# Therapeutic effect of a traditional Chinese medicine, Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to) through controlling Th1/Th2 balance

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## Abstract

It has been reported that Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to, HET) had various effects such as anti-bacteria, anti-virus and anti-tumor effects. Recently, it has also been reported that HET suppressed IgE production. This result suggested that HET would be useful for allergy and atopic disorder. We have recently observed that oral tolerance could not be induced in Th2 cells of germfree (GF) mice because of Th2 predominance of the GF mice. As the predominance of Th2 was thought to be a feature of allergic disorder, we investigated the therapeutic effect of HET on Th1/Th2 balance. When SPF mice were orally administered 5 mg/d of ovalubumin (OVA) for 4 consecutive days as a tolerogen before systemic challenge with OVA (1 $\mu$g/d for 3 times), oral tolerance could be successfully induced both in Th1-mediated IgG2a response and Th2-mediated responses, such as the productions of IgG1 and IgE. In contrast, in GF mice, the oral tolerance could be induced in Th1 cells, but not in Th2 cells, suggesting that Th2 predominated Th1 in GF mice. Staining of intracellular IL-4 and IFN-$\gamma$ in the splenic T cells confirmed the predominance of Th2 in GF condition.When 1 g/kg/d of HET was orally administered everyday for 2 to 3 wks before systemic challenges with OVA, the treatment suppressed IgG1 production. Moreover, the staining of intracellular IL-4 and IFN-$\gamma$ in the splenic T cells demonstrated that HET counterbalanced the Th1/Th2. As Th1 responses played a role in protection against bacteria, virus and tumor, an effect such as enhancing Th1 responses by HET may also explain its various pharmacological activities.

**Key words** Hochu-ekki-to, oral tolerance, mucosal immunity, germfree mouse, Th1/Th2 balance.

**Abbreviations** HET, Hochu-ekki-to; GF, germfree; Ab, antibody; OVA, ovalbumin; i.p., intraperitoneally.

## Introduction

When a high amount of an antigen is orally given, systemic unresponsiveness is obtained. This phenomenon is well known as oral tolerance, which was first described by Wells as a state in which systemic anaphylaxis was prevented by previous feeding of an antigen.[1] Thereafter, there have been reported many factors which contribute to the generation of oral tolerance. Among them, the intestinal bacterial flora and gut-associated lymphoid tissue seem to be major factors.[2] It has previously been demonstrated that intestinal bacterial flora plays a crucial role in the induction of oral tolerance, and thus the oral tolerance could not be induced in germ-free (GF) mice which lacked indigenous intestinal bacterial flora.[3] Failure of oral tolerance induction in GF mice suggested that GF animals can be used as one of the models of food allergy.

Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to, HET), a traditional Chinese medicine, is composed of ten species of medical plants. It has been traditionally used for the various symptoms caused by

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fatigue and for the weakness after severe illness. HET exhibits anti-microbial activity against *Listeria monocytogenes,*[4,5] influenza virus,[6] *Candida albicans,*[7] and cytomegalovirus.[8] It has been also revealed that HET had anti-tumor effect.[9-11] In addition, it has been recently reported that HET could suppress IgE responses,[12,13] and is thus effective for atopic dermatitis.[14] These results demonstrated that HET has various immunomodulating effects, and that it is presumably effective for allergic disorders. In this report, we examined the effect of HET on the oral tolerance induction in GF mice.

## Materials and Methods

*Mice* : Male BALB/c mice raised under specific pathogen-free (SPF) condition were purchased from Japan Clea Inc. (Tokyo, Japan), and kept in our animal facility under SPF condition. GF BALB/c mice were originally obtained from Japan Clea, and were bred in our facility. The GF mice were bred in Trexler-type flexible-film isolators with sterile food and water. Surveillance for bacterial contamination was periodically done by examining the feces. Mice at 5-6 wks of age were used in the present study.

*Hochu-ekki-to (HET)* : HET was manufactured as a spray-dried powder of hot-water extracts from ten species of medicinal plants, and provided from Kanebo Co. (Osaka, Japan). The powder (6400 mg) was extracted from the mixed medicinal plants as below.

| | |
|---|---|
| *Astragali* radix; 4.0 g | *Ginseng* radix; 4.0 g |
| *Atactyloids* rhizoma; 4.0g | *Angelicae* radix; 3.0 g |
| *Zizyphi* fructus; 2.0 g | *Auantii nobilis* pericarpium; 2.0 g |
| *Bupleuri* radix; 2.0 g | *Glycyrrhizae* radix; 1.5 g |
| *Cimicifugae* rhizoma; 1.0 g | *Zingiberis* rhizoma; 0.5 g |

The powder was $\gamma$-irradiated (50 Gy), and then dissolved in sterile water. Mice were orally received 1 g/kg of HET (in 0.5 ml) via a gastric tube everyday for 2 to 3 wks prior to the immunization.

*Immunization and oral tolerance induction* (Fig. 1) : To induce a significant antibody (Ab) in response to ovalubumin (OVA) as an antigen, the mice were intraperitoneally (i.p.) injected with 1 $\mu$g of OVA (Seikagaku Corp., Tokyo) absorbed to 0.1 mg of aluminum hydroxide (alum) every 2 wks starting at day 0. To induce oral tolerance, the mice were orally given 5 mg/day of OVA for 4 consecutive days from day -7 to day -4, and then were immunized i.p. with OVA in alum, as described above. At 5 wks after starting the immunization, blood samples were collected to measure Ab titer. In some experiments, the spleens were collected at the same time to stain intracellular cytokine.

*Determination of the OVA-specific IgG1, IgE and IgG2a in the serum* : Serum levels of OVA specific Abs were determined as reported previously.[3] IgG1 Ab specific for OVA was tested by ELISA. In brief, the serum samples with serial dilutions were placed on the wells of the ELISA plate, which had been coated with OVA. This was followed by the addition of goat anti-mouse IgG1 conjugated with peroxidase (PharMingen, San Diego, CA). After being washed with PBS containing 0.05 % Tween-20, bound Ab was detected by the addition of 0.04 % *o*-phenylendiamine solution. The serum level of OVA-specific IgE was determined by fluorescence ELISA. In brief, a 96-well plate for a fluorescence ELISA (Black Cliniplate, Labsystems Japan, Tokyo) was coated with rat anti-mouse IgE Ab (PharMingen) by incubation overnight at 4°C. After washing, serial dilutions of samples were added to the wells and incubated for 1 hr at room temperature. Next, biotynylated OVA was added to each well, followed by the treatment with streptavidin-$\beta$-galactosidase (Seikagaku Corp.). The enzymatic activity was determined by the addition of 4-methyl-umbelliferyl-$\beta$-D-galactosidase (Funakoshi Pharmaceutical Co., Osaka, Japan) as a substrate, and then measured by fluorescence ELISA reader (Dainippon Seiyaku, Osaka). Anti-OVA IgG2a titer was also measured by fluorescence ELISA using rat anti-mouse IgG2a Ab (PharMingen). In the assay for measuring OVA-specific Ig, the titers of the serum from the mice without oral treatment of OVA (Group 1 and 3 in Exp. 1 and Group 1 in Exp. 2 of Fig. 1) were designated as 100 unit/ml.

*Flowcytometry for determining intracellular cytokines* : To determine Th1/Th2 balance in GF and HET-treated GF mice, intracellular cytokines were stained, and then examined by flowcytometry. The staining procedure was mainly based on the method as

previously reported[15] with some modification according to the CytoStain Kits (PharMingen). The spleens were aseptically collected and the cells were resuspended in a RPMI 1640 medium containing 10 % FCS, 100 u/ml of ampicillin, 40 $\mu$g/ml of gentamycin and 100 $\mu$g/ml of OVA at a final concentration of $5\times 10^6$/ml, and were then incubated in a 24-well plate (1 ml/well) for 3 d. For flowcytometry, the following mAb and materials were commercially purchased: biotin - anti - CD3$\varepsilon$ ( PharMingen ), streptavidin - RED670 (Life Technologies, Gaitherburg, MD), CytoStain Kits, and FITC-anti-IL-4 (PharMingen). Clone R4-6A2, which produced mAb against anti murine IFN-$\gamma$, was obtained from ATCC (Rockville, MD). The hybridoma was cultured in Dubecco's modified Eagle's MEM containing 10 % of fetal bovine serum. The culture supernatant was collected, dialyzed and then conjugated with phycoerythrin (PE). For staining, the cultured cells were at first stained with biotin-CD3$\varepsilon$ for 30 min at 4°C. After washing, the cells were stained with streptavidin - RED670 for another 30 min at 4°C. Then, the cells were fixed and permealized by the solution in Cyto-Stain Kits. Finally, the cells were stained with FITC-IL-4 and PE-IFN-$\gamma$, and analyzed by flowcytometry. In flowcytometry, $CD3^+$ cells were gated, and IL-4/IFN-$\gamma$ ratio was examined.

*Statistical analysis* : Data showed mean±standard deviation. Statystical analysis was done by Student's *t* test.

## Results

*Failure of oral tolerance induction in Th2 cells of GF mice*

In order to examine the role of intestinal bacterial flora on oral tolerance, the serum levels of OVA-specific IgG1, IgG2a and IgE in SPF and GF mice, which had orally received OVA and then were challenged with this antigen, were measured in the first experiment (Fig. 1, Exp.1). As shown in Fig.2A, oral administration of a high amount of OVA significantly suppressed OVA-specific Ab levels in IgG1, IgG2a and IgE (Fig. 2A, shaded columns). Thus, oral tolerance could be successfully induced in SPF mice. On the other hand, oral tolerance could be induced only in

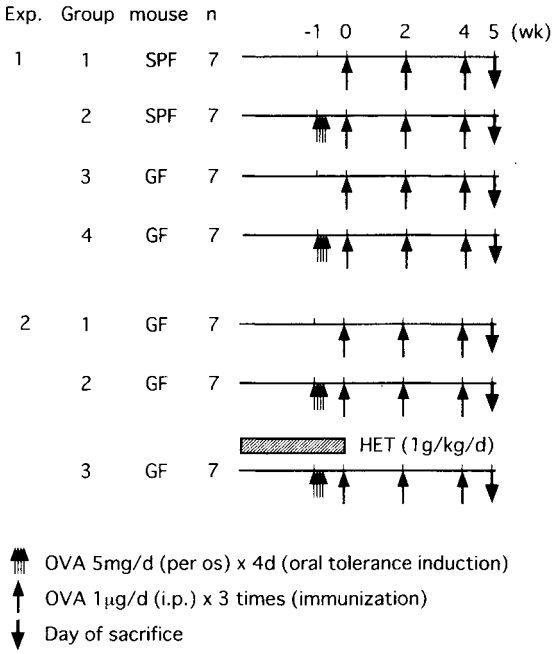


Fig. 1 Experimental design. Male BALB/c mice bred under SPF or germfree (GF) condition were used in this study. The day starting the immunization was designated as day 0, when the mice were 5-6 wk-old of age. Mice were orally administered 5mg/d of ovalubumin (OVA) for 4 consecutive days as a tolerogen before systemic challenge with OVA (1 $\mu$g/d for 3 times at 0, 2, and 4 wks). At 5 wks, the mice were sacrificed for assay. The serum from the mice without oral tolerance (Group 1 and 3 in Exp.1, and Group 1 in Exp.2) were used as controls, and their serum titers against OVA were designated as 100 unit/ml in each experiment. In Exp.2, mice were orally administered irradiated HET (1 g/kg/d) everyday for 2-3 wks until day 0.

IgG2a, but not in IgG1 or IgE in GF mice (Fig. 2A, open columns), whereas the abilities of GF mice to produce IgG1, IgG2a and IgE against challenged antigen were comparable to those of SPF mice.[3] It has been demonstrated that Th cells can be divided into two categories by their patterns of cytokine production.[16] Productions of IL-2 and IFN-$\gamma$ are controlled by Th1, whereas that of IL-4 is by Th2. As for B cell help, Th1 mediates IgG2a production, and Th2 mediates IgG1 and IgE productions. Thus, when Th1/Th2 was considered to be balanced in SPF condition, it seemed that Th2 predominates over Th1 in GF condition. To confirm this, intracellular IL-4 and IFN-$\gamma$ were stained, and then Th1/Th2 balances in SPF and GF mice were examined. The spleens were collected from oral tolerance-induced SPF and GF mice (Exp. 1, Group 2 and 4 in Fig. 1). Then, the spleen cells

Fig. 2A

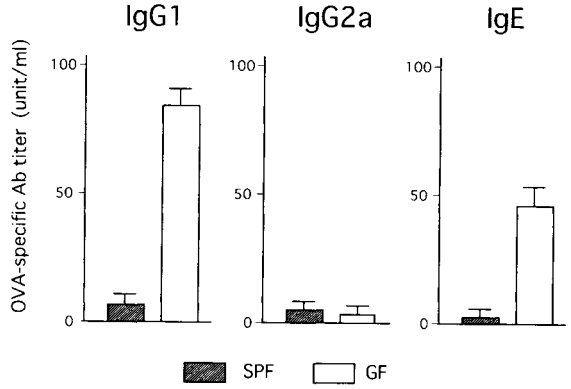


Fig. 2B

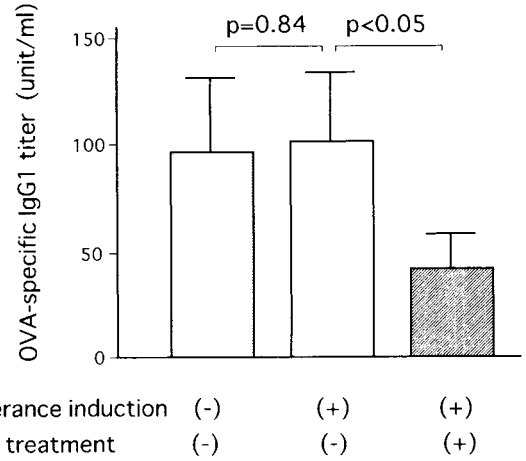


Fig. 2 Ab titers in the serum of mice underwent oral tolerance induction. Protocols were shown as Exp.1 (A), and Exp.2 (B) in Fig. 1. (B) GF mice were administered HET for 2 wks. The bars indicated standard deviations (n=7).

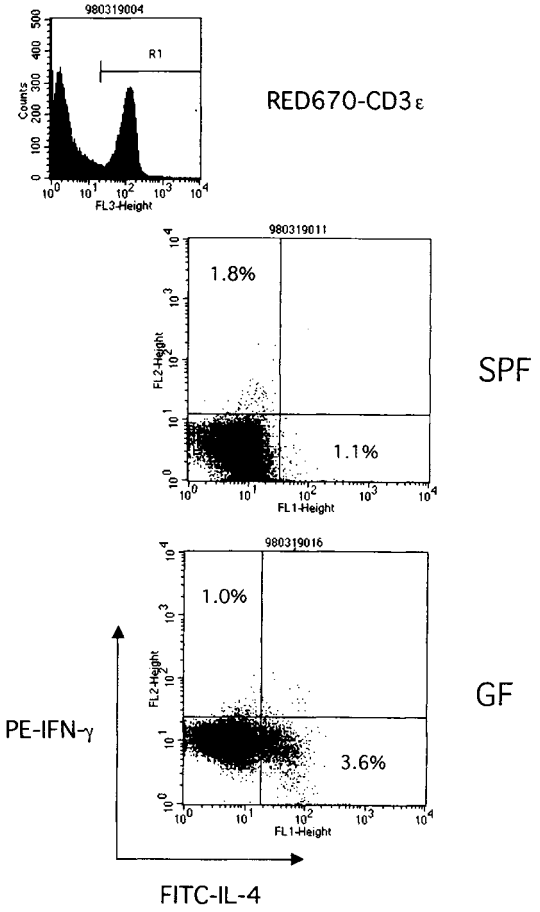


Fig. 3 A representative result of flowcytmetric analysis. The spleens were aseptically collected from SPF and GF mice, for which oral tolerance had been induced and then immunized with OVA. The spleen cells then *in vitro* cultured with OVA for 3 d. After *in vitro* stimulation, the cells were stained with anti-CD3ε. After fixing and permealizing, intracellular IL-4 and IFN-γ were stained to estimate Th1/Th2 balance. Histogram demonstrated the expression level of CD3ε. $CD3\varepsilon^+$ population, R1 in the histogram, was gated in each sample for intracellular cytokine analysis. Dot-plot analyses demonstrated the expression levels of intracellular IL-4 and IFN-γ in the $CD3\varepsilon^+$ spleen cells. The numbers expressed the percentage in each quadrant. In these cases, Th1/Th2 balances in a SPF and GF mouse were estimated as 1.8/1.0=1.8 and 1.0/3.6=0.28, respectively.

were *in vitro* cultured for 3 d with OVA. After the *in vitro* stimulation, the intracellular IL-4 and IFN-γ were then stained for flowcytometric analysis. As a result, Th1/Th2 ratio in SPF (n=7) and GF (n=7) mice were $1.8 \pm 0.3$ and $0.5 \pm 0.7$, respectively ($p < 0.001$, a representative result was shown in Fig. 3). It was thus suggested that the predominance of Th2 resulted in failure of oral tolerance in Th2-responses. Considering that oral tolerance could not be induced in GF mice because of predominance of Th2-type responses, GF mice were supposed to be one of the models for atopic disorders. Therefore, the effect of HET on Th1/Th2 balance in GF mice was examined in the next experiment.

*Counterbalance of Th1/Th2 by HET*

In order to examine the effect of HET on Th1/Th2 balance, GF mice were treated with HET everyday for 2 to 3 wks before starting i.p. immunization with OVA (Fig. 1, Exp. 2). When mice received HET for 3 wks everyday, OVA-specific IgG1 titer was

Table I Th1/Th2 balance in GF mice treated with Hochu-ekki-to (HET)

| wk | HET[a] | IFN-$\gamma^+$ratio[b] ($\times10^{-1}$%) | IL-$4^+$ratio[b] ($\times10^{-1}$%) | Th1/Th2 balance[c] |
|---|---|---|---|---|
| 0 | (−) | 2.7±1.5[d] | 6.7±1.2 | 0.4±0.3 |
| 0 | (+) | 8.3±11.0 | 9.7±3.2 | 0.7±0.9 |
| 5 | (−) | 8.3±1.7[e] | 12.8±1.7 | 0.7±0.1[f] |
| 5 | (+) | 20.3±8.5[e] | 18.8±7.8 | 1.5±0.4[f] |

a) GF mice were orally administered 1 g/kg of HET for 14 consecutive days from day -14 to day 0. The mice also orally received 5 mg of OVA everyday from day -7 to day -4 as a tolerogen, and immunized with 1 $\mu$g of OVA 3 times at 0, 2, 4 wks. Five wks after starting the immunization, the GF mice were sacrificed to collect spleen cells. b) The spleen cells from GF mice were *in vitro* cultured with 100 $\mu$g of OVA for 3d. Then, the cells were stained with biotin anti CD3$\varepsilon$. After washing, the cells were treated with streptavidin-conjugated RED670. Next, the spleen cells were fixed and permealized to stain intracellular IL-4 anf IFN-$\gamma$. The percentages of IL-$4^+$ and IFN-$\gamma^+$ were enumerated by flowcytometry. c) Th1/Th2 balance was calculated in each sample as follows; IFN-$\gamma^+$ ratio/IL-$4^+$ ratio d) mean±Standard deviation (n=7) e) $p<0.005$ f) $p<0.005$

significantly suppressed (Fig. 2B, a right column), whereas the titers in GF mice without treatment (Fig. 2B, a center column) were comparable to those of non-tolerant controls (Fig. 2B, a left column). Thus, the oral tolerance in Th2 cells was seemed to be retrieved in GF mice by treatment with HET. Similar results were obtained by HET treatment for 2 wks (data not shown) starting at 2 wks before OVA immunization. To confirm that such effect of HET was due to its action on Th1/Th2 balance, intracellular IL-4 and IFN-$\gamma$ were stained. As shown in Table 1, HET treatment for 2 wks prior to i.p. immunization with OVA enhanced Th1-type responses, and thus the Th2 predominance in GF mice was improved by HET through its effect to enhance Th1 activity.

## Discussion

In the previous report, we demonstrated that oral tolerance could not be induced in GF mice.[3] Thus, it was expected that a GF mouse would be a model of food allergy. In the present study, we analyzed the precise mechanism from the viewpoint of Th1/Th2 balance. As a result, in GF mice, oral tolerance could be induced in Th1-responses, but not in Th2 responses. From these results, it was supposed that Th2 dominated Th1 in GF mice. Considering that Th2 dominance is one of the characters of atopic disorder, it would be reasonable that a GF mouse is not only a model of food allergy but also a mouse model of atopic disorder. Therefore, in the present study, we measured the serum level of IgG1, which represented Th1 responses, and intracellular IL-4/IFN-$\gamma$ as the markers for Th1-responses and Th1/Th2 balance. As a result, we demonstrated that HET possessed the activity to enhance Th1-type responses, and counterbalance the skewed Th1/Th2 balance (Fig. 3). It has already been reported that HET could suppress IgE production.[12,13] It has also been reported that HET would be effective for atopic dermatitis[14] because of its suppressive effect of IgE production. The present results supported this conclusion from another viewpoint, Th1/Th2 balance.

Considering that Th2 predominated in GF mice, intestinal bacterial flora seemed to counterbalance Th1/Th2 by means of promoting Th1. Actually, Kirjavainen *et al.* reported that bacteria in general promoted the differentiation of Th1-cell lineage.[17,18] It is possible that a number of bacterial factors are involved in keeping Th1 in order to counterbalance Th1/Th2. One of the candidates is a specified CpG motif. This bacterial DNA was originally reported as the anti-tumor DNA derived from *Mycobacterium bovis*.[19] Thus, it was revealed that intestinal bacteria such as E.coli possessed the DNA, and the main feature of the DNA was the palindrome containing 5'-CpG-3'.[20,21] It was also revealed that the CpG motif enhanced IFN-$\gamma$ production and NK activity.[22] Thus,

this may be the reason why the motif demonstrated anti-tumor activity. Considering that both NK cells and IFN-$\gamma$ production are controlled by Th1,[16,23] these reports suggested that the CpG motif regulated Th1 lineage. Actually, it has recently been demonstrated that the CpG motif promoted Th1 switching and its maintenance.[24-27] Moreover, it has also been demonstrated that this motif can act as mucosal adjuvant.[28]

It has been reported that HET exhibited various biological effects such as anti-microbial activity,[4-8] anti-tumor activity,[9-11] and suppression of IgE production.[12,13] Although these various immunomodulating effects of HET seem to be disconcerting, it would be well understood when considered from the viewpoint of Th1/Th2 balance. Mosmann *et al.* demonstrated that NK and CTL activities were mediated by Th1.[29] Moreover, immune responses against influenza virus, *Mycobacterium tuberculosis,* and *Listeria monocytogenes* were fully Th1-type responses, whereas allergic responses including allergy, atopic asthma, vernal conjunctivitis and Omenn's syndrome were Th2-mediated immune responses.[29] Thus, although various immunomodulating effects of HET have been reported, those may be simply explained by the activity of HET to enhance Th1-type immune responses. HET may possess such activity to stimulate Th1 as CpG motif of indigenous bacteria.

In general, Kampo, a traditional Chinese medicine, has various pharmacological activities. This may be one of the reasons which make physicians hesitate to use Kampo. In the present study, we demonstrated that various effects of HET might attribute to its simple effect on Th1 enhancement. Such approach to Kampo may be useful to explain its various effects, and that makes easy to use Kampo for physicians.

## Conclusion

In the present study, we demonstrated that Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to, HET) had an effect to enhance Th1. As a result, HET had a therapeutic effect on allergy, in which Th2 predominated Th1.

## 和文抄録

補中益気湯には抗菌，抗ウイルス，抗腫瘍効果があることが既に報告されている。さらに，最近ではIgE抗体産生抑制効果もあることも明らかになり，アレルギー，アトピーの治療薬としての可能性も示唆されてきた。一方，我々は無菌マウスではIgG1, IgE産生を促すTh2に経口トレランスが誘導できないことから，無菌マウスでは生体のTh1/Th2バランスがTh2側に傾いていることを観察した。そこで本研究ではこの経口トレランス誘導の実験系で補中益気湯投与群を加えることにより，補中益気湯がTh1/Th2バランスに及ぼす効果を検討した。実験には無菌あるいはSPF環境下に飼育したBALB/c雄マウス5～6週齢のマウスを用いた。これらのマウスに卵白アルブミン（OVA）1 $\mu$gを2週間毎（0, 2, 4週とする）に腹腔内に投与し免疫した。経口トレランスの誘導は-1週から連日4日間，5 mgのOVAを経口投与することにより行った。免疫開始5週目に血清を採取し，抗OVA抗体価を測定した。その結果OVA経口投与によりSPFマウスではIgG1, IgG2a, IgE産生ともに非経口投与群の20％以下にまで抑制され，経口トレランスが誘導された。しかし，無菌マウスではIgG1, IgE産生に関しては抑制が認められず，また脾T細胞でのIL-4/IFN-$\gamma$産生細胞の割合の測定から無菌マウスではTh2過剰状態にあることが観察された。そこで，無菌マウスに-2～-3週から0週まで補中益気湯1 g/kgを連日経口投与したところ，OVA特異的IgG1抗体の産生は抑制されていた。T細胞におけるIL-4/IFN-$\gamma$産生細胞の割合を調べたところ，補中益気湯投与群ではTh1/Th2バランスがTh1側に傾くことが明らかとなった。即ち，補中益気湯にはTh1/Th2バランスをTh1側に傾ける効果があるものと考えられた。Th1型ヘルパーT細胞は抗菌，抗ウイルス，抗腫瘍効果に関与していることが知られており，Th1を介して補中益気湯がこのような効果を発現することが示唆された。

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