Influence of γ-aminobutyric acid (GABA) on IgE Production in Ovalbumin-immunized BALB/c Mice

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Summary

γ-aminobutyric acid (GABA) is a kind of amino acid and has been received much attention for their biological activities. In the present study, we examined the influence of GABA on immunomodulatory activities and serum IgE levels, employing ovalbumin (OVA)-immunized BALB/c mice. In cytokine production assay *in vitro*, GABA showed increased IFN-γ production concomitant with decreased IL-4 production by splenocytes from OVA-immunized BALB/c mice. When administrated orally, GABA was effective for decreasing both total and OVA-specific IgE levels in serum. In addition, these decreased IgE levels by administrated GABA were paralleled with increased IFN-γ production and decreased IL-4 production in splenocytes *ex vivo*. It seems that GABA can potentially suppress Th2 responses including IL-4 production via promoting Th1-skewed response *in vivo*, leading to down-regulation of IgE synthesis. These results suggest that GABA might be useful for preventing IgE-mediated allergic diseases.

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Key words : γ-aminobutyric acid (GABA), IgE, IL-4, IFN-γ, Th1/Th2

Many allergic diseases associated with immediate hypersensitivity are closely related to elevated production of serum immunoglobulin E (IgE). It is well known that Type-I allergic reactions are triggered by mast cell degranulation subsequent to antigen cross-linking of IgE molecules that are bound to high affinity IgE receptor FcεRI on the cell surface. IgE is produced by plasma B cells, which is mainly regulated by type 2 helper T cells (Th2) via producing interleukin (IL)-4. IL-4 induces IgE isotype class switching (Coffman *et al.* 1986) and itself promotes Th2 differentiation. It is realized that Th2-biased state is participate in clinical condition of allergic diseases (Romagnani 1994, Cross *et al.* 2004). On the contrary, Th1 produce IFN-γ that plays the opposite role to IL-4, and itself promotes Th1 differentiation. Both Th1 and Th2 subsets are produced from naïve T cells, a non-committed population of precursor T cells. Th1 and Th2 can cross-inhibit each other. The Th1/Th2 balance is considered to be critical for IgE production. Therefore, agents with an ability to induce Th1-biased state, whereby Th2 responses are weakened, are expected to prevent IgE-mediated allergic deceases. Recently, it has been reported that several dietary ingredients including lactic acid bacteria (Matsuzaki *et al.* 1998, Shida *et al.* 2002), saccharides (Nagura *et al.* 2002), polyphenols (Yano *et al.* 2007) and saponins (Katayama *et al.* 2006) are effective for lowering serum IgE levels through modulating Th1/Th2 balance.

γ-amino butyric acid (GABA), which is synthesized by glutamic acid decarboxylase from glutamic acid, is the major inhibitory neurotransmitter on the mammalian central nervous system. GABA is also occurred in various kinds of foods including fermented food products. Administrated GABA has been shown to exert some biological activity including anti-hypertensive (Hayakawa *et al.* 2002, Matsubara *et al.* 2002). However, almost no studies dealing with the immunomodulatory properties of GABA have been reported.

In this study, we investigated the effect of GABA on immunomodulatory activities to induce IFN-γ and IL-4 production by cultured splenocytes from ovalbumin (OVA)-immunized BALB/c mice. We further examined the effect of orally administrated GABA on total and antigen-specific IgE levels in serum and *ex vivo* cytokine production levels in splenocytes.

MATERIALS AND METHODS

Mice

Female BALB/c mice at 5 to 6 weeks of age were purchased from Charles River Japan (Yokohama, Japan) and were maintained conventionally in plastic cages at about 22 ℃ under a 12-h light-dark cycle. The mice were provided with a standard CRF-1 diet (Oriental Yeast, Tokyo, Japan) and allowed *ad libitum* access to autoclaved water throughout the experimental period. This experiment was carried out according to the guidelines laid out by The Ethical Committee for Animal Experiments of Niigata University.

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***In Vitro* Cytokine Production Assay**

To obtain ovalbumin-sensitized splenocytes, female BALB/c mice at 6 weeks of age were intraperitoneally immunized on day 0, 14 and 28 with 100 μg ovalbumin (OVA; Wako, Osaka, Japan) and 1 mg of $Al(OH)_3$ gel (Wako). After checking that the OVA-specific IgE level was rising, mice were sacrificed and their spleens were obtained at day 35 to 42. Single splenocyte suspensions were prepared by crushing and pressing the organs through a nylon mesh and removing red blood cells with lysis buffer followed by washing twice with phosphate buffered saline (PBS). Splenocytes were suspended at 2×10^6 cells/mL in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing 10 % (vol/vol) heat-inactivated fetal bovine serum (FBS; Roche, Mannheim, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin and, in the presence or absence of GABA (Wako), cultivated with 100 μg/mL OVA using 96-well flat-bottomed culture plates (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5 % CO_2 at 37 ℃. Following cultivation for 7 days, culture supernatants were collected to measure the amount of cytokines by ELISA.

***In Vivo* Experiments**

The schedule for *in vivo* experiments is summarized in **Fig. 1**. One and 0.5 mg of GABA in 50 μL sterile water were orally administered to BALB/c mice (n=4 per group) everyday, in parallel with being intraperitoneally immunized with 100 μg OVA and 1 mg $Al(OH)_3$ gel on day 0, 14 and 28. Administration of GABA was started from 1 week before the 1st immunization. As a control, 50 μL of sterile water was orally given to mice. For the measurement of total and OVA-specific IgE level, blood samples were collected from tail bleed. The sera were prepared by centrifugation at 10,000×g at 4 ℃ for 10 min, and then stored at -80 ℃ before use in measurement. On day 35, mice were sacrificed and splenocytes were obtained for *ex vivo* cytokine production assay.

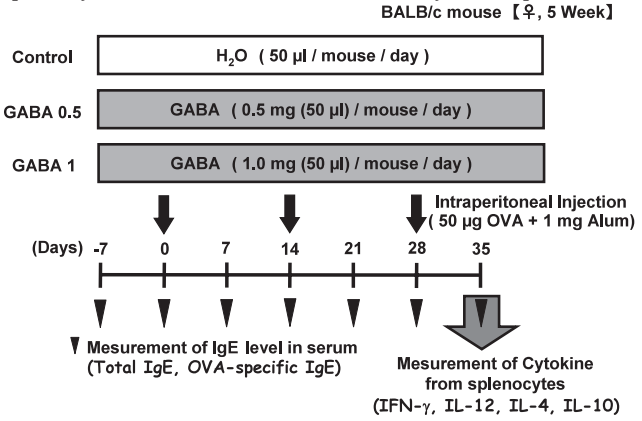

Fig.1 Experimental schedule for immunization with OVA and administration of GABA. Female BALB/c mice (6 weeks of age) were immunized by intraperitoneal injection with 100 μg OVA and 1 mg $Al(OH)_3$ gel on Day 0, 14 and 28. The immunized mice were administrated with GABA or sterile water orally. Serum samples were collected every 7 days from Day 0 to 35 and splenocytes were obtained on Day 35.

***Ex Vivo* Cytokine Production Assay**

Splenocytes obtained from mice given GABA orally were seeded at 2×10^6 cells/mL in 96-well flat-bottomed culture plates and cultivated with 100 μg/mL OVA in RPMI-1640 containing 10 % (vol/vol) heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, under a humidified atmosphere of 5 % CO_2 at 37 ℃. Following cultivation for 7 days, culture supernatants were collected to measure the amount of IgE and cytokines by ELISA.

ELISA

The concentration of IFN-γ and IL-4 were measured using murine Opt EIA ELISA set (BD Biosciences, San Diego, CA) in accordance with the instructions from the manufacture. For determination of total IgE, sandwich ELISA was employed using anti-mouse IgE antibody (LO-ME-2) (ZYMED, San Francisco, CA) as a primary antibody and biotinylated anti-mouse IgE antibody (LO-ME-3) (Acris, Hiddenhausen, Germany) as a secondary antibody in combination with streptavidin-horseradish peroxidase (HRP) conjugate. OVA-specific IgE levels were also assessed by almost same sandwich ELISA system using OVA for coating the ELISA plates instead of primary antibody (LO-ME-2). As HRP substrate, 3,3' ,5,5' -tetramethylbenzidine (Sigma-Aldrich) were used and absorbance at 450 nm was measured using a microplate reader (Model 680, BIO-RAD, Hercules, CA).

Statistical Analysis

Data are represented as the mean ± standard deviation (SD). Statistical analyses were performed by using Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of GABA on *in vitro* production of IFN-γ and IL-4 by splenocytes

To evaluate the potential of GABA to induce IFN-γ production and reduce IL-4 production, splenocytes from OVA-immunized BALB/c mice were cultivated with OVA in the presence of GABA. The amounts of IFN-γ and IL-4 in supernatants of splenocytes cultures were shown in **Fig. 2**. After cultivation without GABA for 7 days, the amounts of IFN-γ and IL-4 were 4.57 ± 0.23 ng/mL and 844 ± 20 pg/mL respectively. These two cytokines in supernatants without OVA were below the limits of detection (data not shown). At a concentration of 1 to 100 μg/ml, GABA caused the increase in the amount of IFN-γ with the concomitant decrease in the amount of IL-4 (**Fig. 2a**), with a significantly higher IFN-γ/IL-4 ratio compared to control ($P < 0.05$) (**Fig. 2b**). Cell viability during the culture period was evaluated by employing MTT assay and no decreased percentages of cell viability were observed in this culture condition with 100 μM

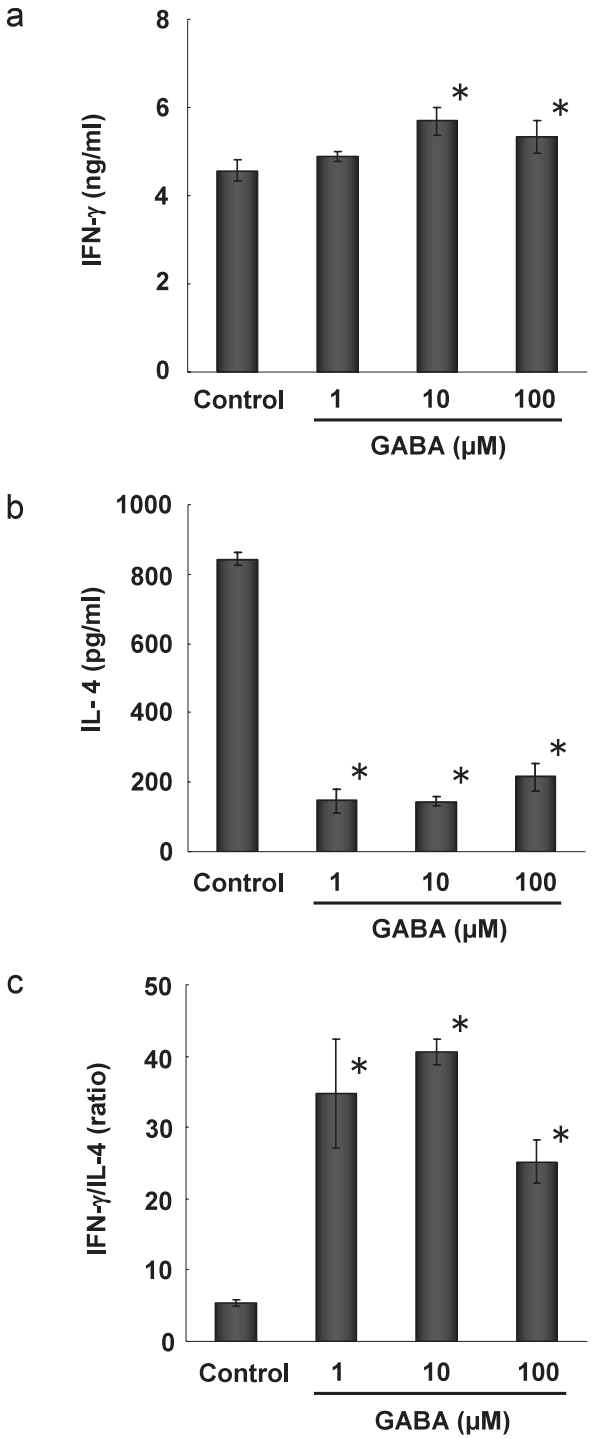

Fig.2 *In vitro* IFN-γ and IL-4 production by splenocytes cultivated with GABA. Splenocytes from OVA-immunized BALB/c mice were cultured with OVA in the absence (control) presence of GABA (1, 10, 100 μg/ml) for 7 days. IFN-γ (A) and IL-4 (B) in culture supernatants were determined by ELISA and IFN-γ/IL-4 ratio (B) were calculated. Data represent mean ± SD (n=4). $^{*}p < 0.05$ compared with control (Student's *t*-test).

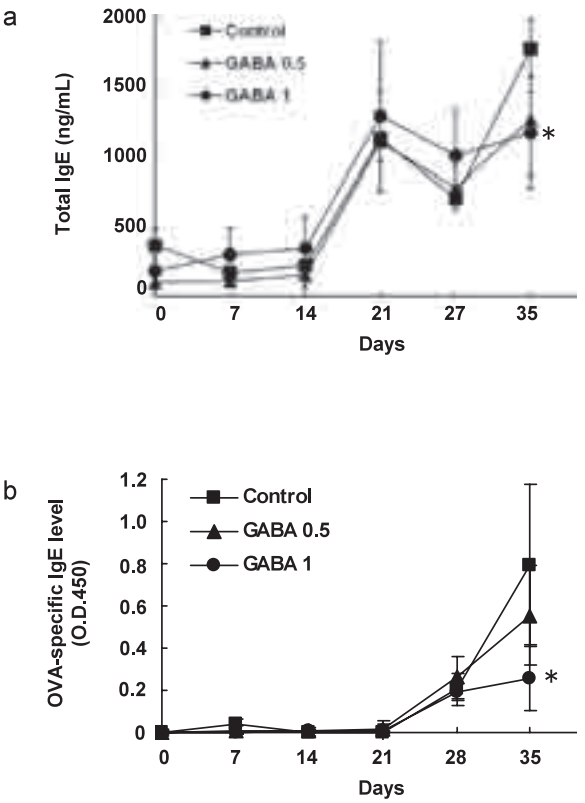

Fig.3 Serum IgE levels in OVA-immunized mice given GABA orally. GABA was suspended in sterile water and administrated orally to mice at 0.5 and 1 mg/mouse/day. Total IgE (A) and OVA-specific IgE (B) levels in serum were determined by ELISA. Data represent mean ± SD of four mice per group (n=4). $^{*}p < 0.05$ compared with control (OVA-immunized mice without GABA) (Student's *t*-test).

GABA (data not shown).

Effect of orally administrated GABA on serum IgE levels

To evaluate whether GABA has the ability to lower serum IgE levels, GABA was administered orally to OVA-immunized mice at 0.5 and 1 mg/day/mouse, and the sera were collected every 7 days during the experiment. The concentrations of total IgE and the levels of OVA-specific IgE in serum were shown in **Fig. 3**. OVA-immunization without administration of GABA resulted in gradual elevation of both total and OVA-specific IgE levels in serum during the experimental period. On day 35, oral administration of GABA suppressed the elevation of total IgE level in serum, with statistical significance at 1 mg/day/mouse ($P < 0.05$) **(Fig. 3a)**. Administrated GABA was also effective for decreasing OVA-specific IgE level in serum with significance at 1 mg/day/mouse ($P < 0.05$) **(Fig. 3b)**. The suppressive efficacy of GABA was observed in a dose-dependent fashion. Non-immunized mice showed almost no changes in total and OVA-specific IgE levels during the experimental period (data not shown).

***Ex vivo* cytokine production by splenocytes from mice given GABA orally**

To evaluate the immunomodulatory activities of GABA *in vivo*, antigen-induced IFN-γ and IL-4 production of splenocytes from mice administrated GABA orally were measured. After confirming decreased levels of total and OVA-specific IgE in serum by GABA administration on day 35, splenocytes were collected from the mice and stimulated with OVA in culture. **Fig. 4** shows the concentration of IFN-γ and IL-4 in culture supernatants of splenocytes. Levels of IFN-γ were significantly higher in splenocytes from GABA-administrated groups than those in control group ($P < 0.05$) **(Fig. 4a)**. Conversely, levels of IL-4 were lower in splenocytes from GABA-administrated groups than those in control group, with statistical significance at 1 mg/day/mouse ($P < 0.05$) **(Fig. 4b)**. Both IFN-γ and IL-4 in supernatants without OVA were below the limits of detection (data not shown). In accordance with *in vitro* cytokine production assay described above, the IFN-γ/IL-4 ratio was significantly higher in the GABA-administrated groups compared to that in the control group ($P < 0.05$) **(Fig. 4c)**.

DISCUSSION

Currently, it is generally accepted that homeostasis between Th1 and Th2 activity is critical for immune regulation. While Th2 is believed to emphasize protection against extracellular pathogens, the Th2 pathway is seen as underlying allergy and IgE-based diseases (Parris 2003). Type-I allergic symptoms are generally caused by antigen cross-linking of IgE on mast cells. It is well known that the Th2 cytokines such as IL-4 and IL-5 are essentially associated with IgE production and IgE-mediated allergy because of their actions to help recruit B cells, mast cells and eosinophils involved in allergic inflammatory reaction (Djukanovic *et al.* 1990, Leung *et al.* 1995, Hamelmann and Gelfand 1999), while the Th1 cytokine, IFN-γ, has an inhibitory effect on IgE production (Pene *et al.* 1988) and Th2 differentiation (Gajewski *et al.* 1988). The balance between Th1 and Th2 cytokines is considered to be critical for IgE production. Since the crucial role of Th2-dominated immune responses in the pathogenesis of allergic diseases is well established, therapeutic interventions which suppress Th2 responses by inducing Th1-polarization might be effective in prevention and treatment of allergic diseases (Durham *et al.* 1998, Bohle 2002).

This study focused on the suppressive effect of GABA on IgE production through its immunomodulatory activity on Th1/Th2 balance. It now seems that Th1 and Th2 responses are heavily related to IFN-γ or IL-4, respectively. At first, GABA was examined with respect to their ability to modulate *in vitro* production of IFN-γ and IL-4 by splenocytes from OVA-immunized BALB/c mice, in order to elucidate its potential to induce Th1-skewed response. After 7 days

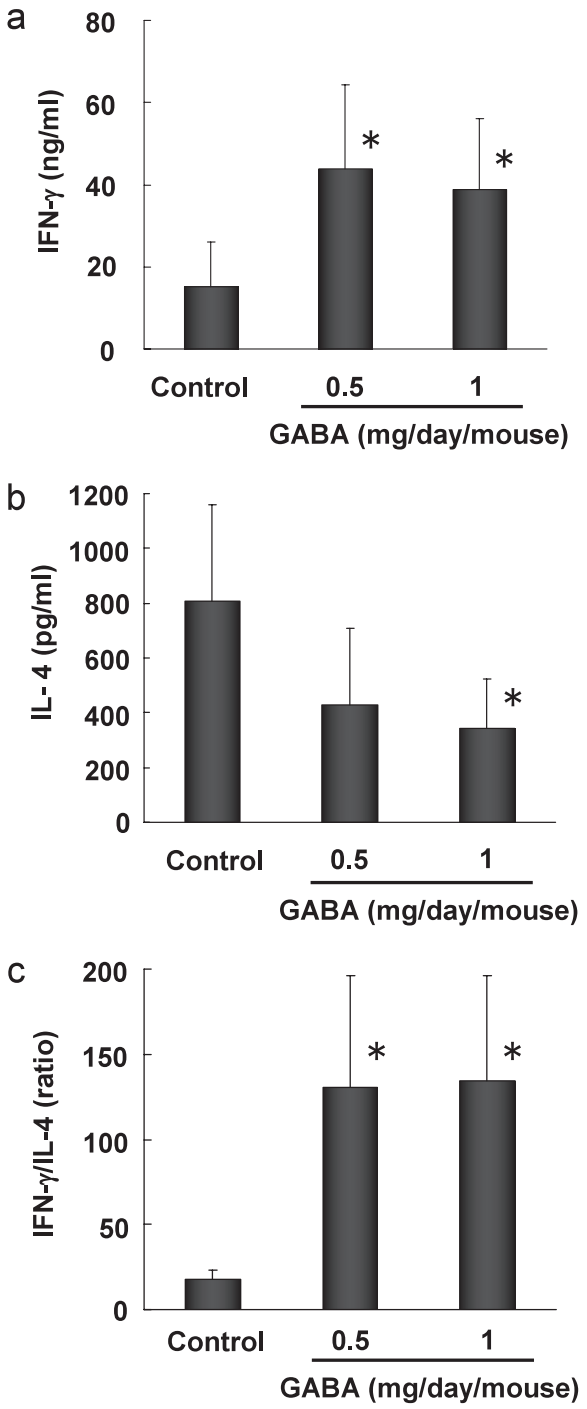

Fig.4 OVA-induced IFN-γ and IL-4 production by splenocytes from OVA-immunized mice given GABA orally. Sprenocytes were obtained on Day 35 and cultured in the presence of OVA (100 μg/ml) for 7 days. The concentrations of total IFN-γ (A) and IL-4 (B) in culture supernatants were determined by ELISA and IFN-γ/IL-4 ratio (C) were calculated. Data represent mean ± SD of four mice per group (n=4). $^{*}p < 0.05$ compared with control (OVA-immunized mice without GABA) (Student's *t*-test).

cultivation, GABA showed increased IFN-γ production concomitant with decreased IL-4 production by splenocytes in responce to OVA **(Fig. 2)**. Next, we evaluated the effect of oral administration of GABA on serum IgE levels *in vivo*. In this trial using OVA-immunized BALB/c mice, GABA was effective for decreasing both total and OVA-specific IgE levels in serum **(Fig. 3)**. In parallel with the suppression of serum IgE levels, orally administrated GABA showed increased IFN-γ production concomitant with decreased IL-4 production by splenocytes upon stimulation with OVA in culture **(Fig. 4)**. This suppression of serum IgE levels could be explained by the down-regulation of IL-4 production since IL-4 signaling is prerequisite for IgE synthesis in B cells. In accordance with our observation in *in vitro* cytokine production assay, oral administration of GABA showed immunomodulatory effect to induce Th1-skewed response in *ex vivo* cytokine production assay. It is possible that GABA can modulate the imbalance between Th1 and Th2 responses through induction of Th1 in systemic immunity, leading to suppression of IgE production.

Although the mechanisms by which GABA suppresses serum IgE levels remain to be elucidated at cellular and molecular levels, the amino acid is expected as a dietary ingredient with a potential to modulate immune responses. This is the first demonstration that orally administrated GABA can lower serum IgE levels. Recently, GABA has been attracting attention as a food ingredient with functions such as improvement of memory and study capability, blood pressure-lowering action (Hayakawa *et al*. 2002, Matsubara *et al*. 2002), renoprotective effect and relaxation (Lyou and Yokogoshi 2004). Besides these biological functions, GABA may have anti-allergic activities. The present study suggests a potential use of GABA in preventing IgE-mediated allergy. Further examination concerning anti-allergic effects of GABA on symptoms in Nc/Nga mice, which are accepted as a suitable model for human atopic dermatitis, are now on going in our research group.

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オバルブミン感作したBALB/cマウスに対するγ-アミノ酪酸（GABA）の血清IgEレベル抑制作用

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要　約

γ-アミノ酪酸（GABA）は，食品に含まれる機能性成分として注目されている。本研究では，オバルブミン（OVA）感作したBALB/cマウスに対するGABAの免疫制御活性について調べ，経口投与による血清IgEレベルの抑制作用について検討した。*in vitro*培養試験において，GABAはマウス脾細胞のインターフェロン－γ（IFN-γ）の産生を促進すると同時にインターロイキン－４（IL-4）の産生を抑制した。GABAを経口投与した結果，OVA感作BALB/cマウスにおける総IgE及びOVA特異的IgEの血清レベルが有意に低下した。さらに，*ex vivo*培養試験において，GABAを経口投与したマウスの脾細胞ではOVA誘導性のIFN-γ産生促進とIL-4産生抑制が認められた。GABAは，Th1の誘導を介してIL-4産生を始めとしたTh2応答を抑制し，IgE産生を抑制する可能性がある。以上の結果から，GABAはIgEが関与するアレルギー疾患の予防に有用であることが示唆される。

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