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Amaranth grain inhibits antigen-specific IgE production through augmentation of the IFN-γ response *in vivo* and *in vitro*

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Abstract

Amaranthus hypochondriacus L. (amaranth) is a nutritionally protein rich plant with a good yield, but there has been no research concerning its immunological effects *in vivo* or *in vitro*. In the present study, we examined the effects of amaranth grain on cytokine and IgE production using *in vitro* helper T cell development and IgE production assays and an animal model of an orally-induced, allergen-specific IgE response. First, we examined the effect of orally administered amaranth on serum IgE concentration which reflects the immune response during allergic disease. We observed significantly decreased ($p < 0.05$) allergen-specific IgE in the blood of mice in our animal model. We found that orally fed amaranth significantly augmented ($p < 0.05$) IFN-γ production of spleen cells. *In vitro* studies demonstrated that the water-soluble fraction of amaranth grain promoted helper T cell type-1 (Th1) phenotype development. Moreover, we found that the amaranth grain extract suppressed antigen-specific IgE production *in vitro*. These data indicate that there is a component in amaranth grain which has a suppressive effect on IgE production and augments Th1 cytokine production. In conclusion, we found that amaranth grain and its extract inhibited antigen-specific IgE production through augmenting Th1 cytokine responses *in vivo* and *in vitro*.

Abbreviations: AM diet – amaranth powder containing diet; ELISA – enzyme-linked immunosorbent assay; IFN – interferon; Ig – immunoglobulin; IL – interleukin; OVA – ovalbumin; Th – helper T cell; TCR – T cell receptor; Tg – transgenic.

Introduction

Amaranthus hypochondriacus L. (amaranth) is a fast-growing, stress-tolerant plant that produces a good yield of small seeded grain in a sorghum-like head. The grain contains high levels of fat, dietary fiber, and protein, which is well balanced in terms of amino acid composition and high in lysine, as compared to conventional cereal grains (National Academy of Science 1984). It is even recommended by the World Health Organization as a source of nutritionally rich protein. In previous studies, amaranth was only researched on nutritional grounds; there have been no reports on the immunological effects of amaranth grain *in vivo* or *in vitro*.

Allergic disease, as represented by asthma or atopic dermatitis, is closely related to allergen-specific immunoglobulin (Ig) E. IgE sensitizes mast cells and basophilic granulocytes. Moreover, the reaction of antigen with cell-bound IgE induces the release of various chemical mediators that cause allergenic reactions. Therefore, inhibition of IgE production could prove as a useful method in the treatment of allergic diseases. IgE synthesis is controlled by helper T (Th) cells that can be classified into two subsets on the basis of cytokine profiles. Type-2 Th (Th2) cells secrete IL-4 that plays a central role in IgE production (Coffman et al. 1988; Pene et al. 1988) and induces IgE isotype class switching. Furthermore, IL-5 and IL-6, which are also secreted by Th2 cells, also enhance IgE production. As opposed to these cytokines, IFN-γ secreted from type-1 Th (Th1) cells suppresses IgE production, inhibits the proliferation of Th2 cells and interferes with IL-4-induced class switching. Mediators of the Th1-type immune response such as IFN-γ, can suppress IgE production as a result of inhibition of the Th2-type response. Regulation of the balance between these two responses is considered to be an important way of both preventing and treating allergic diseases.

In the present study, we examined the immunological effects of amaranth grain on cytokine and IgE production *in vivo* and *in vitro*. In order to evaluate the potential of orally administered amaranth powder to down-regulate IgE production *in vivo* through Th1 cytokine induction, we utilized the ovalbumin $(OVA)_{323–339}$-specific $\alpha\beta$T cell receptor (TCR) transgenic mouse (OVA-TCR Tg) as an animal model of an orally-induced, allergen-specific IgE response (Shida 2000).

Materials and methods

Animals

OVA-TCR Tg mice, OVA 23-3, (female, 6–8 weeks old), transgenic for $OVA_{323–339}$-specific I-A^d-restricted $\alpha\beta$TCR with a BALB/c genetic background (Sato et al. 1994), were used for IgE and cytokine assay after oral administration of OVA, and *in vitro* Th cell development assay. BALB/c mice (female, 6–8 weeks old) purchased from Japan CLEA (Tokyo, Japan) were used as source of splenocytes for antigen presenting cells and splenocytes in the *in vitro* IgE production assay. All experiments were performed in according to the guidelines of the animal laboratory of The University of Tokyo.

Diets

OVA diet (control diet) was composed of 8% albumin from eggs (Wako pure chemical industries, Ltd., contained OVA at 50%), 12.0% casein, 48.17% corn starch, 9% α-starch, 5% sucrose, 5% cellulose, 6% soy bean oil, 5% mineral mixture, 1.3% vitamin mixture, 0.23% choline chloride, and 0.3% methionine. An amaranth powder-containing diet (AM diet) was prepared by adding roasted amaranth powder, which was prepared by industrial crushing and enzyme treatment, at 10% to the above diets in place of casein, cornstarch, and soy bean oil. The final composition of the AM diet was 10% amaranth powder, 8% egg albumin, 10.9% casein, 39.7% corn starch, 9% α-starch, 5% sucrose, 5% cellulose, 5.57% soy bean oil, 5% mineral mixture, 1.3% vitamin mixture, 0.23% choline chloride, and 0.3% methionine. The diets were prepared by Funabashi Farm (Chiba, Japan).

Experimental design for examining effects of oral administration of amaranth

Female OVA-TCR Tg mice were fed the AM diet or control diet. After 14 days, the animals were sacrificed, and spleen cells were taken from each individual mouse and examined for cytokine production *in vitro*. Blood was collected from the tail vein at 14 days, and serum OVA-specific IgE was detected by ELISA.

Spleen cell culture

Spleen cells (2.5×10^6/ml) were stimulated with 1 mg/ml or 0.1 mg/ml OVA (2× crystallized, Seikagaku Co., Tokyo, Japan) in 1 ml of RPMI-1640 medium supplemented with 5% fetal bovine serum (FCS), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin in 48-well culture plates. The cells

were incubated at 37 °C for 48 h in a CO_2 incubator.

Amaranth sample preparations for in vitro *assay*

For *in vitro* assay, defattened amaranth sample was prepared by crushing untreated amaranth grain with food mills after which fat was removed by treatment with acetone. This amaranth preparation was suspended in distilled water at a concentration of 100 mg/ml, autoclaved at 121 °C for 20 min, then centrifuged for 20 min at 800 × *g*, and the supernatant collected. Finally, the supernatant was passed through 0.45 μm filters. The liquid was lyophilized and re-suspended in Dulbecco's phosphate-buffered saline before use.

Th cell development assay

The effect of amaranth samples on Th phenotype development was studied with naive splenic T cells from OVA-TCR Tg mice (Hsieh et al. 1993). Splenocytes were prepared from non-immunized TCR Tg mice. $CD4^+$ T cells were isolated from these cells using MACS (Miltenyi Biotec, Bergish Gladbach, Germany) and the cells obtained were more than 90% CD4 positive as indicated by flow cytometry (FACSort, BD Pharmingen, San Diego, CA). The $CD4^+$ T cells (2.5×10^5/ml) were cultured in 2 ml of culture medium in 24-well plates with OVA (0.1 mg/ml) in the presence or absence of amaranth extract (100 μg/ml) plus mitomycine C (50 μg/ml, Sigma, St. Louis, MO) treated BALB/c splenocytes (1.2×10^6/ml) as antigen presenting cells (primary culture). In order to determine the developing Th phenotype, cultured T cells were harvested on day 7, washed, and re-stimulated at 2.5×10^5/ml in 1 ml in 48-well plates with OVA (0.1 mg/ml) plus mitomycine C-treated BALB/c splenocytes (1.25×10^6/ml) as antigen presenting cells. The supernatants of these cultures were collected at 48 h for quantification of cytokines by ELISA.

In vitro *IgE production assay*

Naive splenic $CD4^+$ T cells from OVA-TCR Tg mice were isolated using MACS. The $CD4^+$ T cells (2.5×10^5/ml) were cultured in 24-well plates with OVA (0.1 mg/ml) and recombinant murine IL-4 (5 ng/ml, Peprotech, Rocky Hill, NJ), plus antigen presenting cells for 1 week in order to induce differentiation of Th cells. After 1 week, T cells were washed and re-stimulated in a 48-well plate with OVA (0.1 mg/ml) plus freshly isolated BALB/c splenocytes (2.5×10^6/ml) in the presence or absence of amaranth samples (100 μg/ml) for 3 days with 10% FCS. The cultured cells were harvested, washed to remove OVA and cultured without OVA in the presence or absence of amaranth samples (100 μg/ml) for a further 4 days. Supernatants were collected for determination by ELISA.

OVA-specific IgE ELISA

Anti-OVA specific IgE levels were detected by ELISA as previously described (Shida et al. 2000). A Maxisorp immunoplate (Nunc, Rosklide, Denmark) was coated with rat anti-mouse IgE monoclonal antibody (clone R35-92, Pharmingen). After blocking with bovine serum albumin (Seikagaku Co.), diluted sample and standard mouse serum were added. Bound antibody was detected by biotinylated OVA, streptavidin–alkaline phosphatase conjugates (Zymed, South San Francisco, CA), with a substrate of *p*-nitrophenylphosphate disodium salt.

Cytokines ELISA

IFN-γ, IL-4 and IL-5 levels in culture supernatants were measured by ELISA. Rat anti-mouse IFN-γ (R4-6A2; Pharmingen), IL-4 (clone BVD4-1D11, Pharmingen) and IL-5 (clone TRFK5, Pharmingen) monoclonal antibodies were used as the capture antibody, and biotin-conjugated rat anti-mouse IFN-γ (clone XMG1.2, Pharmingen) IL-4 (clone BVD6-24G2, Pharmingen) and IL-5 (clone TRFK4, Pharmingen) monoclonal antibodies as the detection antibodies, respectively.

Statistics

Differences between mean values were analyzed by Student's *t*-test. Values of p less than 0.05, which were calculated as two-tailed p values, were considered statistically significant.

Results

Effect of oral administration of roasted amaranth on levels of OVA-specific IgE in peripheral blood in OVA-induced IgE response model

OVA-TCR Tg mice were fed either the control OVA-containing diet, which contained 8% OVA, or the AM diet, which contained 10% amaranth powder and 8% OVA. There was no effect of oral administration of amaranth powder on dietary food intake (data not shown). Body weight was measured for 60 days, but there was no difference between the AM diet group and the control diet group (data not shown). It has been previously shown that feeding an OVA-containing diet to OVA-TCR Tg mice elicits an OVA-specific IgE response (Shida et al. 2000). OVA-specific IgE production in the peripheral blood of the two groups was compared at 14 days. Figure 1 demonstrates that the level of OVA-specific IgE antibody in the blood was significantly decreased in the group fed amaranth powder compared with the control group.

Effect of oral administration of roasted amaranth on cytokine production by splenocytes from OVA fed OVA-TCR Tg mice

We next assessed the OVA-specific IFN-γ production by spleen cells from OVA-TCR Tg mice fed the AM diet or control diet for 14 days. The spleen cells of these mice were stimulated with OVA (0.1 and 1 mg/ml), and IFN-γ and IL-4 production in culture medium was determined by ELISA. The results demonstrate that IFN-γ production in OVA-stimulated spleen cells was significantly ($p < 0.05$) increased in the AM diet group when compared with the control diet group (Figure 2). There was no difference in IL-4 production in cultured splenocytes from either the AM diet group or the control group. IFN-γ production was clearly increased at both doses of 0.1

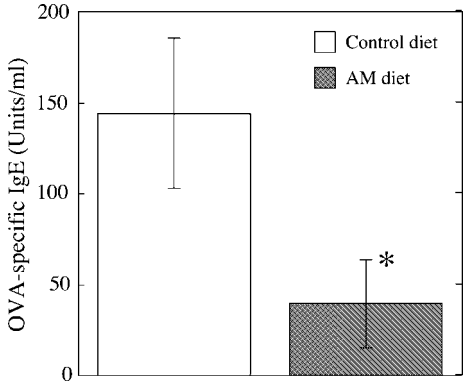

Figure 1. Effect of oral administration of roasted amaranth powder on OVA-specific IgE production in sera of OVA-specific TCR-Tg mice. OVA-specific TCR-Tg mice were orally administered with 10% amaranth and 8% OVA (AM diet), or 8% OVA containing diet (control diet). Blood samples were collected at day 14. OVA-specific IgE levels were determined by ELISA, $n = 5$, $*p < 0.05$. Similar results were obtained in three independent experiments.

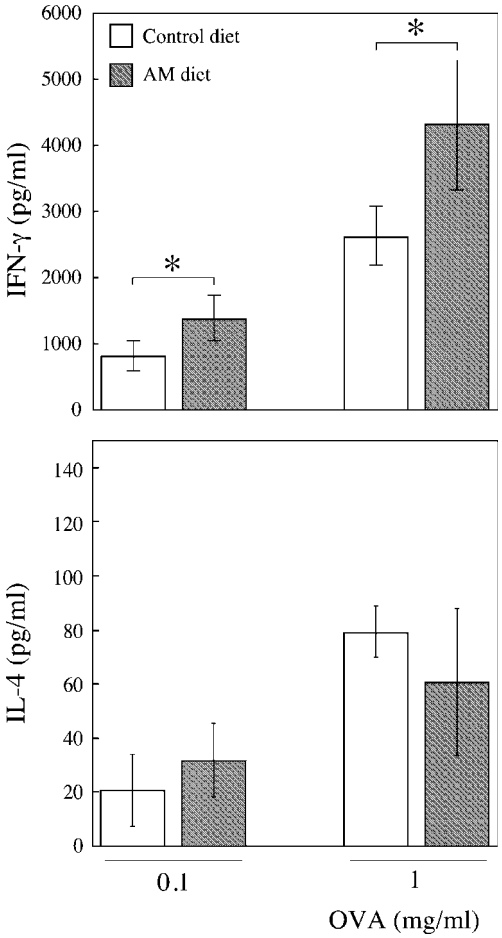

Figure 2. Effect of oral administration of roasted amaranth powder on IFN-γ and IL-4 production of OVA-specific TCR-Tg mice splenocytes. Splenocytes were isolated from OVA-specific TCR-Tg mice that were orally administered with 10% amaranth and 8% OVA (AM diet), or 8% OVA-containing diet (control diet). Splenocytes (2.5×10^6/ml) were cultured in the presence of OVA (0.1 and 1 mg/ml) for 48 h. IFN-γ and IL-4 levels in culture supernatants were determined by ELISA, $n = 5$, $*p < 0.05$. Similar results were obtained in three independent experiments.

and 1 mg/ml OVA, but no difference was observed between the two groups when cultured in the absence of OVA. There were no significant differences between the control group and the AM group in expression of surface markers of CD4 helper T cells such as CD25, CD44, CD45RB, CD62L, and CD69 (data not shown).

Amaranth grain extract causes the development of CD4$^+$ T cells into Th1 phenotype

To examine whether amaranth grain extract promotes Th1 phenotype development, we utilized a Th cell development assay system with naive T cells from OVA-TCR Tg mice. Primary cultured T cells which were isolated from non-stimulated OVA-TCR Tg mice were stimulated with OVA plus irradiated BALB/c splenocytes as antigen-presenting cells in the absence or presence of amaranth grain extract. The culture supernatants were assayed for cytokines at different time points during primary culture (Figure 3a). IFN-γ production at 48 h was increased in the presence of amaranth grain extract compared with control, and the magnitude of this difference increased as the period of culture progressed. Thereafter, in order to determine the type of Th

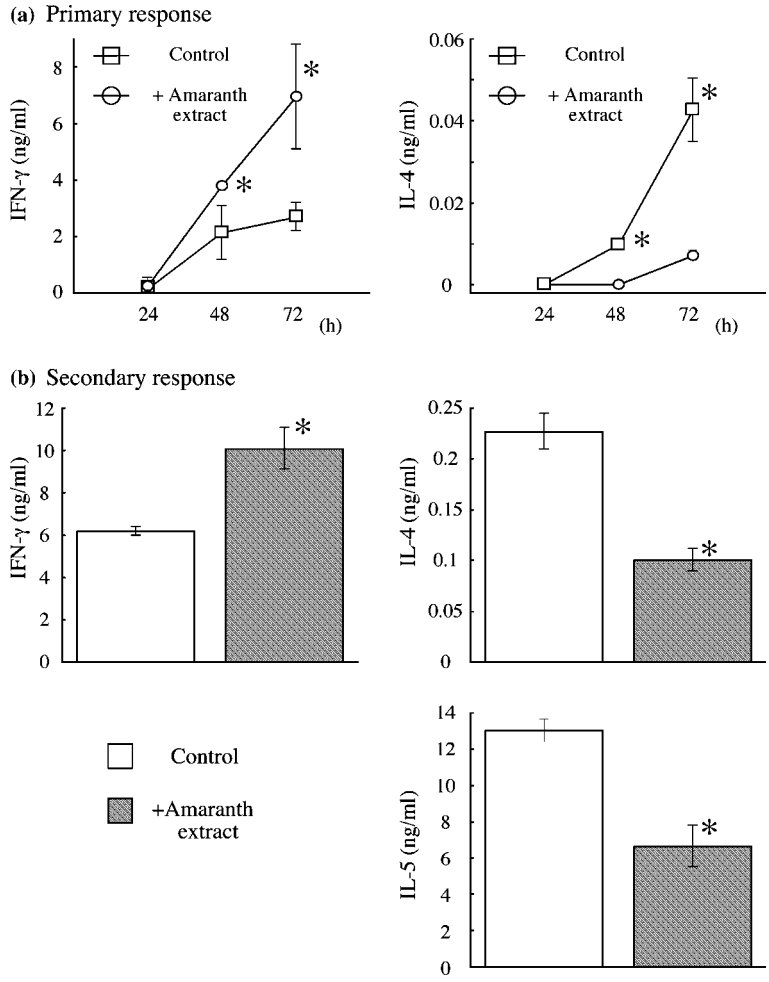

Figure 3. Induction of Th1 development by amaranth extract. TCR-Tg CD4$^+$ T cells were stimulated with OVA (0.1 mg/ml) plus mitomycine C-treated BALB/c mice splenocytes in absence (control) or presence of amaranth extract. (a) Culture supernatants were collected at 48 h and assayed for IFN-γ and IL-4 production. (b) Cultured T cells were harvested on day 7, and re-stimulated with OVA (0.1 mg/ml) plus mitomycine C-treated BALB/c splenocytes without additives. IFN-γ, IL-4 and IL-5 levels in culture supernatants were determined by ELISA. *$p < 0.05$ versus control. Similar results were obtained in three independent experiments.

cells present in these cultures, the resulting Th cells were re-stimulated without the additives and the cytokines in supernatants were assayed (Figure 3b). Addition of amaranth grain extract to the primary cultures initially resulted in the development of Th1 dominant cells that produced increasing amounts of IFN-γ and diminishing amounts of IL-4 and IL-5 in the secondary culture.

Amaranth grain extract inhibits antigen-specific IgE production in a co-culture system with antigen-specific Th cells and naïve BALB/c splenocytes

To investigate the effect of amaranth grain extract on antigen-specific IgE production, freshly isolated splenocytes from untreated BALB/c mice were co-cultured with OVA-specific Th cells induced *in vitro* from TCR-Tg CD4$^+$ T cells and OVA in the absence or presence of the amaranth grain extract. To detect OVA-specific IgE, after stimulation with OVA for 3 days, OVA in the culture medium was removed and culture was continued for a further 4 days after which the level of antigen-specific IgE in collected medium was determined by ELISA. The amaranth grain extract suppressed antigen-specific IgE production *in vitro* (Figure 4). The data indicated that there is a component in amaranth grain, which has a suppressive effect on IgE production by splenocytes.

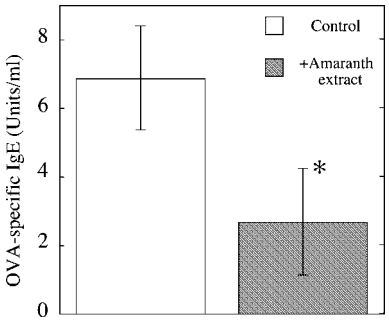

Figure 4. Effect of amaranth extract on antigen-specific IgE production from freshly isolated BALB/c splenocytes co-cultured with antigen-specific Th cells. Th cells induced *in vitro* from TCR-Tg CD4$^+$ T cells (4×10^6/ml) and freshly isolated BALB/c splenocytes (4×10^6/ml) were stimulated in a 48-well plate with OVA (0.1 mg/ml) in the presence or absence of amaranth extract for 3 days. The cultured cells were harvested, washed to remove OVA and cultured without OVA with additives for a further 4 days. OVA-specific IgE were determined by ELISA. *$p < 0.05$ versus control. Similar results were obtained in two independent experiments.

Discussion

The level of antigen-specific IgE in peripheral blood is closely related to the presence of allergic disease. IgE is one of the most important mediators of immediate hypersensitivity; it binds to the high-affinity Fc epsilon receptor I (FcεRI) on mast cells, triggering a release of chemical mediators such as histamine from their granules. Recently, it was shown that IgE regulates survival of mast cells through the FcεRI (Asai et al. 2001). Furthermore, mast cells stimulated by antigen plus IgE release arachidonic acid metabolites such as prostaglandin and leukotriene, and synthesize several cytokines (Mekori and Metcalf 2000). There have been many reports demonstrating the suppression of IgE and Th2 cytokines through augmentation of the Th1 type response by food-related substances such as lactobacilli (Murosaki et al. 1998; Shida et al. 1998), nucleotides (Nagafuchi 2000), raffinose (Nagura et al. 2002) and chitin particles (Shibata et al. 2000).

In this study, we observed the effect of orally administered amaranth grain on experimental allergy models, in which increased allergen-specific IgE levels and Th2 type cytokine production is observed. OVA-TCR Tg mice fed roasted amaranth powder, which was prepared by industrial crushing and enzyme treatment, exhibited significantly ($p < 0.05$) lower anti-OVA specific IgE levels compared with those fed control diet. These changes were accompanied by augmented IFN-γ production by splenocytes, although IL-4 production was not affected. On the other hand, our experiments indicated that the level of expression of CD25, CD44, CD45RB, CD62L and CD69 on the surface of CD4$^+$ T cells did not differ between the AM diet and the control diet group. Generally, CD25 and CD69 are considered to be activated T cell markers, whereas CD62L and CD45RB are naive T cell markers, and CD44 is a memory T cell marker in helper T cells. These results indicate that changes in the ratio of activated or memory T cells did not contribute to the augmentation of the Th1 response in the case of orally administered amaranth powder.

To substantiate the effect of amaranth powder on antigen-specific IgE production and IFN-γ

secretion, we investigated the IgE production and helper T cell differentiation in the presence of a water-soluble extract from defatted amaranth grain *in vitro*. When $CD4^+$ T cells from the spleen of non-sensitized OVA-TCR Tg mice were pre-cultured with recombinant murine IL-4 and OVA, they differentiate into a Th2 dominant population: they exhibit a high level of IL-4 and a low level of IFN-γ production. These Th2-dominant cells and freshly isolated spleen cells from BALB/c mice were co-cultured with OVA in the presence or absence of AM. In this system the B cells in the naive splenocytes were induced by Th cells to secrete antigen-specific IgE. The data indicate that amaranth inhibits OVA-specific IgE production induced in the OVA stimulated co-culture system of Th2-dominant cells and naive splenocytes (Figure 4). This inhibition of IgE was most probably through promoting a Th1 type cytokine production. The results of the Th cell development assay with naive $CD4^+$ T cells from OVA-TCR Tg mice clearly showed the extract of amaranth grain induced Th1 development (Figure 3). In the primary response as well, IFN-γ secretion was augmented while IL-4 secretion was inhibited in the presence of amaranth. Taken together, the results of the *in vitro* experiments suggest that the oral intake of amaranth powder inhibited serum IgE levels through enhancement of IFN-γ production and development of Th1 cells. In another report, an acidic polysaccaharide extracted from the seed of *Celosia argentea*, which is related to amaranth, enhanced IFN-γ production from naive BALB/c mice splenocytes in the presence of mitogen (Hase et al. 1997). There is some possibility that a similar substance is also found in amaranth grain. In any case, our experimental data demonstrated that the substance induced a Th1 type response in an antigen-specific manner.

In conclusion, we found that the OVA-specific IgE antibodies were decreased in the blood of OVA-TCR Tg mice which were fed a diet containing amaranth powder. Furthermore, we observed similar effects of an extract of amaranth grain on OVA-specific IgE production and cytokine production by cultured murine splenocytes. These data show that amaranth grain, or its extract, inhibits antigen-specific IgE production through the induction of dominant Th1 cytokine profiles *in vivo* and *in vitro*. This phenomenon may prove to be a useful tool in the treatment of allergic disease.

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