Highly Oligomeric Procyanidins Ameliorate Experimental Autoimmune Encephalomyelitis via Suppression of Th1 Immunity

Mika Miyake, Katsunori Sasaki, Kazuki Ide, Yasuko Matsukura, Kumiko Shijima and Daisuke Fujiwara

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Extracts of Jatoba, a South American herb, when injected i.p. into a mouse model of experimental autoimmune encephalomyelitis (EAE), inhibited the aggravation of clinical symptoms. At the same time, production of myelin oligodendrocyte glycoprotein Ag-specific IFN-γ and TNF-α by spleen cells was markedly suppressed. After administration of Jatoba there was minimal evidence of the demyelination that is characteristic of the EAE model. Decreases in clinical scores were observed when Jatoba extracts were injected just before Ag. The purified active compounds are likely to be polyphenols that are absorbable to polyvinylpolypyrrolidone. The active compounds were polymerized polyphenol polymers (procyanidins) and at least five degrees of polymerization were necessary for activity. In addition, extracts of other plant materials containing such procyanidins had similar activity. After administration of highly polymerized procyanidins, there was a decrease in both dendritic and CD4+ T cells. Although macrophages were increased in number, the expression of CD80 and MHC class II molecules was depressed indicating that the macrophages were immature. The results indicate that the suppression of development of EAE by the highly polymerized procyanidins resulted from an inhibition of Th1 and the effects might be associated with depression of Ag-presenting capability. The Journal of Immunology, 2006, 176: 5797–5804.

Multiple sclerosis (MS) is an intractable neurologic disease caused by attacks on the myelin sheath of nerve cells forming the CNS. Because the pathogenesis of MS results from excessive function of the immune system, treatment is primarily by a combination of IFN-β and immunosuppressants (1, 2). IFN-β inhibits gelatinase B and matrix metalloproteinase and is thought to improve the disease by inhibiting the migration of T cells to CNS (3–5). Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the CNS that is induced by immunizing laboratory rodents with myelin proteins or peptides emulsified in CFA. EAE is a useful model for MS (6). Immunization results in the induction of myelin-specific Th1 cells that home to the CNS, where they secrete inflammatory cytokines and chemokines, resulting in clinical paralysis and damage to the myelin sheath. There are many studies that support the clinical involvement of TNF-α in EAE. Elevated expression of TNF-α can be found in the CNS during acute episodes of disease. Blockage of either TNF-α or lymphotxin-α with neutralizing Abs and soluble receptors will ameliorate signs of EAE (7–11). Anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β that are produced by Th2, regulatory T, and Th3 cells, can also suppress the development of EAE (12, 13). Because G-CSF reduces the numbers of macrophages, suppresses production of TNF-α and IFN-γ, augments production of IL-4, and decreases the MIP-1α:MCP-1 ratio, improvement of the Th1/Th2 imbalance, reflected by both cytokines and chemokines, may lead to improvement of EAE (14). It has been suggested that the combination of the immunosuppressive drugs vitamin D3 and dexamethasone induced naïve CD4+ T cells to differentiate in vitro into IL-10-producing regulatory T cells (15). The disease is improved through inhibition of differentiation to effector cells. Significant sex differences have been documented with regard to the development of many autoimmune diseases (16, 17). Administration of estrogen improved Th1-mediated autoimmune diseases by increasing regulatory cells and inhibiting the production of TNF-α, a major inflammatory cytokine (18, 19).

All of the treatments for MS involve either augmentation or inhibition of both hormones and cytokines. Therefore, side effects must be considered. Furthermore, protein preparations, including Abs, are expensive and usually require admittance to hospital for injection. Such therapy presents serious problems from the point of view of quality of life. Low molecular mass substances such as statins, which inhibit the hydroxymethylglutaryl-CoA (HMG-CoA)-reducing enzyme, possessed EAE inhibitory effects (20). Although the direct relationship between HMG-CoA-reducing activity and the development of the disease is unclear, the inhibition of Th1 cytokines and enhancement of Th2 cytokines were observed in mice treated with statins. Improvement of the EAE might be related to regulation of Th1/Th2 balance by accelerating differentiation of Th2. Statins also have actions on APCs. They suppress Ag presentation by inhibiting expression of MHC class II, CD40, CD80, and CD86 antigenic markers of macrophage maturity. Thus, it is conceivable that statins exhibited inhibitory effects on the development of EAE through actions both on T and APCs.

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2 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin-3-O-gallate; ECG, (−)-epicatechin-3-O-gallate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LFB-PAS-HE, Luxol Fast Blue/periodic acid-Schiff/hematoxylin; PVPP, polyvinylpolypyrrolidone; EtOH, ethanol; BuOH, butanol; MeOH, methanol.
In recent years, studies of medicinal properties of extracts from plants used in traditional Chinese medicine have made progress. The fern *Polypodium leucotomos* has been used from ancient times by American Indians who empirically recognized that plants had anti-inflammatory actions. The extracts have been used in Spain for treatment of psoriasis, which is an autoimmune disease. Extracts of this fern inhibit Th1 and enhance Th2 in vitro (21).

Studies are also in progress regarding antioxidative 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 antioxidative actions (22). Antiarteriosclerotic, anti hypertensive, and anti tumor effects are well known. Although monomer polyphenols have such effects, highly polymerized polyphenols have stronger antioxidative, anti-mutagenic, and anti tumor activities (23–25). Polyphenols also affect the immune system. For example, the polyphenols derived from tobacco leaf inhibit IgE-mediated mast cell activation (26). Procyanidins derived from cacao and purified on the basis of degree of polymerization revealed inhibitory actions by hexa- to octamer forms on IL-4 production induced by PHA (27). Although (1–3)-mer exhibited enhancing effects on IL-5 production, (6–10)-mer showed inhibitory effects (28). Similarly, while (1–4)-mer exhibited inhibitory effects on the production of IL-1β stimulated by PHA in human PBMC, (5–10)-mer gave enhancing effects (29, 30). Therefore, the functions of procyanidins seem to differ with the degree of polymerization.

In the present study, we found that extracts of Jatoba, an herb that contains highly polymerized polyphenols, possesses EAE inhibitory effects. The active principles were identified as polyphenols. Furthermore, the activity was observed only with highly polymerized procyanidins and not the monomers. The inhibitory effect on disease was brought about through inhibition of enhanced Th1 cell activity that is characteristic of EAE.

**Materials and Methods**

**Mice**

Male C57BL/6 mice were purchased from Charles River Laboratories. All mice were 8–10 wk of age when used.

**Materials**

Herbs. Commercial dry powders of Cat’s claw (*Uncaria tomentosa*), Jatoba (*Hymenaea courbaril*), Jergon Sacha (*Hymenaea courbaril*), Mullaca (*Physalis angulata*), Samambaia (*Polypodium lepidopteris*), and Sidium (*Psidium guajava*) were purchased from Edison SRL. A total of 200 ml of ethanol (EtOH) was added to 10 g of each powder and stirred overnight. After filtration, ethanol extracts were obtained by evaporating the solvent. Each extract was dissolved in 1% EtOH and used as inoculum.

Procyanidins samples were commercial powder of Polyphenon (Tokyo Techo Food), Cacao (Meji Seika kaisha), Applephenon (Asahi Breweries), Gravinol (Kikkoman), Pycnogenol (Sozai Kinou Kenkyujo), Cranberry (Kikkoman), (−)-epicatechin (EC) (Sigma-Aldrich), and procyanidin B2 (Asahi Breweries). Powders were dissolved in 10% ethanol (EtOH). Because the commercial Cranberry powder contains a diluting agent and the amount of procyanidins in the raw material extract was low (data not shown), fractions containing procyanidins were prepared by dissolving 9 g of powder in 600 ml of water for application to a column (Φ 2.6 cm × 72 cm) filled with Sephadex LH-20 resin. After elution with 1 liter each of water and methanol (MeOH) at a speed of 5 ml/min, the column was eluted with 1 liter of 70% acetone/water. The column chromatography was repeated three times.

**Antigen**

Myelin oligodendrocyte glycoprotein 35–55 (MOG 35–55; MEVGYR SFPSPRWHLYRNGK) of murine origin was synthesized using solid phase techniques and was purified by HPLC at Qiagen.

**EAE induction**

Mice were immunized intradermally (i.d.) in the flanks with 200 μg of MOG 35–55 peptide emulsified in IFA (Difco Laboratories) supplemented with 8 mg/ml *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories). Mice were injected i.p. with 200 ng of pertussis toxin (Calbiochem) at days 0 and 3, and examined daily for clinical signs of disease. The clinical signs of EAE were documented according to the following scale: 0, no detectable signs of EAE; 1, complete limp tail; 2, impairment of righting reflex; 3, severe hind limb weakness; 4, complete bilateral hind limb paralysis; 5, complete bilateral hind limb paralysis and unilateral forelimb paralysis; 6, total paralysis and death.

**Jatoba treatment**

EtOH extracts of Jatoba at a stock concentration of 5 mg/ml were injected i.p. to give 1 mg/mouse. In experiments to determine dose dependence, concentrations of 2 and 5 mg/ml and 0.4 and 1 mg were administered i.p. to each mouse. As control, 200 μl of 1% EtOH was administered i.p. For both the measurement of Ag-specific cytokines and the FACS analysis, mice were injected i.p. 5 times from days 0 to 9 and sacrificed on day 11. To observe clinical scores, mice were injected every other day with Jatoba i.p. 10 times from days 0 to 21. In experiments to evaluate the effects of preventive administration, mice were injected with EtOH extracts of Jatoba i.p. 7 times from days 14 to −1, every other day. For histopathology, mice were injected i.p. every other day with EtOH extracts of Jatoba 7 times from days 0 to 14 and sacrificed on day 15.

**Cytokine measurement**

Spleen cells and lymph node cells from inguinal and axillary sites were prepared on day 11, which corresponded to onset of the disease. Five to 15 animals were used per group. Spleen cells were cultured with RPMI 1640 (Sigma-Aldrich) + 10% FCS (Roche) either with or without 2 μM MOG. The concentrations of IFN-γ were measured in supernatants after 1 wk and the concentrations of TNF-α were measured in supernatants after 10 days. TNF-α and IFN-γ levels were determined by using OptEIA ELISA Set (BD Biosciences) following the manufacturer’s protocol.

**Histopathology**

The intact spinal column was removed from mice at the peak of clinical disease on day 15 and fixed in 10% phosphate-buffered formalin. The spinal cords were dissected after fixation and embedded in paraffin before sectioning. The sections were stained with Luxol Fast Blue/periodic acid-Schiff/hematoxylin (LFB-PAS-HE) and examined by light microscopy.

**Butanol (BuOH)-HCl hydrolysis**

Previously described methods (31, 32) were used for the BuOH-HCl hydrolysis. BuOH/concentrated HCl (95:5, 3 ml) and NH₄Fe(SO₄)₂·12 H₂O in 2 M HCl (100 μl) were added to 0.5 ml of Jatoba extract in a tube. After agitation, the tube was heated at 95°C for 5 min. Optical density of the reaction medium was measured at 560 nm.

**Thiols analysis**

Previously described methods (31, 32) were used to perform the thiols analysis with reaction scale down. Each sample was dissolved in MeOH to a concentration of 4 mg/ml. Twenty-five milliliters of the solution was mixed with 25 ml of 3.3% (v/v) HCl in MeOH and 50 ml of 5% (v/v) solution of tolune-α-thiol in MeOH, stirred and heated at 40°C for 30 min. The resulting solution was analyzed directly by HPLC.

**Rapid fractionation**

Fractionation was conducted according to published methods (33). Briefly, Jatoba EtOH extract (60 g) was extracted with ethyl acetate, the water and the ethyl acetate layers were separated, and the water layer was dissolved in methanol. 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 were designated Fr1, 2, 3, and 4. The final supernatant was designated Fr5. The ethyl acetate layer was extracted using the C18 solid phase column (Spectro ENVI 18). The MeOH layer designated Fr6 and diethyl ether layer as Fr7.

**Surface marker and intracellular cytokine**

The expression of surface molecules on the spleen cells was analyzed by FACSort (BD Immunocytometry Systems). Cells were preincubated with CD16/CD32 (rat IgG2b, clone, 93) to block FcγRs. After washing, 5 × 10⁵ cells were stained with specific mAbs for 30 min at 4°C in 50 μl of PBS
containing 5% of FBS and 0.1% of sodium azide. We used FITC- or PE-labeled mAbs for staining of 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), CD40 (rat IgG2a, IC10), CD80 (B7-1) (Armenian hamster IgG, 16-10A1), CD86 (B7-2) (rat IgG2b, GL1), MHC class II (rat IgG2b, M5/114.15.2). FITC- or PE-labeled mouse, rat or Armenian hamster IgG Abs were used as isotype controls.

For measurement of cytokine protein by intracellular cytokine staining, cells were stimulated with 2 μM MOG and 10 μg/ml brefeldin A at 37°C for 4 h. The cells were stained for cell surface markers with PerCP-labeled CD3ε (Armenian hamster IgG 145-2C11) Abs and PE-labeled CD4 (rat IgG2b, GK 1.5) Abs. Cells were washed with staining buffer, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen). Cells were then resuspended in perm/wash buffer (BD Pharmingen) and stained with FITC-labeled Abs specific for IFN-γ (rat IgG1, XMG1.2) or TNF-α (rat IgG1, MP6-XT22) for 30 min at 4°C. All Abs were purchased from eBioscience. Data were analyzed with CellQuest software (BD Biosciences).

Statistical analysis

Differences between groups were analyzed by the Mann-Whitney U test and significant differences were p < 0.01.

Results

Effect of various herbal extracts on EAE

EtOH extracts of Cat’s claw, Jatoba, Jergon Sacha, Mullaca, Samambaia, and Sidium were prepared. Efficacy of the herbal extracts for modifying EAE was monitored by clinical scores and production of Ag-specific cytokines. Extracts from each herb were administered i.p. at a concentration of 1 mg/mouse 10 times from days 0 to 21 every other day starting with the administration of MOG Ag. Mice were examined daily for clinical symptoms and scores were recorded beginning on day 7 before the onset of disease. The strongest inhibitory activity for EAE was observed by injection of Jatoba extracts (Fig. 1). Disease developed on 16.5 days in the control group, but was retarded to day 21 in treated mice. The incidence of disease in the Jatoba control injected mice was 50% as compared with to 100% of the control group. The mean clinical score was 0.2 for the Jatoba group and 2.8 for controls, indicating a marked amelioration of symptoms (Table I).

Extracts of the other herbs had no effects on time of onset of disease (Table I). However, the clinical scores were reduced to 1.3 with Jergon Sacha and Sidium representing a severity of almost half of the control group (Fig. 1, Table I).

Dose dependency of EAE inhibitory effects by Jatoba EtOH extract

Disease onset, as compared with the control group, was retarded by 1 day in the group treated with 0.4 mg/mouse Jatoba and by 13 days in the group given 1 mg/mouse. The maximum scores of clinical symptoms were reduced to 1.9 in the group given 0.4 mg/mouse Jatoba and to 1.1 in the group given 1 mg/mouse Jatoba as compared with 2.6 in the control group. Although the scores were ~1.8 between days 29 and 33 in the control group, they were reduced to almost 0.1 in the mice treated with 0.4 mg and 1 mg of Jatoba. It was suggested that administration of Jatoba could induce remission of the disease (Fig. 2A). In the mice treated with 1 mg of Jatoba preventatively, the onset of disease was retarded by 22 days, indicating that when given before challenge the substance exerted strong EAE inhibitory effects (Table II). Some of the mice in the group were sacrificed on day 11 and spleen cells were prepared to examine the Ag specific production of IFN-γ and TNF-α. Production of 57 ng/ml IFN-γ was observed in the control group after Ag stimulation, whereas the production was reduced to 33 ng/ml with 0.4 mg of Jatoba and 5 ng/ml with 1 mg. Similarly, the Ag specific production of TNF-α was reduced to 117 pg/ml with 0.4 mg of Jatoba and 17 pg/ml with 1 mg, as compared with 176 pg/ml in the control group. Thus, the production of both IFN-γ and TNF-α was inhibited depending on the administered doses of EtOH extracts of Jatoba (Fig. 2B). Ag specific IFN-γ production in both spleen and lymph nodes was inhibited by administering Jatoba (Fig. 2C). We also examined whether Th cells produced these Ag specific cytokines and whether Jatoba inhibited cytokine production by Th cells. Th cells produced Ag specific IFN-γ and TNF-α in the EAE model and Ag-specific cytokine production by Th cells was inhibited by Jatoba (Fig. 2D).

To examine whether the demyelination that results from destruction of the myelin sheath by the autoimmune response can be inhibited by Jatoba, spinal cord was excised on day 15 and demyelination was evaluated using LFB-PAS-HE staining. Marked demyelination was observed in the control group, but almost no demyelination was detected in the group administered with Jatoba. This finding correlates with improvement of clinical symptoms and inhibition of Ag-specific responses of spleen cells after Jatoba treatment (Fig. 2E).

Preventive administration of Jatoba inhibited development of the disease (Fig. 2A). We have examined only the benefits of long-term administration of Jatoba when it was given at the same time that disease was induced, with 10 examinations occurring between days 0 and 21. Therefore, additional conditions for Jatoba administration were evaluated. One group of mice was injected with Jatoba four times from days 0 to 7 on every other day. A second group was injected with Jatoba four times from days 9 to 16 on every other day for 1 wk shortly before the expected time of onset of disease. The results show that short-term administration of Jatoba, even given close to the time of onset of clinical disease, is as effective as long term prior administration (Fig. 2F). Jatoba appears to be effective for inhibiting both the induction and effector phases of disease.
Active components in Jatoba are polyphenols

Because Jatoba extracts were colored with the HCl-BuOH determination method, it was apparent that polymers of polyphenols, (proanthocyanidins) were present (data not shown). So Jatoba extracts were treated with PVPP for efficient extraction of polyphenols. Treatment with PVPP reduced the weight of Jatoba extracts by 87% (data not shown), suggesting that the extracts were rich in polyphenols, that could be adsorbed to PVPP. We tested whether Jatoba extracts treated with PVPP (PVPP-Jatoba) could suppress production of Ag-specific cytokines. The marked inhibition of IFN-γ and TNF-α observed after administration of Jatoba was no longer seen in the mice treated with PVPP-Jatoba group, since the activities were 109 and 83%, respectively, of those observed in the control group (Fig. 3, A and B). These findings suggest that the active components in Jatoba extracts were polyphenols.

The only Amazon herbs for which the procyanidins content and degree of polymerization could be determined were Jatoba and Cat’s claw. The other herbs were under the limits of detection (Table III).

Jatoba were administered seven times during the period of days −14 to −1. Administration was postponed after induction of EAE on day 0. Mean scores are plotted. Each group consisted of five animals. ▲, The time of i.p. administration of Jatoba. B, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. 5 times during the period of days 0–9 at doses of 0, 0.4, 1 mg/mouse. Spleen cells were prepared from mice on day 11 and production by spleen cells of Ag-specific cytokines was studied. Each group consisted of five animals. Means and SE of concentrations of IFN-γ and TNF-α produced are plotted. Significant differences between untreated and treated mice were determined by Mann-Whitney U test (▲, p < 0.01). C, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. five times during the period of days 0–9 at doses of 0, 1 mg/mouse. Inguinal and axillary lymph node cells were prepared from mice on day 11 and production by lymphocytes of Ag-specific cytokines was studied. Each group consisted of six animals. Means and SE of concentrations of IFN-γ and TNF-α produced are plotted. Significant differences between untreated and treated mice were determined by Mann-Whitney U test (▲, p < 0.01). D, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. five times during the period of days 0–9 at doses of 0, 0.4, 1 mg/mouse. Spleen cells were prepared from mice on day 11 and intracellular cytokine production by Th cells of Ag-specific cytokines was studied. Data show the representative cases of each group. ▲, Simultaneously with induction of EAE, EtOH Jatoba extracts were administered i.p. seven times during the period of days 0–14 at doses of 0 and 1 mg/mouse. The spinal cord was dissected out from mice on day 15. Slices stained with LFB-PAS-HE are shown. Data show the representative cases of each group. ▲, EtOH Jatoba extracts were administered i.p. 10 times during the period of days 0–21 at a dose of 1 mg/mouse. Control mice were sham injected. In the group receiving short-term administration, EtOH extracts of Jatoba were administered four times during the period of days 0–7 and days 9–16. Mean scores are plotted. Each group consisted of six animals. ▲, The time of i.p. administration of Jatoba.

FIGURE 2. Dose-dependent inhibition of EAE by EtOH extracts of Jatoba. A, At the same time as induction of EAE, EtOH Jatoba extracts were administered i.p. 10 times during the period of days 0–21 at doses of 0, 0.4, 1 mg/mouse. In groups for preventive administration, EtOH extracts of Jatoba were administered seven times during the period of days −14 to −1. Administration was postponed after induction of EAE on day 0. Mean scores are plotted. Each group consisted of five animals. ▲, The time of i.p. administration of Jatoba. B, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. 5 times during the period of days 0–9 at doses of 0, 0.4, 1 mg/mouse. Spleen cells were prepared from mice on day 11 and production by spleen cells of Ag-specific cytokines was studied. Each group consisted of five animals. Means and SE of concentrations of IFN-γ and TNF-α produced are plotted. Significant differences between untreated and treated mice were determined by Mann-Whitney U test (▲, p < 0.01). C, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. five times during the period of days 0–9 at doses of 0, 1 mg/mouse. Inguinal and axillary lymph node cells were prepared from mice on day 11 and production by lymphocytes of Ag-specific cytokines was studied. Each group consisted of six animals. Means and SE of concentrations of IFN-γ and TNF-α produced are plotted. Significant differences between untreated and treated mice were determined by Mann-Whitney U test (▲, p < 0.01). D, Simultaneously with the induction of EAE, EtOH Jatoba extracts were administered i.p. five times during the period of days 0–9 at doses of 0, 0.4, 1 mg/mouse. Spleen cells were prepared from mice on day 11 and intracellular cytokine production by Th cells of Ag-specific cytokines was studied. Data show the representative cases of each group. ▲, Simultaneously with induction of EAE, EtOH Jatoba extracts were administered i.p. seven times during the period of days 0–14 at doses of 0 and 1 mg/mouse. The spinal cord was dissected out from mice on day 15. Slices stained with LFB-PAS-HE are shown. Data show the representative cases of each group. ▲, EtOH Jatoba extracts were administered i.p. 10 times during the period of days 0–21 at a dose of 1 mg/mouse. Control mice were sham injected. In the group receiving short-term administration, EtOH extracts of Jatoba were administered four times during the period of days 0–7 and days 9–16. Mean scores are plotted. Each group consisted of six animals. ▲, The time of i.p. administration of Jatoba.

Table II. Dose dependency of Jatoba extracts on the suppression of EAE

<table>
<thead>
<tr>
<th>Milligrams</th>
<th>Mean Day of Onset ± SEM</th>
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<tr>
<td>0</td>
<td>11.6 ± 0.5</td>
</tr>
<tr>
<td>0.4</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>24.5 ± 4.7</td>
</tr>
<tr>
<td>1 (preventive)</td>
<td>34 ± 5.7</td>
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</table>

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symptoms and Ag-specific production of IFN-γ were not inhibited after administration of Polyphenon (Fig. 3, C and D). Clinical symptoms were somewhat, but not significantly, aggravated in mice treated with Polyphenon (p = 0.07) when the clinical scores on day 30 were compared with those of the control. These findings suggest that EAE inhibitory effect was observed only with polymer procyanidins, and not the monomer polyphenols.

**Fractionation of procyanidins to determine the structure necessary for EAE inhibition**

The goal of the following experiments was to examine the correlation between the degree of polymerization of procyanidins and the EAE inhibitory activity. Using a rapid fractionation method, procyanidins can be approximately fractionated on the basis of degree of polymerization using a rapid fractionation method (31). The fractions of Jatoba extract (Fr1-Fr7, Table IV), each equivalent to 1 mg of procyanidins, were administered i.p. and EAE inhibitory activity was evaluated using the Ag-specific production of IFN-γ of spleen cells. In fractions 7, as well as Fr6 and Fr5, with mean degree of polymerization as low as 3.2 and 5, there was little inhibitory activity. However, definite inhibition of IFN-γ was observed beginning with Fr4, which contained procyanidins with a mean degree of polymerization >8.3. Therefore a degree of polymerization higher than a certain value is indispensable for procyanidins to exhibit EAE inhibitory activity (Fig. 4A).

To determine the exact degree of polymerization required for inhibitory activity, the purified samples with each degree of polymerization were tested. Trimer to pentamer polymers were prepared from Fr5 and hexamer from Fr4. EC was used as monomer and procyanidin B2 was used as dimer. 1 mg of the mono- to hexamers (1–6 mers) was administered i.p. and significant inhibition of Ag-specific IFN-γ production was observed beginning with the pentamer (Fig. 4B).

**Precise examination of active compounds by comparison with other herbal extracts**

Constituent components and mode of linkage of procyanidins present in nature are quite varied. To examine whether the constituent components and the mode of linkage play a role in EAE inhibitory activity by Jatoba extracts, other samples rich in procyanidins were studied for inhibitory effects. Polyphenon was the negative control and samples for comparison were procyanidins extracted from Cacao, and Applephenon (apple polyphenol), Gravinol (grape seed), Pycnogenol, and Cranberry. The constituent components of monomer polyphenols contained in each sample,

<table>
<thead>
<tr>
<th>Table IV. Mean degree of polymerization of each fraction</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>mDP</td>
</tr>
</tbody>
</table>

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

| Contents (%)         | 18.8 | 71.6 | nd   | nd   | nd   | nd   | nd   |

**Active components in Jatoba are procyanidins**

Polyphenols are present widely in nature. Anti-oxidative activity of CA, EC, (−)-epigallocatechin-3-O-gallate (EGCG), and (−)-epicatechin-3-O-gallate (ECG), monomer polyphenols contained in green tea, has been studied extensively (22). Polyphenol, like green tea, contains monomer polyphenols EGCG. The clinical
The mechanisms of EAE inhibition by highly polymerized procyanidins are unknown. To better understand how the spleen cells involved in immunity were influenced by administration of highly polymerized procyanidins, the populations of spleen cells were analyzed by flow cytometry on day 11 postinduction of EAE. Both CD4\(^+\) T cell and DC decreased significantly in mice treated with Jatoba, while there was a tendency (\(p = 0.099\)) for macrophages to increase (Fig. 6A). The expression of CD80 and MHC class II that is a cell surface marker of macrophage maturity was found to be significantly reduced in the Jatoba-treated group as compared with the controls, indicating that the increased macrophages were immature phenotypes (Fig. 6, B and C).

**Table V. Mean degree of polymerization, component, and unit linkage of each sample**

<table>
<thead>
<tr>
<th>Natural Substances</th>
<th>mDP</th>
<th>Procyanidins (%)</th>
<th>Components</th>
<th>Unit Linkage</th>
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<tbody>
<tr>
<td>Jatoba</td>
<td>8.2</td>
<td>71.6</td>
<td>EC</td>
<td>B type</td>
</tr>
<tr>
<td>Polyphenon</td>
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<td>31.8</td>
<td>CA, EGC, EC, ECG</td>
<td>B type</td>
</tr>
<tr>
<td>Cacao</td>
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<td>53.8</td>
<td>CA, EC</td>
<td>B type</td>
</tr>
<tr>
<td>ApplePhenon</td>
<td>3.3</td>
<td>51.2</td>
<td>CA, EC</td>
<td>B type</td>
</tr>
<tr>
<td>Gravinol</td>
<td>3.7</td>
<td>59.1</td>
<td>CA, EC ECG</td>
<td>B type</td>
</tr>
<tr>
<td>Pycnogenol</td>
<td>5.2</td>
<td>45.7</td>
<td>CA, EC</td>
<td>B type</td>
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<tr>
<td>Cranberry-LH20</td>
<td>8.8</td>
<td>67.8</td>
<td>CA, EC ECG</td>
<td>A type, B type</td>
</tr>
</tbody>
</table>

\(^a\) mDP, Mean degree of polymerization; LH20, purified using LH20.

**FIGURE 5.** Precise examination of active compounds by comparison with other herbal extracts. EtOH extracts of Jatoba, Polyphenon, Cacao, Applephenon, Gravinol, Pycnogenol, and Cranberry-LH20 were administered 10 times i.p. during the period of days 0–20 each at a dose corresponding to 1 mg/mouse procyanidins to mice in which EAE was induced. Clinical scores were recorded until day 25. Each group consisted of five animals. Means of scores are plotted.

The main active components of Jatoba were highly polymerized procyanidins. However, administration of polyphenols with strong antioxidative actions, such as polyphenols of green tea, tended to aggravate the disease. Therefore, antioxidative actions did not contribute to EAE inhibitory activity produced by highly polymerized procyanidins. It appeared that procyanidins with the degree of polymerization over five represented the main active component. As highly polymerized procyanidins exhibit a high hydrophilicity, action could be on the cell surface to influence cell-cell interaction rather than on the cytosol. A receptor through C4→C8 and/or C4→C6 bonds (B-type). These flavanyl-3-ol units can be doubly linked C4→C8 bond and an additional ether bond between O7→C2 (A-type) (34). Because extracts from some plants like Cranberry that contained A-type links (Table V) had inhibitory activity, the mode of linkage was not determining for activity. The degree of polymerization was a necessary and sufficient condition for EAE inhibitory activity of highly polymerized procyanidins (Fig. 5).
specific for EGCG, a monomer polyphenol, a 67-kDa laminin receptor has been found (35). It suggests that the presence of receptor for each polyphenol. It is possible that receptors exist that recognize substances like highly polymerized procyanidins. The inhibition of Th1 cytokine production was found in polymers larger than pentamer. Furthermore, there were no specific requirements for the components of procyanidins and patterns of catechin.

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DISCLOSURES

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REFERENCES