Effects of the Aqueous Extract of Epimedii Herba on the Induction of Oral Tolerance in Mice

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We investigated the effects of the aqueous extract of Epimedii Herba (AEEH) on the induction of oral tolerance. Oral tolerance was induced in mice by giving an oral administration of 20 mg ovalbumin (OVA) 7 d before immunization with the antigen. AEEH at 40 mg/kg was given orally daily for 6 d from 24 h after the feeding of OVA. The results showed that oral administration of OVA greatly suppressed total serum and antigen-specific immunoglobulin (Ig) levels, phagocytic activity and delayed-type hypersensitivity (DTH) reaction to the antigen. The suppression of these immune responses to OVA by the oral antigen was associated with a marked reduction of the production of interferon-γ (IFN-γ) and interleukin-4 (IL-4) from spleen cells. However, AEEH treatment significantly blocked the suppression of total serum and antigen-specific IgG2a antibodies, phagocytic activity and DTH response by the oral OVA. The suppression of IFN-γ production by the oral antigen was also greatly decreased by AEEH treatment. Therefore, AEEH appears to be effective in preventing the induction of oral tolerance to OVA.

Key words oral tolerance; ovalbumin; mouse; Epimedii Herba

It has long been recognized that oral administration of an antigen induces immunological unresponsiveness to the antigen, this is termed oral tolerance.[1,2] Induction of oral tolerance is proposed to be an immunotherapy of allergic and autoimmune diseases.[3—5] The physiological significance of oral tolerance is not yet clear, but possibilities include anergy and deletion of antigen-specific lymphocytes[6,7] and suppression by inhibitory cytokines including transforming growth factor-β and interleukin-4 (IL-4) secreted from regulatory T cells.[8,9] For instance, Garside *et al.*[10] showed that feeding of 25 mg ovalbumin (OVA) reduced production of Th2 cytokines including IL-4 as well as the Th1 cytokine IFN-γ. Similar results were observed by Yoshino and Ohsawa.[11] Melamed *et al.*[12] also found that continuous feeding of OVA decreased IL-4 secretion. In particular, it was demonstrated that feeding of antigen at high doses favored anergy in mice.[13] These findings suggest that high doses of oral antigen may suppress the secretion of various cytokines. Modulation of oral tolerance by drugs and chemicals is believed to promote allergic responses *via* enhanced production of antibodies to the antigen in inhibitory modulation, or to be available for immunotherapy of allergy in stimulatory modulation. In a previous study, we showed that cyclophosphamide (CP) abrogates oral tolerance to OVA in mice.[14] CP also can enhance delayed-type hypersensitivity responses through the elimination of suppressor T cells in BALB/c mice by oral OVA[15,16]; it thus could be a modulator of oral tolerance. Nevertheless, the use of CP as a modulator of the oral tolerance *in vivo* has a disadvantage since CP is a potent pharmacological agent with a wide range of actions, including a potential for damaging the intestine.[17] Therefore, it would be important to modulate an animal model of oral tolerance using agents with a more physiological action, with little or no adverse side-effects to promote immune responses *via* enhanced Th cell functions in inhibitory modulation.

Epimedii Herba is the dry aboveground part of *Epimedium koreanum* NAKAI (Berberidaceae) collected in summer and fall when the plant is mature. It is an important traditional Chinese herbal medicine widely used as a tonic and in the treatment of rheumatic.[18,19] The main constituent in *Epimedium* species is icariin, a glycoside of prenylflavones.[20—23] The icariin decreased the production of T suppressor cells and antibody titer was thus greatly elevated.[24] It has been shown that a polysaccharide with a molecular weight of about 75000 isolated from *E. koreanum* may be used as an immune adjuvant.[25] The methanol extract of *E. sagittatum* also significantly augmented the leukocyte count and lymphocyte transformation rate of patients with vital energy deficiency,[26] and further promoted phagocytic activity of the reticuloendothelial system in mice and humans.[27,28] We demonstrated that the fractions of *E. koreanum* have immunostimulating effects in mice.[29] In particular, it was shown that 40 mg/kg aqueous extract of Epimedii Herba (AEEH) at an effective dose significantly enhanced the production of cytokines and antibodies in mice.[30] In view of the above reports, it is speculated that AEEH may modulate oral tolerance without causing any side effects, although little is known about these effects on oral tolerance.

The purpose of the present study, therefore, was to determine the effects of AEEH on the induction of oral tolerance to OVA in mice.

MATERIALS AND METHODS

Animals Male BALB/c mice were purchased from Dae Han Animal Center (Chungbuk, Korea). Rodent laboratory chow (Cheil Chedang, Korea) and tap water were provided *ad libitum*. Mice were used for experiments at 6 weeks of age after acclimatization for 7 d.

Sample and Preparation of Extract Epimedii Herba was standardized in the Korean Pharmacopoeia (KP). AEEH was prepared according to the method of Ono *et al.*[31] Briefly, the dried leaves of *Epimedium koreanum* NAKAI (400 g, Hanjung Pharmaceutical Co., Korea) were extracted with dis-

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tilled water (6500 ml) at 100 °C for 3 h. After filtration and centrifugation (1700×***g***, 30 min), the solution was concentrated at 40 °C with rotary evaporators under reduced pressure. The precipitate was collected and freeze-dried to give AEEH. The dried extract (42.8 g; yield 10.7%) was obtained as a yellowish brown powder after lyophilization, and then dissolved or suspended in distilled water (pH 7.8) and used for the actual experiment at suitable concentration.

Antigen OVA (chicken albumin, grade V) purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). OVA was dissolved in sterile distilled water (20 mg/ml) for oral administration to mice or in complete Freund's adjuvant (CFA) (1 mg/ml) for the immunization.

Induction of Oral Tolerance to OVA and Chemical Treatment To induce oral tolerance, mice were given an oral administration of 20 mg OVA in 1 ml of sterile distilled water. Control animals were given a corresponding volume of sterile distilled water. Seven days later they were immunized with an i.p. injection of 0.1 mg OVA in CFA. On day 7 after the immunization, blood samples for antibody determinations were collected by heart puncture. After centrifuging the samples, sera were collected and stored at −70 °C until assay of antibody levels. For modulation of the tolerance, AEEH was tested using the most potent dose and treatment conditions in a previous report on AEEH to enhance immune response.[30] AEEH was orally given six times at a daily dose of 40 mg/kg in distilled water 24 h after the feeding of OVA in order to avoid affecting OVA uptake in the gut. Control mice were given the corresponding volume of distilled water.

Measurement of Delayed-Type Hypersensitivity On day 7 after immunization, 10 μg of OVA dissolved in 20 μl of phosphate-buffered saline (PBS) was injected *s.c.* into the right footpad. As a vehicle control, 20 μl of PBS was injected *s.c.* into the left footpad. The thickness of the right and left footpads was measured using dial gauge calipers calibrated with 0.01 mm graduations (Mitutoyo Mfg. Co., Ltd., Japan) immediately before and 24 h after the challenge injection. The increase in left footpad thickness was subtracted from the increase in right footpad thickness to give the value due to the specific response to the antigen. In unsensitized mice, responses to OVA and PBS were essentially equivalent.

Measurement of Total Serum IgG, IgG1, IgG2a and IgM Antibodies Serum samples were analyzed by an enzyme-linked immunosorbent assay (ELISA).[32] Briefly, 96-well microtiter plates were coated overnight at 4 °C with 100 μl/well of primary antibody appropriately diluted in PBS. As a primary antibody we used affinity-puried goat $F(ab)_2$ anti-mouse IgG (Caltag, Burlingame, CA, U.S.A.), IgG1, IgG2a, or IgM (PharMingen, San Diego, CA, U.S.A.) and incubated overnight at 4 °C. The wells were then washed three times with PBS containing 0.05% Tween (PBS-Tween) and blocked with 1% bovine serum albumin (BSA)/PBS at room temperature for 2 h. This buffer solution was also used as a diluent in all subsequent steps of the ELISAs. After three washings of the blocked wells with PBS-Tween, 100 μl of appropriately diluted serum sample was added to duplicate wells. As a standard serum we used 10 serial two-fold dilutions of a pooled mouse serum standard containing known concentrations of IgG (Pierce, Rockford, IL, U.S.A), IgG1, IgG2a, or IgM (PharMingen), added to duplicate wells (100 μl/well). The plates were then incubated at room temperature for 1 h before washing, as described above. Aliquots of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Caltag), IgG1, IgG2a (PharMingen), or IgM (Caltag) diluted with 1% BSA/PBS were added to each plate. The plates were further incubated for 1 h at room temperature. After washing, peroxidase activities were assayed as follows: 100 μl of substrate solution (10 mg of *o*-phenylenediamine and 8 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate-phosphate buffer, pH 5) was added to each well of the plate. The plates were incubated for 15 min at room temperature, and enzyme reaction terminated by adding 50 μl/well of 1 N H_2SO_4. Optical density (OD) at 490 nm of each well was then measured with a microplate spectrophotometer (Sunnyvale, CA, U.S.A.). OD values of blanks containing no serum were subtracted from those of standards and unknowns. The concentrations of the various samples were obtained by interpolation on standard curves. The final concentration in each sample was calculated as the mean of the results of duplicate cultures.

Measurement of Anti-OVA IgG, IgG1, IgG2a and IgM Antibodies Anti-OVA IgG, IgG1, IgG2a and IgM antibodies were measured with ELISA.[32] In brief, 96-well microtiter plates were coated with 100 μl/well of OVA (100 μg/ml) dissolved in PBS and incubated at 4 °C for 2 h. The wells were then washed three times with PBS-Tween and blocked with 1% BSA/PBS at room temperature for 2 h. This buffer solution was also used as a diluent in all subsequent steps of the ELISAs. After three washings of the blocked wells with PBS-Tween, the serum samples were diluted with 1% BSA/PBS at 1/40 for IgG and IgG2a measurements and 1/10 for IgG1 and IgM measurements, in order to obtain an OD reading on a linear curve from the serial dilutions. One hundred μl of each sample was added to the plate well and incubated at room temperature for 1 h before washing again. Aliquots of 100 μl of HRP-conjugated goat anti-mouse IgG (Caltag), IgG1, IgG2a (PharMingen), or IgM (Caltag) diluted with BSA/PBS were added to each plate. The plates were further incubated for 1 h at room temperature. After washing, peroxidase activities were assayed as follows: 100 μl of substrate solution (10 mg of *o*-phenylenediamine and 8 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate–phosphate buffer, pH 5) was added to each well of the plate. The plates were incubated for 15 min at room temperature, and enzyme reaction terminated by adding 50 μl/well of 1 N H_2SO_4. OD at 490 nm of each well was then measured with a microplate spectrophotometer. OD values of blanks containing no serum were subtracted from those of samples. Serum levels of each anti-OVA Ig subclass were represented as mean OD values at 490 nm of duplicate determinations. In this assay, sera obtained from unprimed mice gave OD readings comparable to blanks.

Assay of Phagocytic Activity Phagocytic activity was determined by the modified method of Biozzi *et al.*[33] In brief, phagocyte activity was determined on day 7 after the immunization. For the preparation of colloidal carbon solution, rotring ink® was diluted 1/6 with 1% gelatin and kept in a stopple tube at 37 °C during the experiment. To measure the phagocytic activity, separate groups of mice were challenged *via* the lateral tail vein using a 1 ml syringe with 26 gauze needle at the dose of 0.01 ml of colloidal carbon solution per gram of mouse. At the interval of 10, 20 or 30 min,

20 μl of blood sample was obtained from the retro-orbital venous plexus. The collected blood samples were expelled into individual vials containing 2 ml of 0.1% sodium carbonate, and the contents were well mixed for the lysis of erythrocytes. The absorbance of the colloidal carbon contained in blood was measured with a spectrophotometer (Varian, Cary 219) at 600 nm using water as blank. Ten times of density readings were converted into a logarithmic scale and plotted against time. The slope of the line was called phagocytic coefficient K. Mice were killed, and the weights of spleen and liver were measured. The corrected phagocytic index is a measure of phagocytic activity per unit weight of tissue.

$$\text{corrected phagocytic index} = [\text{body wt.}/(\text{spleen wt.} + \text{liver wt.})] \times \sqrt[3]{K}$$

Cytokine Measurement Mice were killed 7 d after immunization and the separated splenocytes were prepared and resuspended at a final concentration of 5×10^6 cells/ml and cultured for 72 h in RPMI 1640 media mixed with activating mitogen of concanavalin A (Con A) at 5 μg/ml. Supernatants were harvested after 48 h. Secretion of IFN-γ and IL-4 was quantified using sandwich ELISA techniques. In brief, 96-well microtiter plates were coated overnight at 4 °C with 100 μl/well of 0.1 M phosphate buffer containing antibodies (4 μg/ml) against IFN-γ and IL-4 (PharMingen). The plates were blocked by incubation with 150 μl/well of 1% BSA/PBS at 37 °C for 1 h. After blocking, the plates were washed three times, and samples or standards (recombinant mouse IFN-γ and IL-4; PharMingen) were added to each well in a volume of 100 μl and incubated at 37 °C for 1 h. The plates were washed three times and 100 μl/well of biotinylated anti-mouse IFN-γ and IL-4 (2 μg/ml) antibodies (PharMingen) diluted in 1% BSA/PBS was added. After incubation at 37 °C for 1 h, the plates were washed three times, and 100 μl/well of streptavidin–alkaline phosphatase (PharMingen) was added at 2 μg/ml. The plates were washed before 100 μl of *p*-nitrophenylphosphate was added to each well, then read at 405 nm using a microplate spectrophotometer. Cytokine levels were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines, and results are expressed in pg/ml.

Statistical Analysis Results are expressed as the mean and standard error (S.E.). Statistical significance of the differences among groups was examined at a 5% level of significance by ANOVA analysis.

RESULTS

Effect of AEEH on DTH Responses to OVA in Orally Tolerant Mice To investigate whether AEEH modulates cell-mediated immune responses in orally tolerant animals, mice were fed OVA before immunization with the antigen. As shown in Fig. 1, feeding of OVA was observed by marked suppression of DTH response to OVA. Oral administration of AEEH and OVA blocked the suppression of DTH response by the oral antigen. This finding indicates that AEEH has a modulating effect against oral antigen on footpad DTH response, associated with cell-mediated immune response.[15,16]

Effect of AEEH on Humoral Immune Responses to OVA in Orally Tolerant Mice The effect of AEEH on humoral immune responses to OVA was investigated by measuring levels of total serum and antigen-specific Ig antibodies

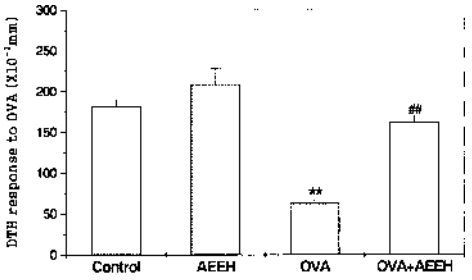

Fig. 1. Effects of the Aqueous Extract of Epimedii Herba on the Delayed-Type Hypersensitivity Response to Ovalbumin by the Oral Antigen

All groups of mice were immunized with an i.p. injection of 0.1 mg ovalbumin (OVA) in CFA 7 d after 20 mg OVA feeding. Seven days before the immunization, all groups were orally given distilled water alone (control) or OVA alone (OVA), or AEEH for 6 consecutive days (40 mg/kg/d) from 24 h after an oral OVA (OVA+AEEH) or AEEH alone for 6 d without the oral OVA (AEEH). Footpad delayed-type hypersensitivity (DTH) responses to OVA were tested on day 14 as described in Materials and Methods. Each column represents the mean±S.E. of 5 to 6 mice. Significantly different from control at ** $p<0.01$. Significantly different between OVA and OVA plus AEEH groups at ## $p<0.01$.

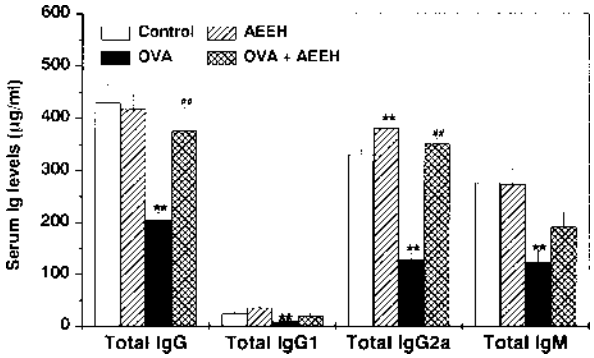

Fig. 2. Effects of the Aqueous Extract of Epimedii Herba on the Total Serum Ig Levels in ICR Mice While Inducing Oral Tolerance to OVA

All groups of mice were immunized with OVA as described in Fig. 1. Seven days before the immunization, all groups were orally given distilled water alone (control: □) or OVA alone (OVA: ■), or AEEH for 6 consecutive days (40 mg/kg/d) from 24 h after an oral OVA (OVA+AEEH: ▨) or AEEH alone for 6 d without the oral OVA (AEEH: ▨). Serum antibodies were measured using ELISA. Results represent the mean± S.E. of duplicate samples from 5 to 6 mice. Significantly different from control at ** $p<0.01$. Significantly different between OVA and OVA plus AEEH groups at ## $p<0.01$.

in mice receiving oral OVA and AEEH prior to the antigen immunization. Figures 2 and 3 show the effects of AEEH on total serum and anti-OVA IgG levels in orally tolerant mice. Oral administration of OVA markedly decreased levels of both total serum and antigen-specific IgG antibodies. As shown in the OVA+AEEH group, mice receiving oral AEEH after feeding of OVA had significantly higher levels of total serum and anti-OVA IgG than tolerant mice (OVA group). These findings indicate that AEEH modulates IgG antibody production tolerant by OVA feeding.

Concerning IgG subclasses, anti-OVA IgG2a and IgG1 antibodies as well as total serum IgG2a and IgG1 antibodies were further measured to investigate the effects of AEEH and OVA given orally on Th1 and Th2 cell responses, respectively.[34,35] The data are shown in Figs. 2 and 3. Markedly reduced production of total serum and anti-OVA IgG2a antibodies was observed in mice fed OVA. Oral administration of AEEH and OVA greatly blocked the suppression of total serum and anti-OVA IgG2a antibody production by the oral antigen. Oral administration of OVA markedly reduced the production of total serum and anti-OVA IgG1 antibody by

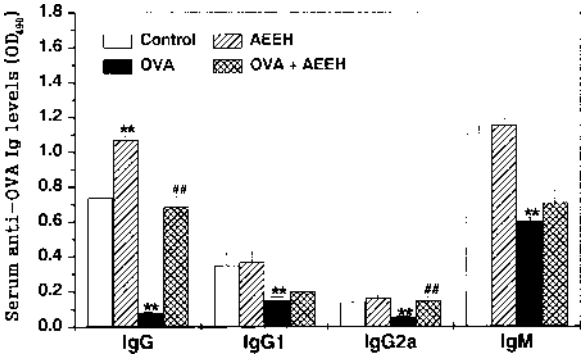

Fig. 3. Effects of the Aqueous Extract of Epimedii Herba on the Antigen-Specific Antibody Levels in ICR Mice While Inducing Oral Tolerance to OVA

All groups of mice were immunized with OVA as described in Fig. 1. Seven days before the immunization, all groups were orally given distilled water alone (control: □) or OVA alone (OVA: ■), or AEEH for 6 consecutive days (40 mg/kg/d) from 24 h after an oral OVA (OVA+AEEH: ⊠) or AEEH alone for 6 d without the oral OVA (AEEH: ▨). Serum anti-OVA Ig levels were measured using ELISA. Each column represents the mean±S.E. of duplicate samples from 5 to 6 mice. Significantly different from control at ** $p<0.01$. Significantly different between OVA and OVA plus AEEH groups at ## $p<0.01$.

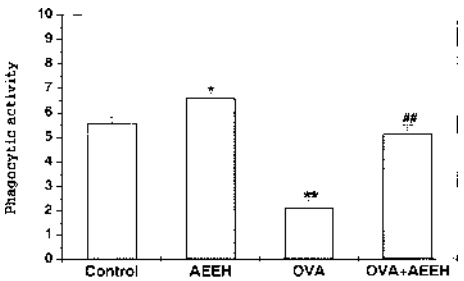

Fig. 4. Effects of the Aqueous Extract of Epimedii Herba on the Phagocytic Activity in Orally Tolerant Mice

All groups of mice were immunized with OVA as described in Fig. 1. Seven days before the immunization, all groups were orally given distilled water alone (control) or OVA alone (OVA), or AEEH for 6 consecutive days (40 mg/kg/d) from 24 h after an oral OVA (OVA+AEEH) or AEEH alone for 6 d without the oral OVA (AEEH). Phagocytic activity was tested on day 14 as described in Materials and Methods. Results represent the mean±S.E. of 5 to 6 mice. Significantly different from control at * $p<0.05$; ** $p<0.01$. Significantly different between OVA and OVA plus AEEH groups at ## $p<0.01$.

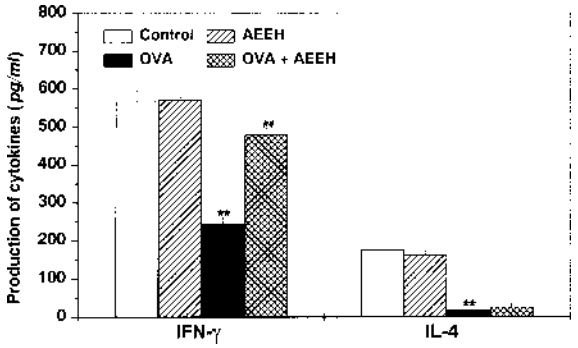

Fig. 5. Effects of the Aqueous Extract of Epimedii Herba on the Production of Interferon-γ and Interleukin-4 in Orally Tolerant Mice

All groups of mice were immunized with OVA as described in Fig. 1. Seven days before the immunization, all groups were orally given distilled water alone (control: □) or OVA alone (OVA: ■), or AEEH for 6 consecutive days (40 mg/kg/d) from 24 h after an oral OVA (OVA+AEEH: ⊠) or AEEH alone for 6 d without the oral OVA (AEEH: ▨). IFN-γ and IL-4 contents were measured by sandwich ELISA as described in Materials and Methods. Results represent the mean±S.E. of duplicate samples from 5 to 6 mice. Significantly different from control at ** $p<0.01$. Significantly different between OVA and OVA plus AEEH groups at ## $p<0.01$.

the oral OVA. However, AEEH combined with OVA did not significantly inhibit the suppression of total serum or anti-OVA IgG1 antibody production by the oral antigen; this was associated with significant recovery of IgG2a but not IgG1 levels.

Similar to IgG1 levels, total serum and anti-OVA IgM antibodies were strongly reduced in mice fed the antigen. However, AEEH combined with OVA did not significantly affect total serum or anti-OVA IgM antibody in the tolerant mice, but seemingly had a tendency toward slight induction of total serum and anti-OVA IgG1 levels. The above results thus indicate that AEEH has a restorative effect against oral antigen on humoral immune response such as total serum and anti-OVA IgG2a levels.

Effect of AEEH on Phagocytic Activity in Orally Tolerant Mice The effect of AEEH on phagocytic activity in orally tolerant mice was examined. When compared with controls, OVA feeding resulted in marked suppression of this activity (62.1% suppression), as shown in Fig. 4. However, AEEH combined with OVA significantly reduced the suppression of phagocytic activity by the oral antigen. Suppression rate of the activity in mice given OVA with AEEH was 8%.

Effect of AEEH on the Production of IFN-γ and IL-4 in Orally Tolerant Mice IFN-γ and IL-4 are known as Th1 and Th2 cytokines, respectively.[36,37] We also examined whether the blockade by AEEH of suppression of immune responses to OVA in orally tolerant mice was associated with Th1 and Th2 type of $CD4^+$ T cell responses. OVA feeding markedly suppressed IFN-γ (down to 60% of control value) as shown in Fig. 5. Strong suppression of IL-4 production was also observed in OVA-fed animals (down to 90% of control value). AEEH combined with OVA significantly diminished the suppression of IFN-γ production by oral OVA, but it failed to modulate the reduction in IL-4 secretion by the oral antigen. These findings indicate that AEEH prevented Th1 function suppressed by OVA.

DISCUSSION

In general, oral administration of an antigen induces a state of unresponsiveness in cellular and humoral immune responses to the antigen, termed oral tolerance.[1,4,38] Modulation of oral tolerance by drugs and chemicals can promote immune responses *via* enhanced production of antibodies to the antigen in inhibitory modulation. Thus, the purpose of the present study was to determine the modulating effects of AEEH on oral tolerance to OVA.

Several experimental schedules have been described for the induction of oral tolerance to soluble protein antigens in mice. For instance, it was shown that feeding high doses (more than 5 mg) of antigen appeared to induce anergy.[13] Hanson and Miller[39] also found that oral administration of antigen was effective in inducing oral tolerance to the antigen in mice; similar results were seen in our previous studies.[14,40] Thus, we used a single dose of 20 mg OVA for oral administration to mice, which is known as the most efficient way to induce oral tolerance. To investigate the modulating effects of this tolerance, on the other hand, we selected the same dose from an immunological study of AEEH previously re-

ported by Kim *et al.*[30] They showed that AEEH at 40 mg/kg was quite effective in enhancing the production of cytokines (IFN-γ, IL-4) and antibodies (IgG, IgG2a, IgG1, IgM) in mice, without causing any side effects.

Oral tolerance was suggested to be induced by mechanisms such as suppressor T (Ts) cell activity and/or anergy of Th cells, B cell anergy, and production of tolerogens. The evidence implicating Ts cells has been obtained in studies on mice.[41—43] However, a number of studies recently showed that oral tolerance has focused mainly on defective Th cell function rather than on active Ts cells.[44,45] Further, it has been shown that murine Th1 cells mediate DTH *via* IL-2 production and regulate IgG2a production *via* IFN-γ production, whereas Th2 cells regulate IgG1 and IgE production *via* IL-4, IL-5, IL-10 and IL-13.[46—50] In addition, our previous study showed that antigen-specific IgG level was more sensitive in oral tolerance than total serum IgG and IgE levels.[14] Similarly, the present study also showed that the induction of oral tolerance was more effective in antigen-specific antibody levels (Fig. 3) than total serum antibody levels (Fig. 2). Thus, in this study the results of antigen-specific antibody levels but not total serum antibody levels have been discussed. It was further shown that the feeding of OVA greatly suppressed phagocytic activity (Fig. 4) and mitogen-stimulated cell proliferation (data not shown) as well as both Th1 and Th2 functions, as shown by our experiments (Figs. 1, 3, 5). Therefore, we developed an improved model of the induction of oral tolerance to OVA using mice; this was confirmed by marked reduction in cytokine production and phagocytic activity. The model was applied to detect modulating effects of a selected substance, AEEH.

Th1 cells, a subset of $CD4^+$ T cells, have been shown to play a role in the induction of DTH and IgG2a antibody production *via* IL-2 and IFN-γ production, respectively (Th1 responses).[51,52] In the present study, AEEH combined with oral OVA strongly elevated anti-OVA IgG2a levels and DTH response compared with those in tolerant mice (Figs. 1, 3), and it was also effective in enhancing of IFN-γ secretion (Fig. 5) as well as T cell proliferation (data not shown). These results indicate that AEEH has a restorative effect against oral antigen on IFN-γ production as well as humoral and cellular immune responses, and that it may prevent Th1 function suppressed by oral antigen.

Th2 cells regulate IgG1, IgM and IgE production *via* IL-4, IL-5, IL-10 and IL-13.[46—49] Similar to Th1 function, the feeding of OVA to mice also greatly suppressed Th2 function as demonstrated in the decreases of IL-4 production or anti-OVA IgG1 and IgM levels (Figs. 3, 5). These were similar to the findings that a single high dose of antigen reduced the production of antigen-specific IgG1 antibodies.[10,14] On the other hand, we recently found that AEEH enhanced the production of antibodies and cytokines in mice.[30] However, our data showed that AEEH combined with oral OVA did not significantly affect anti-OVA IgM and IgG1 levels as well as the production of IL-4 (Figs. 3, 5), in spite of the enhanced B cell proliferation (data not shown) as compared with those in orally tolerant mice. Thus, AEEH treatment in the present study may not restore the suppression of Th2 function induced by OVA.

Phagocytes such as macrophages and polymorphonuclear leukocytes play a significant part in antigen recognition and processing with subsequent interaction with T and B cells to initiate cellular and humoral immune responses.[53,54] They are also known to release many cytokines that have important roles in maintaining homeostasis. In the present study, it has been shown that AEEH combined with oral OVA restore the majority of phagocytic activity in orally tolerant mice (Fig. 4). In view of reports described above and the data shown in Fig. 4, it is believed likely that AEEH also strongly restores the reduction of the reticuloendothelial system, including macrophages in orally tolerant mice by blocking the decrease of IFN-γ by OVA. Furthermore, these findings and our data showing the prevention of oral tolerance by AEEH suggest that Epimedii Herba may act as one of the substances playing an important role in blocking immunologic tolerance in humans without causing any side effects. Further studies are needed to clarify the precise mechanism of AEEH on Th1 cell function in immunologic tolerance, and also the differences in mechanism according to animal species.

In conclusion, AEEH treatment seems to be effective in blocking the suppression of phagocytic activity and Th1 responses including DTH response to OVA and antigen-specific IgG2a antibody production as well as IFN-γ secretion in orally tolerant mice.

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