

Allergy International (2000) 49: 37–45

Original Article

Suppressive effects of the Chinese herbal remedy *Tripterygium wilfordii* Hook f on eosinophilia and IgE hyperproduction in mice

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ABSTRACT

The effects of chloroform extract of *Tripterygium wilfordii* Hook f (TWH extract) on eosinophilia and IgE hyperproduction induced by *Mesocestoides cortii* infection were examined in BALB/c mice. Mice were infected with *M. cortii* by intraperitoneal injection of 500 tetrathyridia. The number of peripheral blood eosinophils and IgE levels were examined 21 days after infection. Oral administration of TWH extract once per day for three weeks dose-dependently suppressed peripheral blood eosinophilia and serum IgE levels, which are enhanced by *M. cortii* infection. Significant suppression of both eosinophilia and serum IgE levels were first noted in mice treated with 300 µg/kg of the extract. The maximum inhibition was observed when mice were treated with 410 µg/kg TWH extract. The ability of spleen cells to produce interleukin (IL)-4 and IL-5 in response to *M. cortii* somatic antigen was also significantly suppressed when donor mice were treated with more than 400 µg/kg of the extract. Extract treatment at a daily dose of 400 µg/kg suppressed costimulatory molecule (CD40 and CD86) expression on spleen cells induced by *M. cortii* infection. These results may suggest that TWH extract will be a good candidate for immunotherapy in allergic diseases.

Key words: eosinophilia, immunoglobulin E, *Mesocestoides cortii*, mouse, suppression, *Tripterygium wilfordii*.

INTRODUCTION

The importance of inflammation in the pathogenesis of allergic diseases, such as bronchial asthma, atopic dermatitis and urticaria, has recently been stressed.^{1–3} Considerable progress has been made to determine the effector mechanisms behind the allergic diseases and has revealed that the development of the diseases is regulated by a vast array of mediators. Generally, these may be divided into two broad groups. Chemical mediators, such as serotonin, histamine, leukotrienes and other various chemotactic factors, may be primarily involved in the early events of acute inflammatory reactions. A second group of mediators, growth factors and interleukins, which are the essential vectors in the cellular communication system, may be involved in long-term tissue alterations in the inflammatory diseases.^{3–5}

Anti-allergic agents (e.g. sodium cromoglycate, azelastine, terfenadine etc.) are effective in the treatment of allergic disorders.^{1–5} These agents are thought to act at least in part by stabilizing the cell membrane of effector cells, such as mast cells and eosinophils, and by preventing release of chemical mediators from the cells.⁶ While the treatment with these agents is reported to provide symptomatic relief and to speed resolution of the early phase of allergic responses, it fails to prevent or reverse the inflammation, which can lead to irreversible tissue damage. Emphasis is therefore now being placed on more aggressive institution of anti-inflammatory therapy in allergic diseases to prevent the tissue destruction

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Received 1 May 1999. Accepted for publication 12 August 1999.

caused by unmitigated inflammation.^{7,8} From this point of view, immunosuppressive agents, such as cyclosporine A, FK506 and spagualin, are recommended in the treatment of the diseases and clinical trials have reported the attenuating effects of these immunosuppressive agents on clinical conditions of allergic diseases.^{7,8}

Tripterygium wilfordii Hook f (TWH) is a traditional Chinese herb grown in the south of China. Extract from TWH, obtained either by chemical or by water-boiling methods, has been widely used in the treatment of some immune related diseases (e.g. rheumatoid arthritis, Bechet's disease, system lupus erythematosus etc.) and satisfactory results have been obtained.⁹⁻¹¹ In addition, animal experiments have demonstrated that TWH extract significantly inhibits mitogen-induced T cell proliferation, mixed lymphocyte reaction and cytotoxic T cell activity.¹²⁻¹⁴ Although TWH extract is also reported to inhibit helper T-cell mediated immune responses¹⁵ and to prolong skin allograft survival in mice,¹³ the therapeutic efficacy of the extract on allergic disorders has not been reported.¹⁶

Infection with parasites, especially larval worm, is well known to induce IgE hyperproduction and peripheral blood eosinophilia in mammalian hosts, including humans and rodents.¹⁷⁻¹⁹ Because these findings are quite similar to those in allergic diseases,¹⁷⁻¹⁹ the parasite/host system may be considered to provide a suitable model in which to examine the efficacy and the mode of action of the agents on allergic diseases.^{20,21} *Mesocostoides cortii* is a common parasite in dogs, cats and man in North and Central America. The preadult worm of this cestoda parasite is called tetrathyridial larva and occurs in the peritoneal cavity of wild rodents. Infection of rodents with tetrathyridial larva is well accepted to produce peripheral blood eosinophilia and IgE hyperproduction.²² In the present study, we have therefore used the *M. cortii*/mouse system and examined the influence of TWH extract on peripheral blood eosinophilia and IgE hyperproduction.

METHODS

Mice

Male BALB/c mice were purchased from Charles River Japan Inc. (Atsugi, Japan). They were allowed at least 1 week to adapt to the environment ($25 \pm 3^\circ\text{C}$, $55 \pm 5\%$ humidity and a 12 h light/dark cycle) and were used at 6 weeks of age.

Agent

T2 tablets, chloroform methanol extracts of TWH, were purchased from Zhuzhou Pharmaceutical Co. (Zhuzhou, Hunan, China) as an oral administration grade for humans. Each T2 tablet contained 30.0 μg extract and did not show the antihelminthic and parasitocidal activities *in vivo*.¹⁵ For administration into mice, randomly selected tablets were ground and suspended in normal saline at a concentration of 30.0 $\mu\text{g}/\text{mL}$.

Parasite and parasitologic technique

Mesocostoides cortii kindly donated by Dr A Niwa (School of Medicine, Kinki University) was maintained in mice by intraperitoneal injection of 50 μL packed tetrathyridia. For preparation of *M. cortii* somatic antigen, tetrathyridia were washed out with phosphate-buffered saline (PBS) from the peritoneal cavity of mice on day 30 after infection.²² Worms were washed 5 times with ice-cold PBS to remove unwanted materials, such as peritoneal leukocytes, and then disrupted with a sonic dismembrator (Artek System Corp., Farmingdale, NY, USA) at 60 W in an ice-cold water bath for 60 min. The homogenate was centrifuged at 50 000 g for 60 min at 4°C and the supernatant obtained. After measuring its protein concentration using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA), the preparation was sterilized by passing through a 0.22 μm filter (Nihon Millipore Corp., Yonezawa, Japan) and stored at -40°C until use.¹⁵

Cell preparation

Spleens were removed from mice killed under ether anesthesia. The organs prepared from five individual mice were pressed through a 60 gauge steel mesh to obtain single cell suspensions and washed once with PBS. After lysing red blood cells with 0.15 mol/L NH_4Cl -Tris buffer, the cell suspension was filtered through a 200 gauge steel mesh to remove cell clumps and debris. The cells were washed 4 times with RPMI-1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, NSW, Australia) and the final concentration of cells was adjusted to 5×10^6 cells/mL.

Cell culture and culture supernatants

A total of 5×10^6 cells was cultured in 24-well plates (IWAKI GLASS Co. Ltd, Tokyo, Japan) in the presence or

absence of 100 µg/mL specific antigen.¹⁵ Culture supernatants used for cytokine assay were obtained 48 h after incubation.

Cytokine assay

Interleukin (IL)-4 and IL-5 levels in culture supernatants were assayed by mouse cytokine enzyme-linked immunosorbent assay (ELISA) Test Kits (Genzyme Corp., Cambridge, MA, USA). The ELISA were done in duplicate according to the manufacturer's recommendation and the results were expressed as mean pg/mL ± SD of five mice.

Assay for immunoglobulin E

Blood was obtained from retro-orbital plexus in a volume of 50 µL. After clotting, the serum was obtained and the total serum IgE levels were assayed by mouse IgE ELISA Test Kits (Yamasa Co. Ltd, Chiba, Japan). The ELISA was done in duplicate according to the manufacturer's recommendation and the results were expressed as mean ng/mL ± SD of five mice.

Eosinophil counts

Blood was smeared on microscope slides and stained with Giemsa solution. A total of 300 nucleated cells was counted and percentage of eosinophils calculated.

Flow cytometry

Spleen cells (1×10^6 /mL) were first incubated for 5 min at 4°C with 10.0 µg monoclonal antibody (mAb) against mouse CD16 (rat IgG2b; Pharmingen, San Diego, CA, USA) to block FcγIII/II receptors. After two washes with PBS, the cells were incubated for another 25 min at $25 \pm 2^\circ\text{C}$ with 10.0 µg of either fluorescein isothiocyanate (FITC)-conjugated rat antimouse CD40 (rat IgG_{2a}; Pharmingen) or FITC-conjugated hamster antimouse CD86 (hamster IgG; Pharmingen). After two washes, the cells were resuspended in fresh PBS at a concentration of 0.5×10^6 cells/mL and analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Downers Grew, CA, USA). Dead cells were gated out by means of forward scatter and sideward scatter. Fluorescence of 10^4 cells was recorded and the data were analyzed with Consort 30 software (Becton Dickinson).

Statistical analyses

The significance of differences in mean values between two groups was examined by the Mann–Whitney *U*-test.

RESULTS

Influence of *Mesocestoides cortii* infection on eosinophilia and IgE hyperproduction

The present study was designed to examine whether *M. cortii* infection could affect the number of peripheral blood eosinophils and IgE production. BALB/c mice were infected with *M. cortii* by intraperitoneal injection of various numbers of larvae. Counting the number of peripheral blood eosinophils and examining IgE levels were performed daily after infection. As shown in Fig. 1a, infection of mice with *M. cortii* caused a gradual increase in the number of peripheral blood eosinophils, peaked on day 14 and plateaued thereafter. Likewise, the amount of IgE in peripheral blood rose to a level more than 30-fold that observed in non-infected control (approximately 100 ng/mL; Fig. 1b) from day 14 of the infection onwards. The data in Fig. 1a,b also clearly show that there is no apparent difference between these two immunologic responses and number of worms used to infect.

Effect of TWH extract on eosinophilia and IgE production

The present study was designed to examine the influence of TWH extract on eosinophilia and IgE production. BALB/c mice were infected with *M. cortii* by intraperitoneal injection of 500 *M. cortii* larvae on day 0. The mice were treated orally with various concentrations of TWH extract once per day for 21 days, starting on the day of infection. Samples for examining the number of eosinophils and IgE levels were obtained 21 days after infection. The TWH extract inhibited an increase in eosinophil numbers induced by *M. cortii* infection in a dose-dependent manner. As the concentration of TWH extract administered was increased, the number of eosinophils decreased and complete inhibition, not significant compared with the non-infected control ($P > 0.05$), was observed when mice were treated with the extract at a single dose of 410 µg/mL (Fig. 2). Although administration of TWH extract also dose-dependently suppressed IgE hyperproduction, complete suppression, not significant compared with the non-infected control ($P > 0.05$), was first observed when the mice were treated with 400 µg/mL TWH extract (Fig. 3).

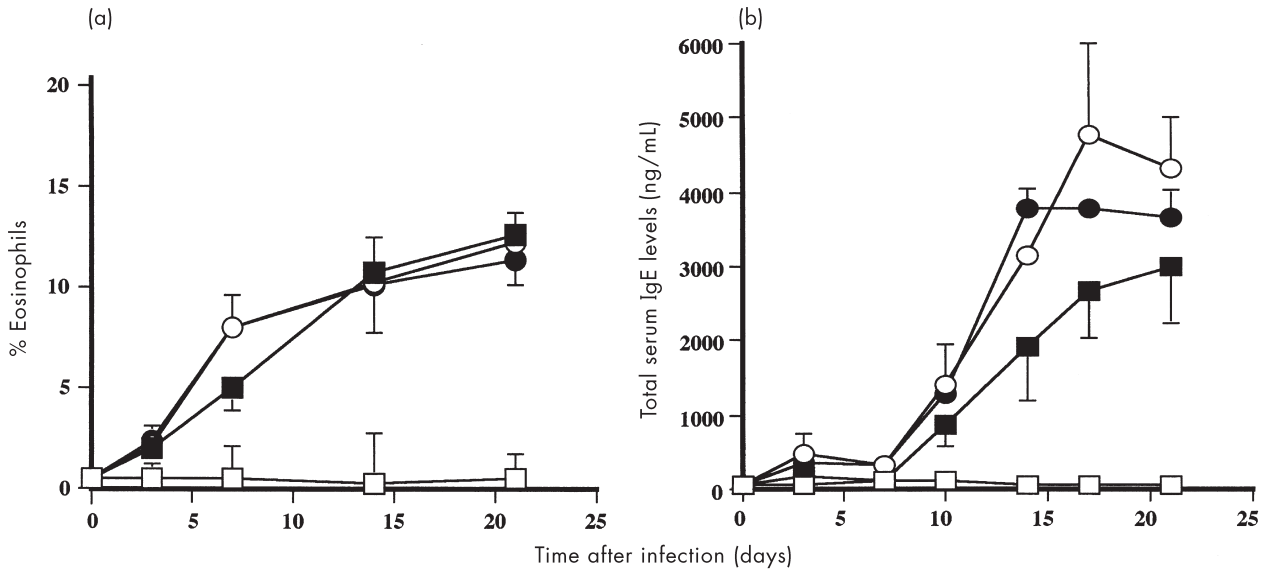


Fig. 1 Influence of *Mesocestoides cortii* infection on peripheral blood eosinophilia and serum IgE levels in mice. BALB/c mice were infected with *M. cortii* by intraperitoneal injection of various numbers of tetrahyridia on day 0. Samples for examining number of eosinophils and serum IgE levels were obtained as indicated. The results are expressed as the mean \pm SD of five individual mice. (□), Non-infected control; (■), infected with 100 tetrahyridia; (●), infected with 500 tetrahyridia; (○), infected with 1000 tetrahyridia.

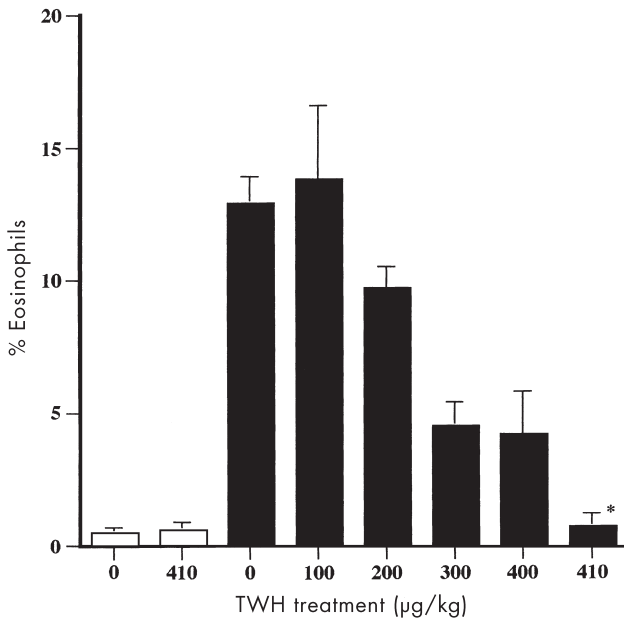


Fig. 2 Influence of *Tripterygium wilfordii* Hook f (TWH) extract on peripheral blood eosinophilia. BALB/c mice were infected with *Mesocestoides cortii* by intraperitoneal injection of 500 tetrahyridia on day 0 (■). (□), Non-infected. The mice were treated orally with various doses of TWH extract once per day for 21 days. The number of eosinophils was examined on day 21 and the results were expressed as the mean (%) \pm SD of five individual mice. * $P > 0.05$ (not significant compared with non-infected control).

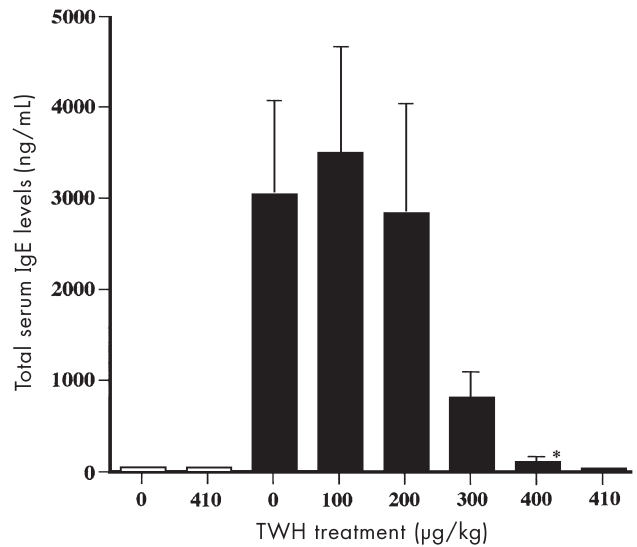


Fig. 3 Influence of *Tripterygium wilfordii* Hook f (TWH) extract on serum IgE hyperproduction. BALB/c mice were infected with *Mesocestoides cortii* by intraperitoneal injection of 500 tetrahyridia on day 0 (■). (□), Non-infected. The mice were treated orally with various doses of TWH extract once per day for 21 days. Serum IgE level was examined on day 21 by enzyme-linked immunosorbent assay and the results were expressed as the mean (ng/mL) \pm SD of five individual mice. * $P > 0.05$ (not significant compared with non-infected control).

Effects of TWH extract on IL-4 and IL-5 production from spleen cells in response to antigenic stimulation *in vitro*

Because IL-4 and IL-5 are well known to be essential factors for IgE production and eosinophil growth,^{23,24} the present study was undertaken to examine whether TWH extract suppresses the production of these cytokines and results in inhibition of both IgE production and eosinophilia. BALB/c mice were intraperitoneally infected with 500 larvae on day 0. These mice were administered orally with various doses of TWH extract once per day for 21 days, starting on the day of infection. Spleen cells were prepared on day 21 and cultured *in vitro* in the presence of *M. cortii* somatic antigen for 48 h. The culture supernatants were obtained for examining the levels of IL-4 and IL-5. As shown in Fig. 4a,b, treatment of mice with 100 µg/kg per day TWH extract did not affect the ability of spleen cells to produce IL-4 and IL-5 in response to antigenic stimulation *in vitro*. However, administration of TWH extract at a single dose of 200 µg/kg significantly suppressed production of both IL-4 ($P < 0.05$) and IL-5 ($P < 0.01$) and the minimum dose of TWH extract to completely inhibit the production was 400 µg/kg per day (Fig. 4a,b).

Effect of TWH extract on the expression of costimulatory molecules on spleen cells

It is well known that costimulatory signals provided by the interaction of T cell surface molecules with their ligands, such as CD40 and CD86, expressed on antigen-presenting cells (APC) are essential for successful activation of T cells. We therefore finally investigated whether TWH extract can suppress costimulatory molecule expression induced by *M. cortii* infection and result in inhibition of the immune responses as stated earlier. BALB/c mice were infected with 500 *M. cortii* larvae on day 0. These mice were treated orally with 400 µg/kg per day TWH extract for 21 days. On day 21 after infection, spleen cells were obtained from three individual mice and examined for CD40 and CD86 expression. Administration of TWH extract into non-infected mice scarcely affected CD40 expression on spleen cells (Fig. 5a,b). However, TWH extract caused suppression on CD40 expression enhanced by *M. cortii* infection; the ratio (small and large cells) of CD40-positive cells in infected and saline-treated mice was reduced from 4 to 1 by treatment with TWH extract (Fig. 5c,d). As shown in Fig. 6a,b, TWH extract also produced no apparent effects on CD86 expression when the extract was administered into

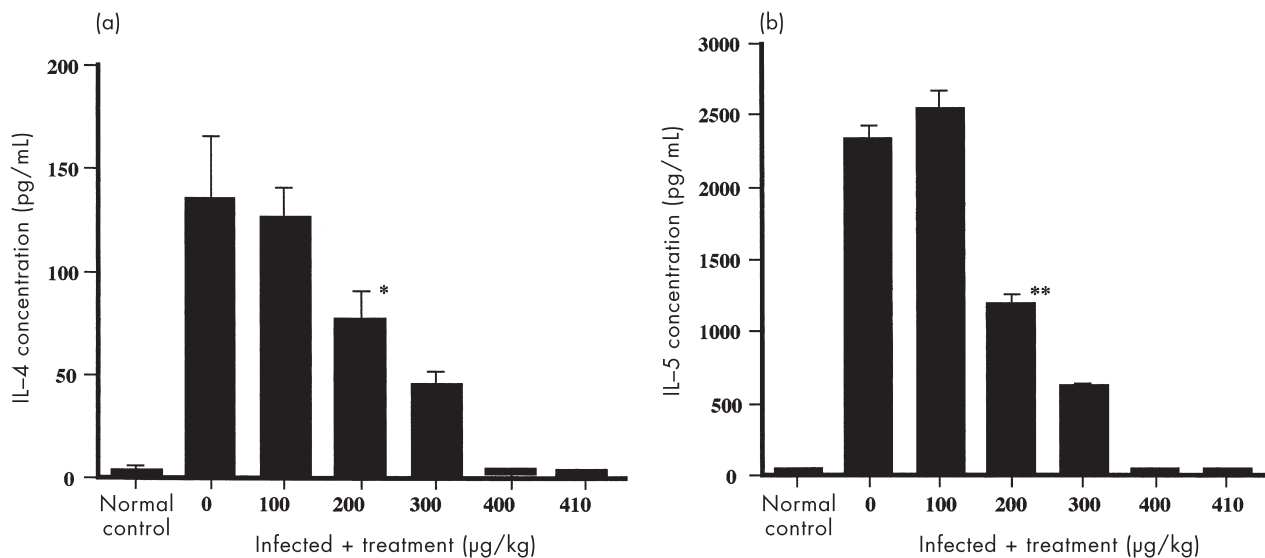


Fig. 4 Influence of *Tripterygium wilfordii* Hook f (TWH) extract on the ability of spleen cells to produce IL-4 (a) and IL-5 (b) in response to antigenic stimulation *in vitro*. BALB/c mice were infected with *Mesocostoides cortii* by intraperitoneal injection of 500 tetrahyridia on day 0. The mice were administered orally with various doses of TWH extract once per day for 21 days. Spleen cells were prepared from mice on day 21 and cultured *in vitro* with *M. cortii* somatic antigen. After 48 h, the culture supernatants were obtained and cytokine concentrations were examined by enzyme-linked immunosorbent assay. The results were expressed as the mean (pg/mL) \pm SD of five individual mice. * $P < 0.05$, ** $P < 0.01$.

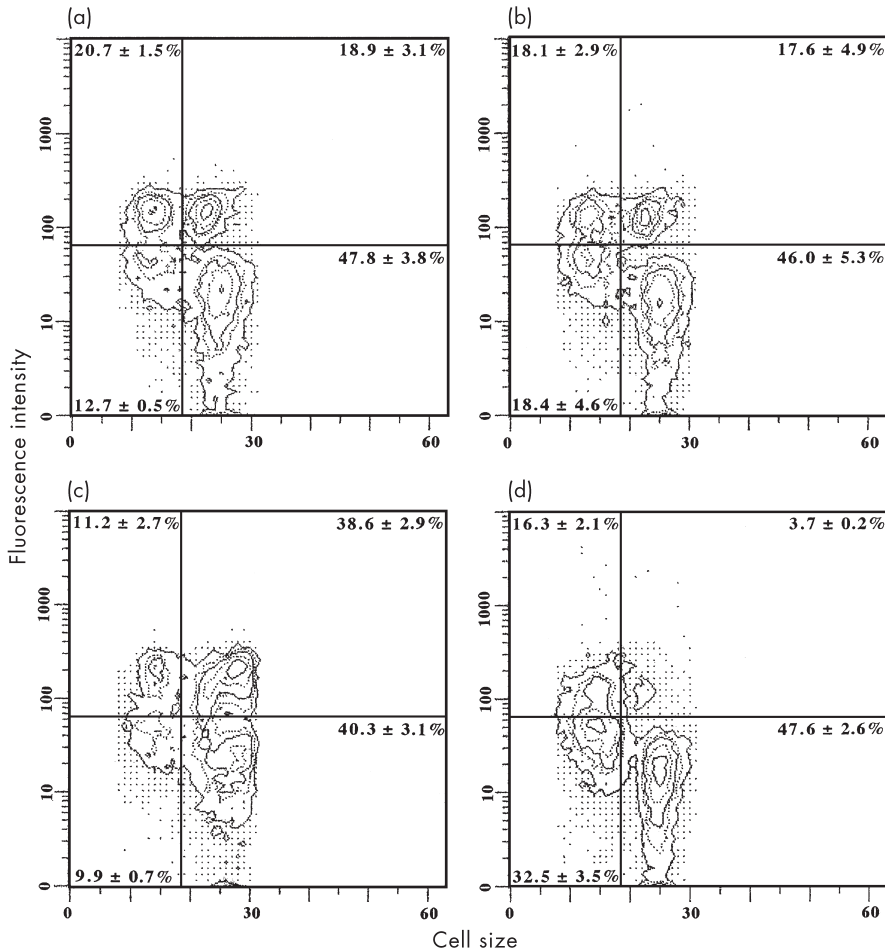


Fig. 5 Suppressive effect of *Tripterygium wilfordii* Hook f (TWH) extract on CD40 expression on spleen cells. BALB/c mice were infected with *Mesocostoides cortii* by intraperitoneal injection of 500 tetrathyridia on day 0. The mice were administered orally with 400 µg/kg TWH extract once per day for 21 days. Spleen cells were prepared from mice on day 21 and examined for CD40 expression by flow cytometry. The numbers in each quadrant represent mean percentage ± SD of CD40-positive cells of three mice. (a) Non-infected, saline-treated control; (b) non-infected, TWH extract-treated control; (c) infected, saline-treated; (d) infected, TWH extract-treated.

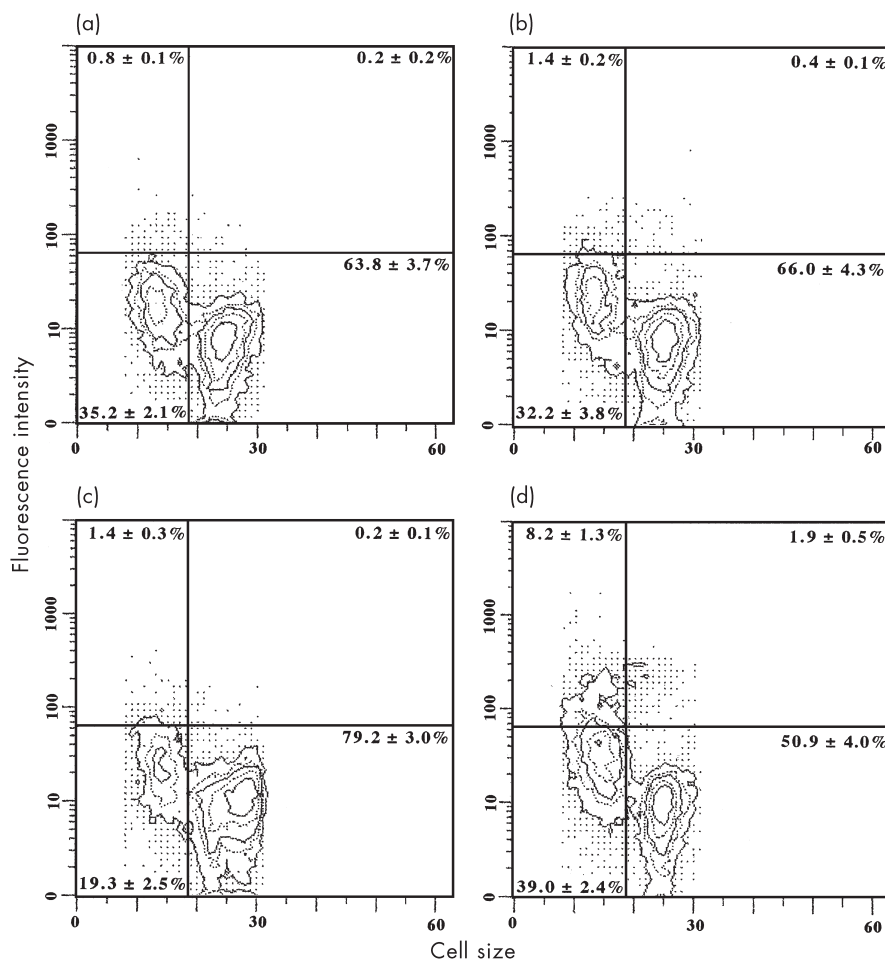
non-infected mice and examined for the expression of CD86 on spleen cells. In contrast, TWH extract showed strong suppressive effects on CD86 expression induced by *M. cortii* infection; the ratio (small and large cells) of CD86-positive cells in infected and saline-treated mice was reduced from 4 to 1 by treatment with TWH extract (Fig. 6c,d).

DISCUSSION

A variety of preparations of a Chinese traditional medicine, TWH extract, have been reported to have therapeutic effects on autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus and Behcet's diseases, among others.⁹⁻¹¹ Because of the beneficial clinical impact, many efforts have been made to understand how these preparations exert their therapeutic function. Results obtained from these studies have shown that TWH is able to suppress inflammatory process induced by a variety of stimuli.^{12-14,25} Recently, we have

reported that TWH extract can suppress the development of the chronic graft-versus-host reaction (GVHR) induced in (C57BL/6 × DBA/2) hybrid mice by injection of DBA/2 spleen cells.²⁶ The early events in the chronic GVHR are reported to contain similar mechanistic elements to those seen in allergic responses characterized by elevating IgE production,^{27,28} suggesting the efficacy of TWH extract in the treatment of allergic responses associated with IgE hyperproduction. However, there is no direct evidence showing the protective activity of TWH extract in allergic diseases. The experiments presented here characterize the effects of TWH extract on allergic immune responses, especially eosinophilia and IgE hyperproduction, during normal *in vivo* immune responses and evaluate the possibility of clinical use in the treatment and the management of allergic diseases as an anti-allergic agent. The model examined is that of eosinophilia and IgE hyperproduction during *M. cortii* infection. In the present study, we examined first the influence of TWH extract on eosinophilia and IgE hyperproduction. The data obtained clearly show that

Fig. 6 Suppressive effect of *Tripterygium wilfordii* Hook f (TWH) extract on CD86 expression on spleen cells. BALB/c mice were infected with *Mesocostoides cortii* by intraperitoneal injection of 500 tetrathyridia on day 0. The mice were administered orally with 400 $\mu\text{g}/\text{kg}$ TWH extract once per day for 21 days. Spleen cells were prepared from mice on day 21 and examined for CD86 expression by flow cytometry. The numbers in each quadrant represent mean percentage \pm SD of CD86 positive cells of three mice. (a) Non-infected, saline-treated control; (b) non-infected, TWH extract-treated control; (c) infected, saline-treated; (d) infected, TWH extract-treated.



TWH extract can suppress these two immune responses induced by *M. cortii* infection in dose-dependent manner: although administration of TWH extract at a single dose of 100 $\mu\text{g}/\text{kg}$ did not influence the *M. cortii*-induced immune responses, treatment of mice with more than 200 $\mu\text{g}/\text{kg}$ extract caused significant suppression of the responses. Complete suppression was observed when mice were treated with the extract at a single dose of 400 $\mu\text{g}/\text{kg}$ for 3 weeks, which is one-fourth of the recommended therapeutic dose in human autoimmune diseases, such as RA, systemic lupus erythematosus and psoriasis.⁸⁻¹⁰ The most striking feature of the histopathology of allergic diseases is the intense infiltration with eosinophils, macrophages and lymphocytes.²⁹⁻³¹ Eosinophils can secrete a number of lipid mediators and proteins, which may have a role to play in the pathophysiology of the allergic diseases.³⁰ The overproduction of IgE is also generally recognized as a common feature of the allergic diseases and the participation of this anti-

body in the diseases is well documented.²⁹ From these reports, the present results may suggest that TWH extract is a good candidate for the agent in the treatment and management of allergic diseases.

The TWH extract has been reported to suppress immune responses through the inhibition of T cell function, especially cytokine secretion, and to have a slight effect on B cell proliferation and antibody formation.^{15,32,33} Although experimental and clinical data suggest that IgE synthesis in B cells is a complex process involving several cellular and molecular interactions, there is an established concept that IL-4 is the most essential cytokine for the generation of IgE.²³ Eosinophils are granular cells that arise principally in the bone marrow. Eosinophil differentiation, like that of all other leukocytes, is influenced by several types of cytokines, such as IL-3, IL-5 and granulocyte-macrophage colony stimulating factor.^{30,31} Of these cytokines, IL-5 is accepted to be specific for eosinophils and the most

important cytokine for terminal differentiation of the committed eosinophil precursors.²⁴ It is also accepted that IL-5 can lead to the specific recruitment of eosinophils, enhanced eosinophil cytotoxicity and prolonged eosinophil survival.²⁴ My unpublished data (Asano, 1999) shows no toxic effects of TWH extract on granulocytes, such as mast cells and eosinophils, when these cells were cultured in the presence of 10.0 µg/mL extract. Earlier reports and our unpublished data may suggest that the suppressive effects of TWH extract on eosinophilia and IgE hyperproduction is owing to inhibitory action of TWH extract on IL-4 and IL-5 production from T cells.^{25,26} This suggestion may be supported by the observation that TWH extract can suppress the ability of spleen cells to produce IL-4 and IL-5 when the cells are prepared from *M. cortii* infected and TWH extract-treated mice and cultured in the presence of specific antigen. There is now extensive evidence implicating allergen-specific type 2 helper T cells (Th2) as key orchestrators of the chronic inflammatory responses that underlie many allergic diseases.^{23,30} Optimal T cell activation requires not only interaction between antigen peptide bound to MHC molecules of APC and the T cell receptor, but also additional signals, termed costimulation.³⁴ A number of recent reports have shown that ligation of costimulatory molecules, especially CD28 and CD86, is essential for Th2 cell activation and production of IL-4 and IL-5.³⁵⁻³⁷ Interaction between CD40 on B cells and CD40 ligand (CD40L) on T cells has been also reported as an important costimulatory signal required for switch recombination to IgE synthesis in the presence of IL-4.³⁸ These reports prompted us to examine the influence of TWH extract on costimulatory molecule expression. The present results clearly show that TWH extract exerts suppressive effects on CD40 and CD86 expression on APC in response to *M. cortii* infection, when mice were treated with TWH extract at a daily dose of 400 µg/kg once per day for 3 weeks.

Although the present study clearly shows that treatment of mice with TWH extract can suppress eosinophilia and IgE hyperproduction through inhibition of both Th2 cell cytokine production and costimulatory molecule expression on APC, the precise mechanisms by which TWH extract can suppress cytokine production and the expression of cell surface molecules are not clear at present. Further experiments are needed to clarify this point. A number of compounds has been isolated from TWH extract or cultured plant cell lines and structurally identified, but the active compounds of the extract has not been completely delineated.¹⁴ Diterpenoid compounds, such

as tripchloride, triptolide and triptolide, have been isolated from TWH and reportedly have anti-inflammatory and immunosuppressive effects.³⁹⁻⁴¹ It has also been reported that the suppressive potential of triptolide is slightly greater than that of triptolide and the combination of triptolide and triptolide at a 1:1 molar ratio causes more inhibition of immune responses, including cytokine production and lymphocyte proliferation *in vitro*.¹⁴ From these reports, it may be suggested that the immunosuppressive activity in *M. cortii*-infected mice is brought about by the combination of both triptolide and triptolide. Biochemical analysis of the constituents of TWH extract will be important in further delineating its mechanisms of action in allergic immune responses.

In conclusion, the findings of the present study demonstrate that TWH extract can inhibit development of *M. cortii*-induced immune responses, which are quite similar to those of allergic diseases. Our previous reports^{15,26} show that TWH extract does not show adverse effects, such as loss of appetite, ruffled fur and a hunched posture, when mice are treated with the extract for more than 3 weeks. The present results therefore may suggest that the extract will be a useful drug in the treatment and prevention of allergic diseases.

ACKNOWLEDGEMENTS

This study was supported, in part, by The Japan Private School Promotion Foundation.

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