific cytokines was studied. Each group consisted of five animals. The mean and SE of the concentration of IFN-γ produced are shown. C, Simultaneously with the induction of CIA, the EtOH extract of Jatoba was injected i.p. 6 times during the period from day 0 to day 11 into mice at a dose of 1 mg/mouse. Spleen cells were prepared from the mice on day 14, stained with mAbs to CD3ε, CD4, CD8α, CD11b, CD11c and B220, and analyzed by flow cytometry. Significant differences between the untreated and treated mice were determined by the Mann-Whitney U test (*, $p < 0.01$).

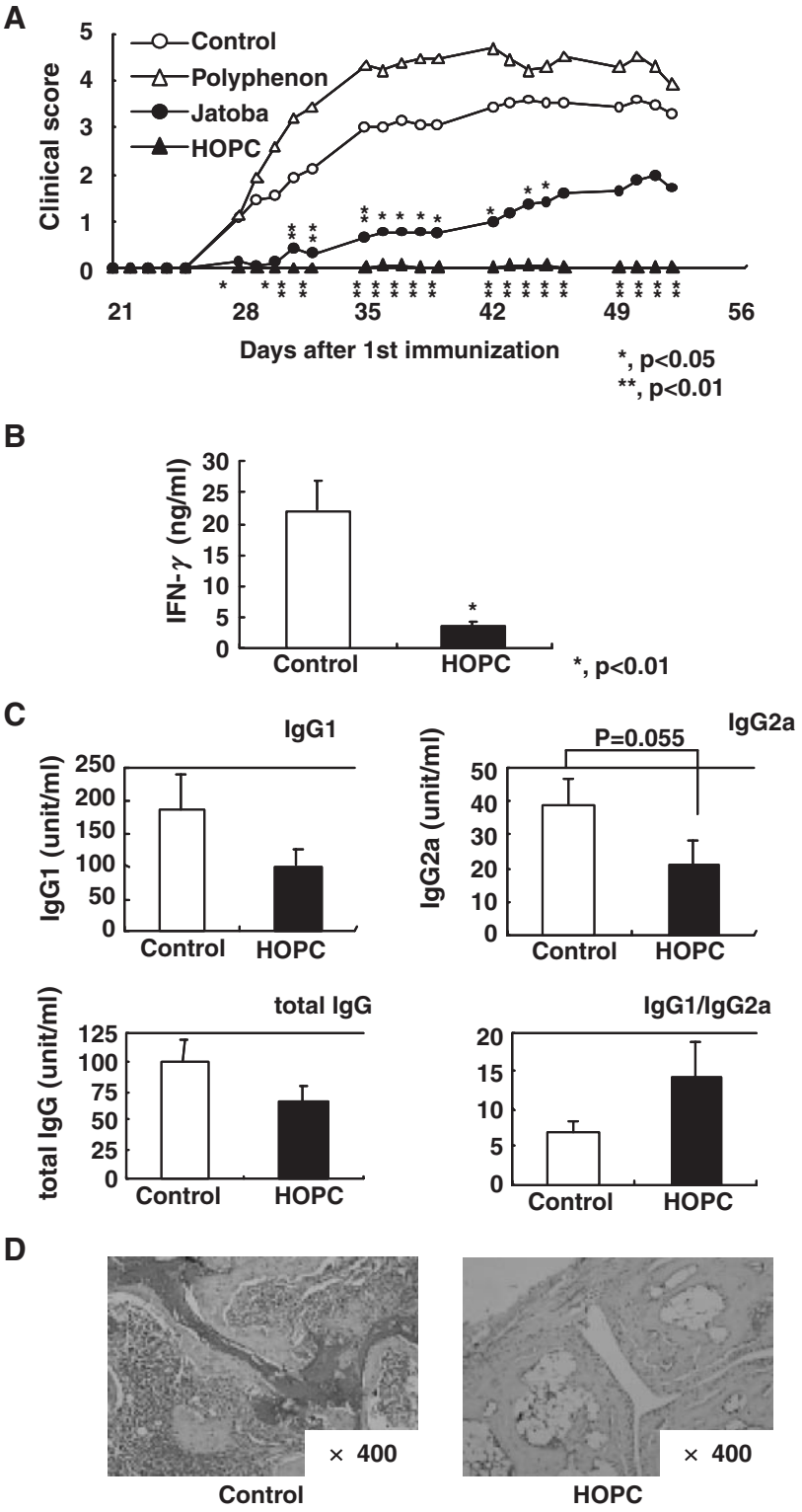

Fig. 2. Improvement of CIA Scores by the Oral Administration of Jatoba.

A, The EtOH extract of Jatoba, HOPC and green tea polyphenols were administered orally at a dose of 12.5 mg/mouse 35 times during the period from day 0 to day 48 to mice in which CIA had been induced. Each group consisted of 15 animals. Clinical scores were recorded until day 53, mean scores being plotted in the graph. Significant differences between the untreated and treated mice were determined by a Mann-Whitney U test (**, $p < 0.01$; *, $p < 0.05$). B, Simultaneously with the induction of CIA, HOPC was administered orally 10 times during the period from day 0 to day 12 to mice at a dose of 12.5 mg/mouse. Spleen cells were prepared from the mice on day 14, and the production of Ag-specific cytokine was studied. Each group consisted of 16 animals. The mean and SE of the concentration of IFN-γ produced are plotted. C, Serum was prepared after recording the clinical score. The serum from the mice was analyzed by ELISA to quantify the anti-CII Ab levels (see the materials and methods section). Each group consisted of 15 animals. The mean and SE of the level of each antibody are plotted in the graph. D, After recording the clinical score, the mice were sacrificed and histological sections of the paw were evaluated. Data show representative cases in each group.

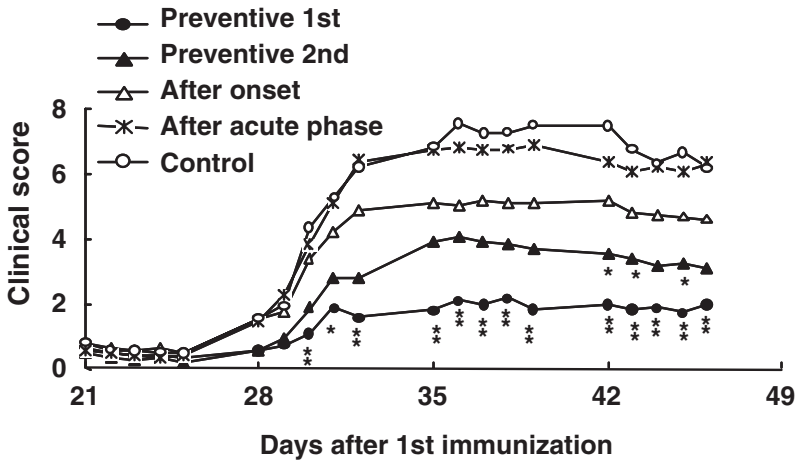

Fig. 3. Jatoba as a Potential Therapeutic Agent for CIA.

To further investigate the therapeutic potential of HOPC, we performed different commencing times for the HOPC administration. HOPC was administered orally at a concentration of 12.5 mg/mouse, starting with the 1st administration of CII Ag (from day 0, preventive 1st), the 2nd administration of CII Ag (from day 21, preventive 2nd), a week after the 2nd administration of CII Ag (from day 28, after the onset), or 2 weeks after the 2nd administration of CII Ag (from day 35, after the acute phase). The mice were examined daily for clinical symptoms, and scores were recorded after the 2nd immunization. Mean scores are plotted in the graph. Significant differences between the untreated and treated mice were determined by a Mann-Whitney U test (**, $p < 0.01$; *, $p < 0.05$).

Table 1. Incidence of CIA

	Incidence (%)
Control	78.57
Green tea polyphenol	64.39
Jatoba	37.5
HOPC	7.69

Table 2. Incidence, Maximal Score and Mean Day for Onset of Each Group

	Incidence (%)	Maximal score	Mean day for onset
Control	100	7.6	26.1 ± 0.99
After acute phase	100	6.8	26.4 ± 0.97
After onset	93.3	5.2	25.2 ± 1.18
Preventive 2nd	92.86	4.1	25.9 ± 1.14
Preventive 1st	50	2.2	24.4 ± 1.62

IgG2a and total IgG with the HOPC-administered group were low. In particulars, serum IgG2a tended to be inhibited by the HOPC administration; as a result, the mean value of IgG1/IgG2a increased. (Fig. 2C).

We next evaluated the cartilage and bone integrity histologically (Fig. 2D). The progression of synovial hyperplasia, and of cartilage and bone erosion was significantly suppressed by HOPC.

Administration of HOPC after CIA onset had been established

To further investigate the therapeutic potential of HOPC, we administered HOPC at different times. HOPC was administered orally at a concentration of 12.5 mg/mouse starting with the 1st administration of CII Ag (preventive 1st), 2nd administration of CII Ag (preventive 2nd), a week after the 2nd administration of CII Ag (after the onset), or 2 weeks after 2nd administration of CII Ag (after the acute phase). The mice were examined daily for clinical symptoms, and scores were recorded after the 2nd immunization.

The incidence of disease was not affected by the different administration times. However, the maximal clinical score was reduced in the 'preventive 2nd' and 'after onset' groups. These results suggest that HOPC would be a potential therapeutic agent for the treatment of CIA (Fig. 3, Table 2).

Discussion

Our results demonstrate that the Jatoba extract exerted an inhibitory effect on a mouse model of rheumatoid arthritis. We have shown in our previous study that HOPC ameliorated EAE *via* the suppression of Th1 immunity. In this model, HOPC acted to inhibit IFN-γ production and increase macrophages in the splenocyte population. Moreover, we identified the increased macrophages from the administration of HOPC as the immature phenotype which decreased the level of surface Ag expression.[19)]

In this present study, we showed that the same compound has also effective on CIA. We also identified the same two effects on the two different diseases by using procyanidins: inhibition of IFN-γ production by splenocytes and a change in the splenocyte population.

We thus conclude that procyanidins ameliorated both diseases through the same mechanism which prevented the development of the disease by lowering the Ag-

presenting capability of macrophages and by inhibiting their activation.

We initially thought that the inhibitory effect of procyanidins on autoimmune disease was demonstrated by the inhibition of Th1 cytokine, like IFN-γ. In fact, IFN-γ is known to induce a proinflammatory effect, for example macrophage activation,[25] MHC class II Ag expression in variety of cells,[26,27] and secretion of IgG2a *in vivo* and *in vitro*.[28] IFN-γ has received considerable attention concerning its role in the autoimmune process. Irmler *et al.* have recently reported that antigen-induced arthritis was exacerbated in IFN-γ deficient mice because of an unrestricted IL-17 response.[29] Moreover the suppression of CIA in IL-17-deficient mice have been reported by Nakae *et al.*[30] These studies have indicated that IFN-γ had an anti-inflammatory effect on these autoimmune diseases. In this present study, we only evaluated IFN-γ production by using a spleen 10 d after the 1st immunization. Based on these factors, we want in the future to evaluate IL-17 and the types of Th cells that will be induced by APCs differentiated by a procyanidin administration.

We identified in this study an increase in macrophages by the oral administration of procyanidins. Based on a result from the previous study,[19] we considered that the increased macrophages were of the immature type. Although we could explain that the decrease in lymphocytes had been caused by the decreased antigen-presenting ability of the macrophages, this couldn't explain why DC decreased. We therefore don't have a clear answer. Decrease of DC may be observed one as the result in which macrophage increased because DC shares progenitor with a macrophage.

We have also established the effectiveness of an oral administration of procyanidins. Since most types of therapy for autoimmune diseases are associated with pain, high cost and side effects, we need to develop an oral medication, or functional food, that can be better tolerated by patients. We have already established that an oral treatment with procyanidins was effective for treating autoimmune diseases in mice, and we conclude that procyanidins may be the most useful material for an oral medication. Procyanidins are water soluble and could be administered as tea. In fact, an extract of Jatoba has been drunk as tea by people in South America for thousands of years and is noted for its anti-fatigue and nutritional powers.

We have established in our previous study that the inhibitory activity of EAE was dependent on the degree of polymerization of procyanidins. Thus, the polyphenols from green tea, *e.g.*, EC, have no inhibitory activity. On the other hand, there is a report that an oral treatment with green tea polyphenols prevented CIA in mice.[20,31] We therefore compared the inhibitory activity of CIA by green tea polyphenols with that of procyanidins. Surprisingly, our results demonstrate that green tea polyphenols tended to aggravate the disease. This result supports findings from our previous investigation into EAE. We have not yet established why there is a contradiction in these results, although one explanation may lie in differences between the components of the green tea polyphenols used. In our previous study, we evaluated EC as a monomeric polylphenol to prove that the degree of polymerization of procyanidins would be important for the inhibitory activity of EAE. However, almost all of the green tea polyphenol was a (−)-epigallocatechin-3-*O*-gallate (EGCG). In this present study, we used Polyphenon™ as the green tea polyphenols, which contained 58.4% EGCG, 9% (−)-epigallocatechin (EGC), 11% EC and other green tea polyphenols.[32,33] If the green tea polyphenol we used contained more EGCG and less EC, this may explain the discrepancies in our results.

We next examined the optimum time for the procyanidin administration. We set the period of administration from the 1st immunization through to the end of the study since we wanted to establish the effective treatment period for procyanidins. We conclude that "preventive 2nd" was the most effective time for administration and that administration during the acute phase was ineffective. Some autoimmune diseases repeat the cycles of remission and relapse, so treatment with procyanidins during the remission phase may prove useful in inhibiting a relapse.

In conclusion, we demonstrated that an oral treatment with highly oligomeric procyanidins ameliorated collagen-induced arthritis in a mouse model. Procyanidin has a variety of activities, *e.g.*, anti-oxidative, anti-inflammatory, tyrosinase inhibitory *etc.*, and there is a distinct possibility that effective inhibition of many autoimmune diseases may be achieved by a compound with such activities.

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