



Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies

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ABSTRACT

Background: Food Allergy Herbal Formula-2 (FAHF-2) prevents anaphylaxis in a murine model of peanut allergy. Multiple food allergies (MFA) are common and associated with a higher risk of anaphylaxis. No well-characterized murine model of sensitization to multiple food allergens exists, and no satisfactory therapy for MFA is currently available.

Objective: To determine the effect of FAHF-2 in a murine model of MFA.

Methods: C3H/HeJ mice were orally sensitized to peanut, codfish, and egg concurrently. Oral FAHF-2 treatment commenced 1 day after completing sensitization and continued daily for 7 weeks. Mice were subsequently orally challenged with each allergen.

Results: Antibodies in sera from mice simultaneously sensitized with peanut, codfish, and egg recognized major allergens of all 3 foods, demonstrating sensitization to multiple unrelated food allergens (MFA mice). Sham-treated MFA mice exhibited anaphylactic symptoms accompanied by elevation of plasma histamine and hypothermia. In contrast, FAHF-2-treated MFA mice showed no anaphylactic symptoms, normal body temperature, and histamine levels after challenge with each allergen. Protection was accompanied by reduction in allergen-specific immunoglobulin E levels. Allergen-stimulated Th2 cytokine interleukin-4 and interleukin-13 production levels decreased, whereas the Th1 cytokine interferon- γ levels were elevated in cultured splenocytes and mesenteric lymph node cells in FAHF-2-treated mice.

Conclusion: We established the first murine model of MFA. FAHF-2 prevents peanut, egg, and fish-induced anaphylactic reactions in this model, suggesting that FAHF-2 may have potential for treating human MFA.

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Introduction

Allergy to multiple foods does not appear to be rare.¹ Although investigation into multiple food allergies (MFA) is limited, a clinical study by Wang et al² found that 13 of 19 (68%) participants were allergic to at least 3 foods, and 15 of 19 (80%) had allergy to at least 2 different foods.² A study in a school setting by McIntyre et al³ found that approximately two thirds of anaphylactic reactions occurred in students with multiple food allergies, and students with multiple allergies (including foods) had more serious grades of reactions.³ MFA is also more strongly associated with atopic disease, as shown in the study by Schroeder et al,⁴ in which children with symptomatic food allergy to multiple foods exhibited a higher

incidence of asthma.⁴ Strict avoidance is the only option for managing food allergy, and as expected, MFA poses more obstacles to disease management. Planning diets to accommodate avoidance of multiple foods is challenging and may impact a patient's nutritional status.^{5,6} Additionally, risk for accidental ingestion is greater in MFA, creating higher anxiety levels for patients and their families.^{7,8} A recent study reported that parental burden scores increased significantly as the number of foods the child reacted to increased.⁹

Anti-IgE administration is beneficial in a limited population of food-allergic patients but has recently been reported to have safety concerns.^{10,11} Oral or sublingual immunotherapy using multiple foods in the same individual has not been tested. These findings suggest that MFA individuals represent a more vulnerable yet understudied population of food-allergic individuals, and therapies for MFA are urgently needed.

Although murine models are not identical to human diseases, those closely mimicking human food allergy such as peanut allergy are useful tools for initial investigation of potential therapies for food allergy.^{12–16} We previously showed that Food Allergy Herbal Formula-2 (FAHF-2) protected against peanut-induced anaphylactic symptoms in the murine peanut (PN) allergy model^{17,18} and that

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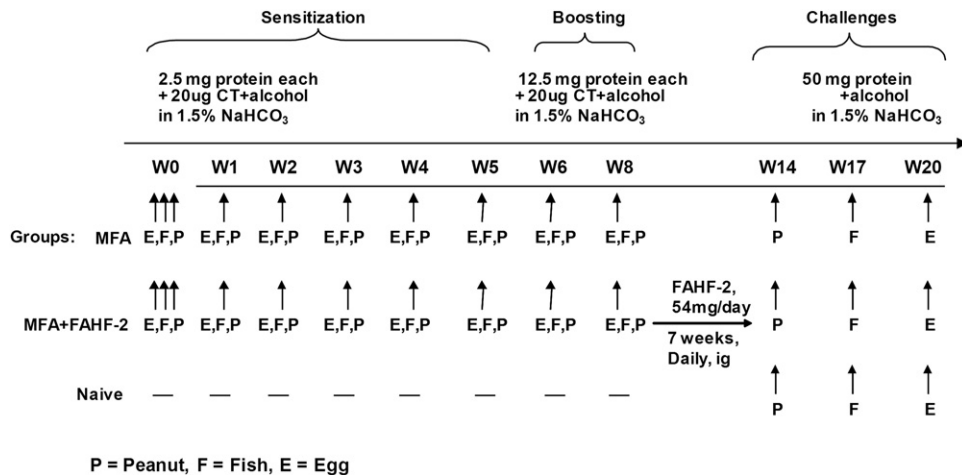


Fig. 1. Generation of MFA mice. C3H/HeJ mice were orally sensitized with a mixture of peanut, codfish, and egg along with cholera toxin (CT). After boosting at week 8, some mice were given daily oral treatment with FAHF-2 for 7 weeks. Untreated mice received water (vehicle). Naive mice were not sensitized or treated. All mice were challenged with peanut at week 14, fish at week 17, and egg at week 20 ($N = 5$ mice per group).

this protection was long lasting.¹⁹ Mechanisms underlying therapeutic efficacy of FAHF-2 included suppression of PN-specific IgE and PN-stimulated Th-2 cytokine production along with increased specific-IgG2a production. A key feature of FAHF-2 therapy is persistent elevation of antigen-specific interferon- γ (IFN- γ) production, which we found to be attributable mainly to CD8-T cells.^{17,19} Our previous study also showed that neutralization of IFN- γ or depletion of CD8+ T cells by co-administration of anti-IFN- γ or anti-CD8 antibodies blocked FAHF-2 suppression of IgE and Th2 cytokine production, but not protection from anaphylaxis that persisted up to 4 weeks after therapy.⁵ We recently found that FAHF-2 directly inhibits degranulation of murine mast cells and basophils, suggesting that FAHF-2-mediated protection from anaphylaxis is multifaceted.²⁰ FAHF-2 also has a high safety profile.^{18,19} Our recent controlled phase I trial in patients with peanut and multiple food allergies also showed that FAHF-2 was safe and well tolerated over a 1-week course of treatment and that in vitro treatment of patient peripheral blood mononuclear cells with FAHF-2 reduced IL-5 and increased IFN- γ and IL-10 secretion.²¹ Consistent with the findings in mouse model of peanut allergy, a 6-month extended phase I study showed that FAHF-2 treatment reduced peripheral blood basophil activation by *ex vivo* antigen stimulation.²² Given the broad immunotherapeutic effects of FAHF-2, we hypothesized that FAHF-2 may be effective for treating MFA. However, an animal model that mimics human MFA is as of yet unreported.

In this study, we first established a murine model of MFA created by concurrent oral sensitization of C3H/HeJ mice with 3 common but unrelated allergenic foods: peanut, codfish, and egg, and investigated whether FAHF-2 treatment of MFA mice protected against anaphylaxis after separate oral challenges with PN, fish, and egg allergens.

Methods

Mice

Five-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, Maine) were maintained in pathogen-free facilities at the Mount Sinai vivarium on PN-free chow according to standard guidelines for the care and use of animals.²³

Concurrent sensitization to peanut, codfish, and egg

Mice were intragastrically sensitized with homogenized peanut, codfish, and egg containing 2.5 mg protein for each food (extract preparation described in eMethods), in 0.5 mL phosphate-

buffered saline (PBS) containing 75 mg sodium bicarbonate, 20 µg of the mucosal adjuvant cholera toxin, 16.5 µL (1.1 µL/g body weight) 80 proof Stolichnaya Vodka (Allied DOMECQ Spirits, Westport, Connecticut), a source of food grade ethanol) to neutralize stomach pH and to increase gastrointestinal permeability,²⁴ weekly from weeks (w) 0 to 5 (Fig 1). Mice were boosted with homogenized peanut, codfish, and egg containing 12.5 mg protein for each food as described. Peanut, fish, and egg concurrently sensitized mice, and naive mice ($N = 5$ mice/group) were challenged intragastrically separately with PN, fish, and egg allergens (50 mg protein each) at 3-week intervals beginning with PN at week 14 then codfish at week 17 and finally egg at week 20 (Fig 1). Three-week intervals were used to ensure that reactions to the preceding challenge were completely resolved. Challenge solution did not include cholera toxin. Mice were given a sensitization dose of fish and egg as described, 48 hours after PN challenge, and a sensitization dose of egg 48 hours after fish challenge to maintain reactivity over the duration of the experiment.

FAHF-2 formula and treatment

FAHF-2 was obtained from Xiyuan Chinese Medicine Research and Pharmaceutical Manufacturer, Chinese Academy of Chinese Medicine Sciences, Beijing China. Herbal components of FAHF-2 were identified as *Prunus mume*, *Zanthoxylum schinifolium*, *Angelica sinensis*, *Zingiber officinale*, *Cinnamomum cassia*, *Phellodendron chinense*, *Coptis chinensis*, *Panax ginseng*, and *Ganoderma lucidum*. Extensive quality control, analytical chemistry data, and batch consistency of the formula have been published previously.^{21,22} Endotoxin level in FAHF-2 was measured using Pyrogen Plus assay kit (Lonza, Hopkinton, Massachusetts) and found to be below 0.03 EU/mL, which is the limit of sensitivity for this kit. FAHF-2 was administered intragastrically at 27 mg/in 0.5 ml drinking water twice per day for 7 weeks. The daily dose of FAHF-2 used in this study was determined by a conversion table of equivalent human-to-animal dose ratios based on body surface area.²⁵ FAHF-2 treatment commenced at week 8.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of peanut, cod fish, and egg proteins

Gel separation of food proteins was done using standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques followed by visualization of transferred bands by stain-

ing with SimplyBlue (Invitrogen, Grand Island, New York) as described in the eMethods.

IgE immunoblot

Immunolabeling was performed using pooled sera from MFA mice harvested before week 14 challenge as shown in the protocol (Fig 1). Normal mouse sera were used as a negative control. The detailed protocol is described in the eMethods.

Preliminary identification of codfish IgE-binding proteins

Bands corresponding to IgE-binding proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel were excised and sent to the Wistar Institute, (Philadelphia, Pennsylvania), for identification by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS).

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30 to 40 minutes after oral challenge. Visually observed symptoms were scored, using the scoring system described previously²⁶(0): no symptoms; (1) scratching and rubbing around the snout and head; (2) puffiness around eyes and snout, pilar erecti, reduced activity, or decreased activity with increased respiratory rate; (3) wheezing, labored respiration, cyanosis around mouth and tail; (4) no activity after prodding, or tremor and convulsion; (5) death. Cage identities were concealed during visual assessment of anaphylactic symptoms. Rectal temperatures were measured after scoring using a rectal probe (Harvard Apparatus, Holliston, Massachusetts).

Measurement of plasma histamine levels

As described previously, blood was collected immediately after recording symptoms, and plasma was harvested within 20 minutes of blood collection and stored at -80°C until used.¹⁸ Histamine was measured using an enzyme immunoassay kit (ImmunoTECH Inc., Marseille, France), as described by the manufacturer.

Measurement of food specific-IgE and IgG2a. Peanut, cod, and egg-specific IgE and IgG2a levels in serum from the same sensitized mice were measured as reported previously¹⁷ and are described in eMethods.

Cell culture and cytokine measurements

After final challenge at week 20, splenocytes from individual animals and pooled MLN cells were prepared as previously described and cultured in the presence or absence of 50 μg PN, codfish, or egg protein extracts.¹⁷ Some cultures were stimulated with concanavalin A (2.5 $\mu\text{g}/\text{mL}$; data not shown).¹⁷ Culture supernatants were collected after 72 hours. Cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D systems, Minneapolis, Minnesota, for IL-13, BD Biosciences, San Jose, California, for all others) and reported after subtraction of basal cytokine levels of cultures stimulated with medium alone.

Statistical analysis

Data were analyzed using SigmaStat statistical software (Systat Inc., Chicago, Illinois). Symptom scores, plasma histamine, body temperature, and immunoglobulin data were analyzed by one way ANOVA on ranks followed by Student-Newman-Kuels posttest. Splenocyte cytokine levels were analyzed by one-way ANOVA followed by Bonferroni's *t* test. *P* values $< .05$ were considered significant. The minimal number of mice in each group required to detect statistically significant differences in symptom scores is 4 to 5, as determined by power analysis (power, 0.8, with $\alpha = .05$).

Results

Mice concurrently sensitized to peanut, egg, and codfish generated IgE against all three food allergens

Peanut, egg, and codfish specific IgE levels in sera from sensitized individual mice were detected by ELISA and compared with naïve mice at week 14 before challenge. Specific IgE to peanut, codfish, and egg was detected in serum of MFA mice but not in

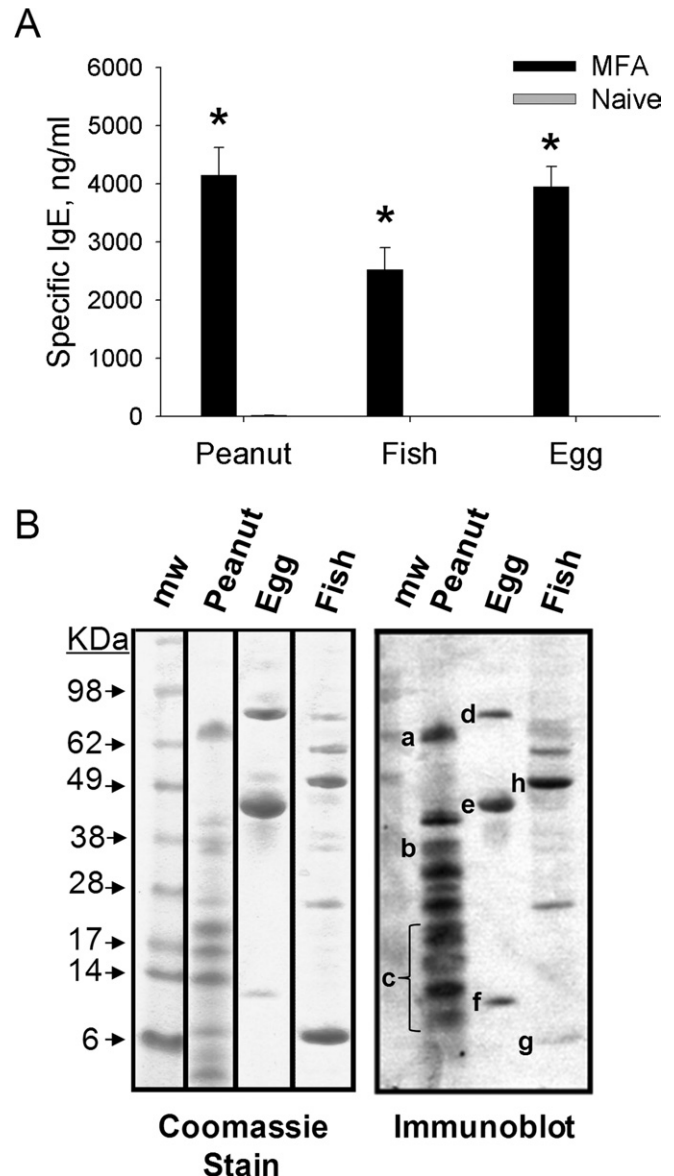
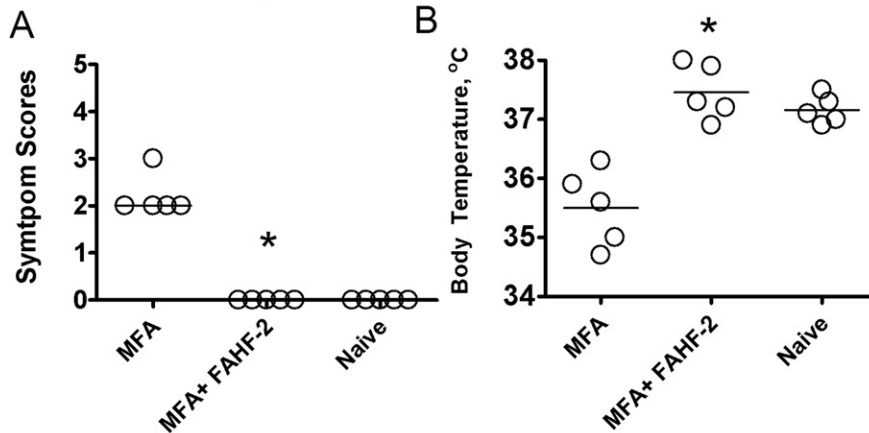


Fig. 2. Food-specific IgE and SDS-PAGE of protein extracts and IgE immunoblot. (A) Serum IgE specific to peanut, codfish, and egg were detected in serum of mice tested individually in duplicate by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean $\pm$ SEM. **P* $< .05$, *N* = 5 mice/group. (B) Protein extracts of peanut, codfish, and egg were analyzed by SDS-PAGE, and bands were observed by Coomassie blue stain (left panel). After transfer of proteins to nylon membranes, IgE binding (right panel) was evaluated by immunoblotting with pooled sera from multiple food-allergic mice followed by detection using horseradish peroxidase (HRP)-labeled anti-mouse IgE antibodies and chemiluminescence. Band-a: immunobinding at known molecular weight for Ara h 1; Band-b: immunobinding at known molecular weight for Ara h 4; Bands included in c: likely binding to Ara h 2, 5-11; Band-d: immunobinding at known molecular weight for conalbumin; a, Band -e: Likely immunobinding to ovalbumin and ovomucoid; Band-f: Binding at known molecular weight for Lysozyme; Band- g: immunobinding to parvalbumin confirmed by LC-MS/MS; Band-h: b: immunobinding to protein that is a close match for salmon and puffer fish enolase by LC-MS/MS analysis followed by SEQUEST search.

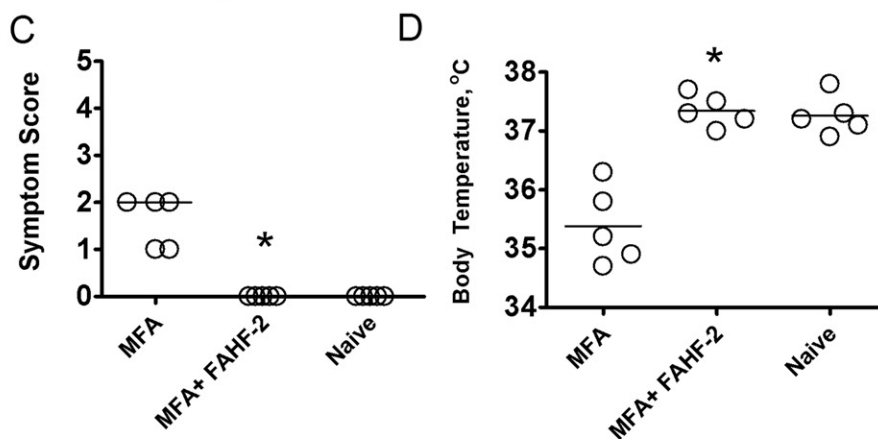
naïve animals ($P < .05$, Fig 2A). The IgE binding patterns detected by immunoblot (Fig 2B, right panel) corresponded well to established food allergens recognized by humans, as catalogued by the International Union of Immunological Sciences' Allergen Nomenclature Sub-Committee.²⁷ We observed more protein (Fig 2B, left panel) and IgE-binding bands for PN than for egg white or codfish. The IgE immunoreactivity to PN in MFA mice appears to be in good agreement with the human profile, as bands were observed at or very

close to the established molecular weight for Ara h 1 (64 kDa, band-a) and Ara h 4 (37 kDa, band-b). Multiple immunoreactive bands were identified between 17 and 6 kDa (bands indicated by c). These are likely to include binding to the major peanut allergen Ara h 2 or minor allergen Ara h 8 (17 kDa), Ara h 5, and Ara h 6 (15 kDa for both), Ara h 11 (14 kDa), and Ara h 9 (9.8 kDa). IgE binding bands to egg white proteins were consistent with previously described egg allergens recognized by humans, namely, conalbumin (78 kDa,

Peanut Challenge



Fish Challenge



Egg Challenge

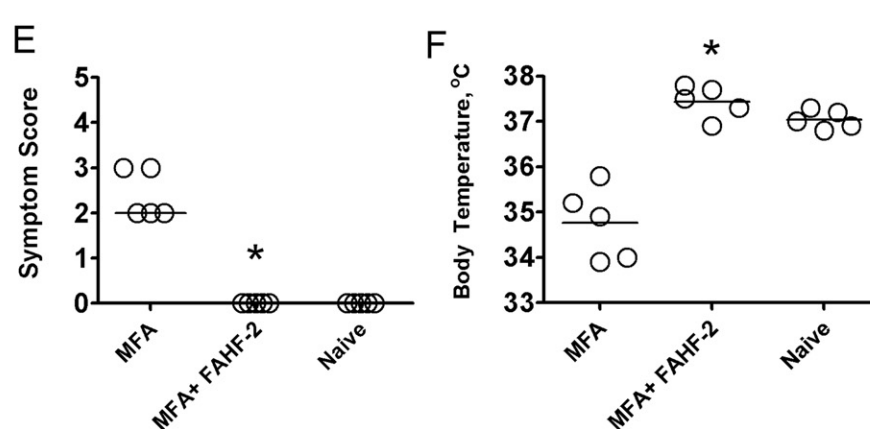


Fig. 3. Anaphylactic responses. (A, C, E) Symptoms were visually assessed 30 minutes after challenge and assigned scores based on system described in the Methods. Bars represent group medians. (B, D, F) Body temperatures were measured after scoring by rectal thermometer. Bars represent group means. * $P < .05$; $N = 5$ mice/group.

band-d) ovalbumin (28 kDa), and ovomucoid (44 kDa), which run together on the gel at approximately 40 kDa (band-e), as also reported by others,²⁸ and lysozyme (14 kDa, band-f). The known major allergen in cod is the 12 kD protein, parvalbumin (Gad c 1).²⁹ We suspected that the IgE-binding band observed at approximately 6 kDa (band-g) was parvalbumin, and there was an unknown band with strong IgE binding at 49 kD (band-h). Tandem LC-MS/MS analysis confirmed that the 6-kD band was indeed parvalbumin, and the 49-kD band was indentified to be a close match with salmon enolase and related to enolases of zebrafish (*Danio rerio*) and puffer fish (*Tetraodon nigroviridis*) when queried against known protein sequences by SEQUEST search.

FAHF-2 prevented anaphylaxis in MFA mice after oral challenges with PN, fish, and egg

MFA and naïve mice were subjected to separate oral food challenges with PN, fish, and egg at 3-week intervals beginning with PN at week 14 (Fig 1). Consistent with our previous publications on murine model of PN-allergy,^{17–19} untreated MFA mice showed obvious symptoms of anaphylaxis, lower core body temperatures than naïve mice, indicating systemic anaphylaxis (Fig 3A, B). A similar trend was observed in untreated mice challenged 3 weeks later with fish (week 17) and further 3 weeks later with egg (week 20) (Fig 3C–F), indicating MFA. Seven weeks of FAHF-2 treatment completely protected MFA mice against symptoms of anaphylaxis and decreased core body temperatures. In addition, plasma histamine levels, which are elevated during anaphylaxis, were dramatically lower in FAHF-2-treated MFA mice as compared with untreated mice (Fig 4A–C). Together these data show that FAHF-2 treatment completely protected MFA mice from anaphylactic reactions to all the foods against which they were sensitized.

FAHF-2-treated MFA mice showed sustained suppression of food-specific IgE and increased IgG2a levels

We previously showed that FAHF-2 therapeutic effects are associated with decreased allergen-specific IgE and elevated allergen-specific IgG2a levels.^{17,19} In the present study, we found that peanut, fish, and egg IgE levels were significantly reduced after FAHF-2 treatment at week 12 (Fig 5A, C, E). This reduction persisted throughout a 14-week posttherapy period. In contrast, all food-specific IgG2a antibodies were elevated in FAHF-2-treated mice as compared with untreated animals (Fig 5B, D, F). These results show that treatment exerted a similar beneficial anti-allergic effect on immunoglobulin profiles of all foods to which they were allergic, and that FAHF-2 is an effective antigen-nonspecific therapy in this model.

FAHF-2-treated MFA mice showed reduced Th-2 Cytokine and Increases interferon- γ responses

Cytokine levels in splenocyte or mesenteric lymph node cultures from untreated MFA mice showed increased production of Th-2 cytokines IL-4 and IL-13 in response to restimulation with peanut, fish, and egg proteins (Figs 6 and 7) as compared with naïve mice, which was consistent with their allergic status, because IL-4 and IL-13 are well-established pro-allergic cytokines.^{30,31} Restimulation with fish yielded slightly lower cytokine levels as compared with peanut and egg. FAHF-2 treatment significantly reduced splenocyte culture IL-4 and IL-13 levels in response to restimulation with all food antigens ($P < .05$ – 0.01). A similar trend of FAHF-2-induced reduction was observed for peanut, fish, and egg-restimulated IL-10 production ($P < .05$ – 0.01 , Fig 6D), but no significant change was observed in antigen-restimulated transforming growth factor beta levels (Fig 6F). In contrast, IFN- γ production after peanut, egg, and fish stimulation were elevated by FAHF-2 treatment in splenocytes cultures ($P < .01$ – $.001$; Fig 6E).

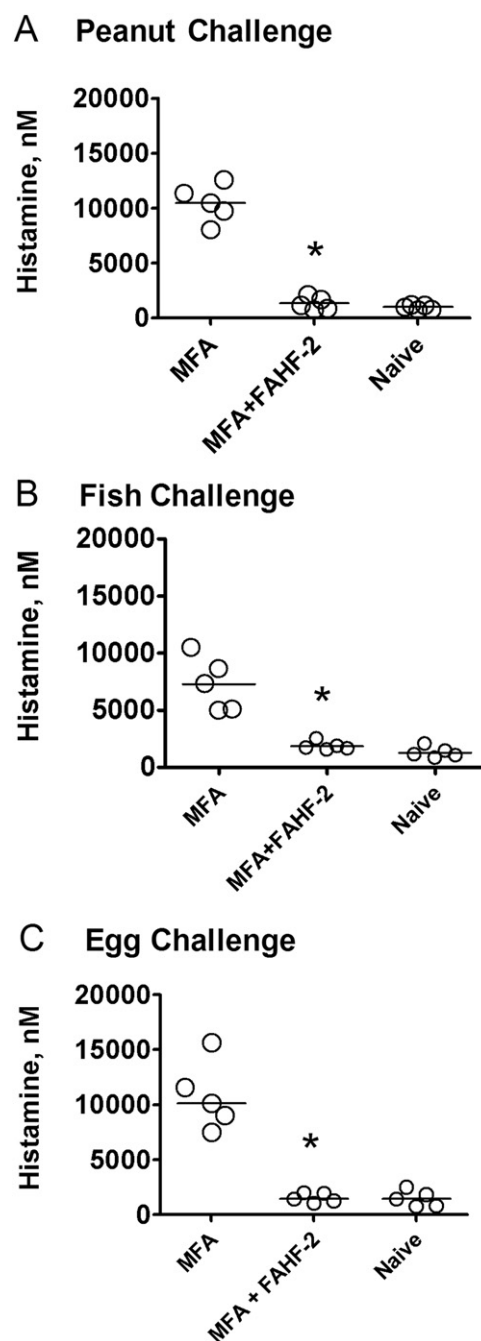


Fig. 4. Plasma histamine. Histamine in plasma harvested 30 minutes after each challenge was measured by ELISA. Bars represent group means of individual samples assayed in duplicate. * $P < .05$; $N = 5$ mice/group.

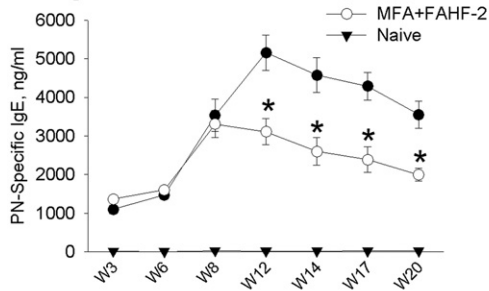
Cytokine profiles in MLN cultures were essentially similar to those in splenocyte cultures (Fig 7A–E), where a reduction of 40% to 70% was observed for Th2 cytokines, and a 73% increase in IFN- γ was observed in FAHF-2-treated MFA mice. These findings show that FAHF-2 generated beneficial immunomodulatory effect on Th-1/Th-2 responses in mice with multiple food allergies.

Discussion

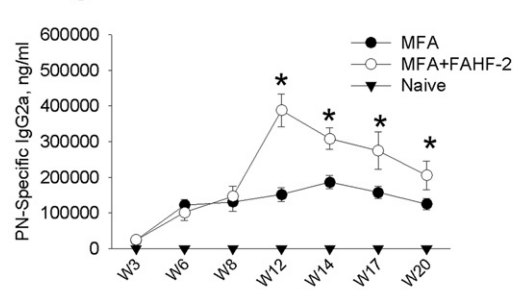
MFA is not rare; emerging evidence suggests that it is associated with a higher risk of anaphylaxis and atopic disease.^{4,32} Immunotherapeutic approaches for MFA have not been previously reported. As a first attempt to develop interventions for treating MFA, we generated MFA mice that developed specific IgE to peanut, fish, and

Peanut Challenge

A IgE

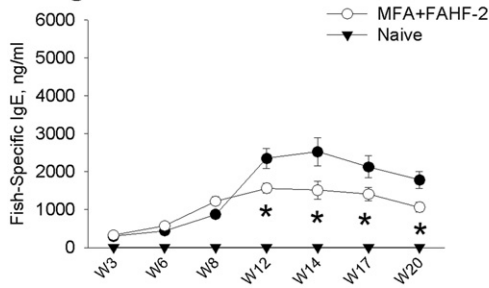


B IgG2a

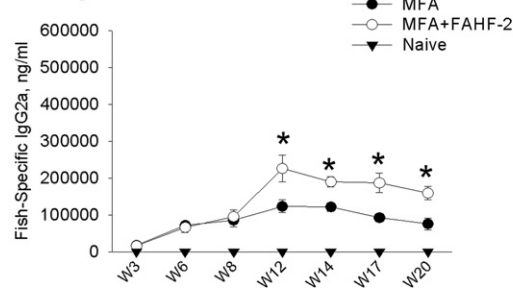


Fish Challenge

C IgE

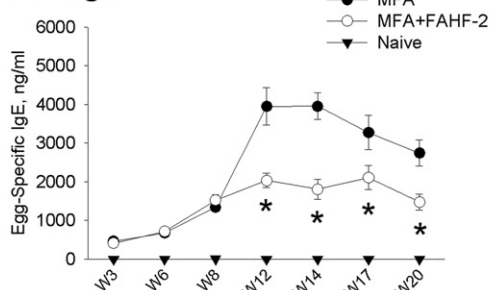


D IgG2a



Egg Challenge

E IgE



F IgG2a

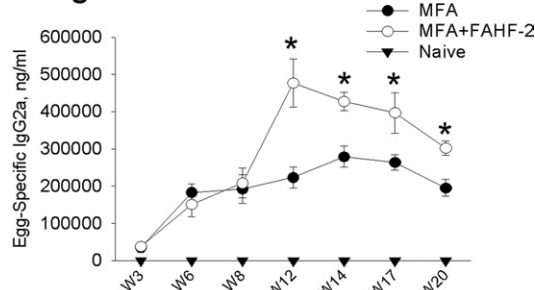


Fig. 5. Food-specific IgE and IgG2a. (A, C, E) Specific serum IgE and (B, D, F) IgG2a for peanut, fish, and egg were evaluated at times indicated by ELISA. Data shown as mean $\pm$ SEM of individual samples assayed in duplicate. * $P < .05$; $N = 5$ mice/group.

egg. Immunoblot analysis showed close agreement between the IgE-binding bands recognized by mice and those established for humans. Interestingly, we found that in addition to the known fish allergen parvalbumin, mice showed strong IgE-binding to a codfish protein that was a close match for enolase from salmon and puffer fish. Enolases, although not yet identified as fish allergens, are well-known major allergens in mold, yeast, and recently described in latex.^{33–35} Previous reports have shown IgE-binding in fish allergic subjects to protein bands at approximately 49 kDa, similar to our finding in mice, although those bands were not analyzed further to establish protein identity.^{36–38}

Separate peanut, codfish, and egg challenges of MFA mice induced anaphylactic reactions to each of the 3 foods. In addition, we found robust Th-2 cytokine responses to restimulation with peanut, fish, and egg proteins in splenocyte and MLN cultures from untreated MFA mice, implying maintenance of systemic and mucosal T-cells specific for peanut, fish, or egg proteins for up to 3 months after the last boosting.

Therapy/management of MFA in humans is complicated by the need to simultaneously avoid several foods. Specific oral or sublingual immunotherapy currently under investigation has not yet included treatment of concurrent reactivity to multiple foods. Other nonspecific therapies such as anti-IgE are currently not approved for use in food allergy. Effective treatments for MFA are urgently needed. Using a novel murine model of MFA, we hypothesized that FAHF-2 would be protective in the MFA setting. MFA mice given FAHF-2 treatment were completely protected from anaphylaxis during all oral food challenges. FAHF-2-induced significant and sustained reduction of food-specific IgE, and Th-2 cytokine levels after stimulation with each of the 3 food antigens. Consistent with earlier findings, FAHF-2 caused significant elevations in the Th-1 cytokine IFN- γ , and IgG2a specific to all 3 foods but a reduction in IL-10 and no change in TGF- β , suggesting that a redirection of Th-2 biased immune responses to Th-1 rather than a Treg-mediated mechanism likely contributes to the beneficial effects of FAHF-2. Previously reported FAHF-2 inhibition of mast

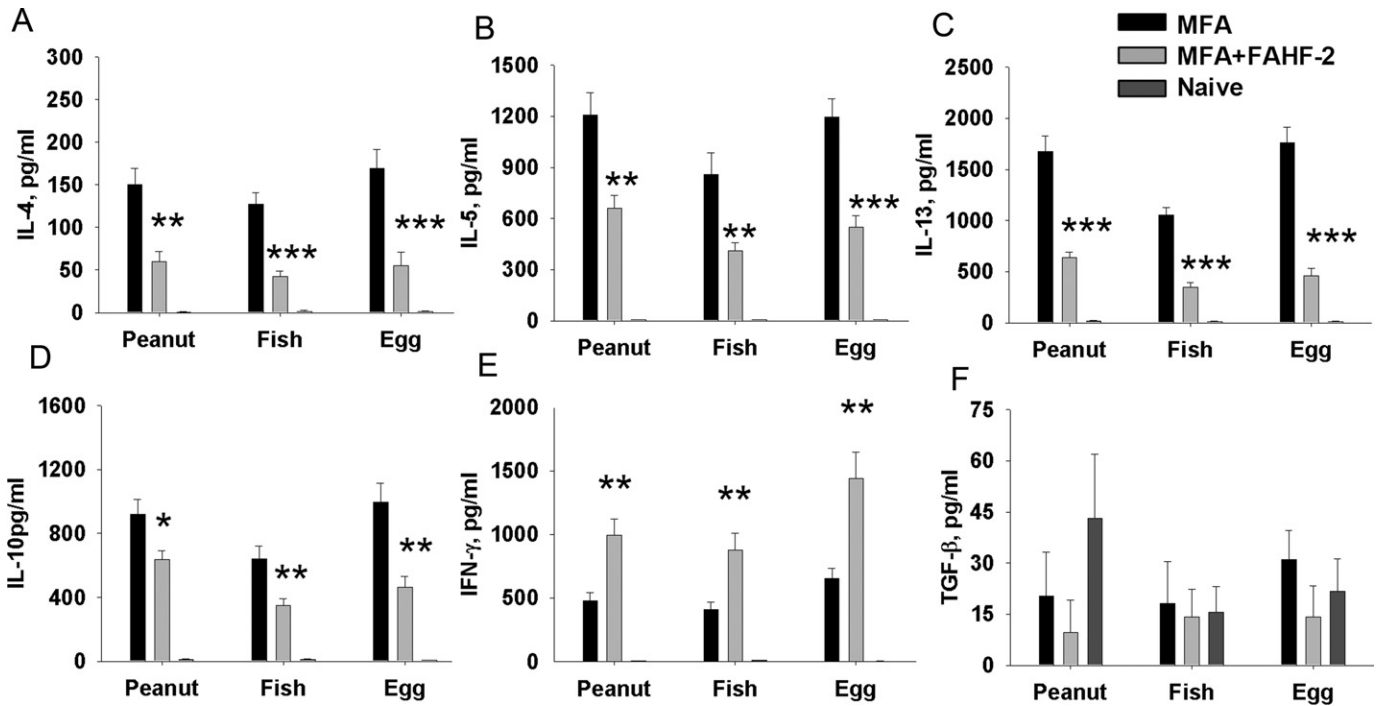


Fig. 6. Food protein restimulated cytokines in splenocytes cultures. Cytokine production by cultured splenocytes (A-F) stimulated with protein extracts of peanut, fish, or egg (50 µg protein for each) were measured by ELISA. Data shown as mean ± SEM of individual samples assayed in duplicate. Values are reported after subtraction of baseline levels measured in cultures stimulated with medium alone. * $P < .05$; ** $P < .01$; *** $P < .001$; $N = 5$ mice/group.

cell/basophil degranulation likely contributes to the complete protection from anaphylaxis observed in this study.^{20,22} As shown by Arias et al.³⁹ anaphylaxis involves multiple cell types and pathways, including mast cells, basophils, and macrophages. FAHF-2 reduction of antigen-specific IgE and pro-allergic cytokine levels

makes it an ideal therapeutic agent that impacts multiple aspects of the anaphylactic reaction. Research is underway to identify key chemical components of FAHF-2 responsible for its therapeutic effects and the signaling pathways involved. Butanol-extracted FAHF-2, which is devoid of proteins, peptides, and carbohydrates,

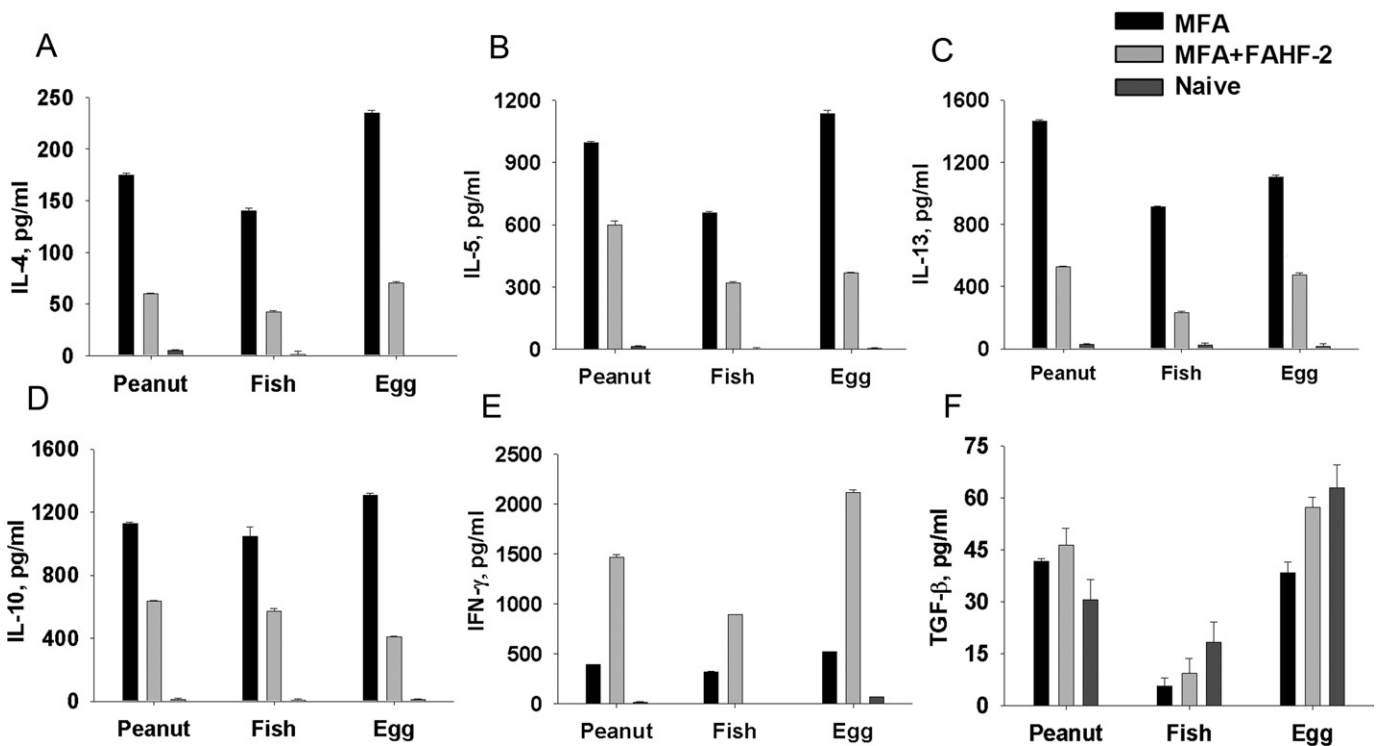


Fig. 7. Food-protein restimulated cytokines in mesenteric lymph node cultures. Cytokine production by pooled cultures of MLN cells (A-F) stimulated with protein extracts of peanut, fish, or egg (50 µg protein for each) were measured by ELISA. Data shown as mean ± SEM of pooled samples assayed in triplicate. Values are reported after subtraction of baseline levels measured in cultures stimulated with medium alone. $N = 5$ mice/group.

was found to be as effective as the unrefined formula,⁴⁰ suggesting that FAHF-2 activity is mediated by small organic molecules. We recently found that anti-mast cell/basophil properties of FAHF-2 are attributable to the alkaloid compounds berberine, palmatine, and jatrorrhizine.²⁰ A combination of these compounds was shown to inhibit syk phosphorylation in response to IgE binding in RBL-2H3 cells.²⁰ FAHF-2 active compounds involved in cytokine regulation and their molecular targets are being actively investigated. Taken together, FAHF-2 therapy may prove to be a comprehensive antigen-nonspecific therapeutic option for treatment of MFA.

In summary, we, for the first time, generated a murine allergy model with reactions to multiple foods. FAHF-2 completely blocked anaphylactic reactions to peanut, egg, and fish challenges and showed favorable immunotherapeutic effects on cytokine and antibody production. These findings provide a rationale to investigate FAHF-2 for treating MFA in humans.

References

- [1] Hill DJ, Hosking CS, Heine RG. Clinical spectrum of food allergy in children in Australia and South-East Asia: identification and targets for treatment. *Ann Med*. 1999;31:272–281.
- [2] Wang J, Patil SP, Yang N, et al. Safety, tolerability, and immunologic effects of a food allergy herbal formula in food allergic individuals: a randomized, double-blinded, placebo-controlled, dose escalation, phase 1 study. *Ann Allergy Asthma Immunol*. 2010;105:75–84.
- [3] McIntyre CL, Sheetz AH, Carroll CR, et al. Administration of epinephrine for life-threatening allergic reactions in school settings. *Pediatrics*. 2005;116:1134–1140.
- [4] Schroeder A, Kumar R, Pongracic JA, et al. Food allergy is associated with an increased risk of asthma. *Clin Exp Allergy*. 2009;39:261–270.
- [5] Miz-Echevarria L, Bilbao A, Andrade F, et al. Fatty acid deficiency profile in children with food allergy managed with elimination diets. 1. *Acta Paediatr*. 2008;97:1572–1576.
- [6] Noimark L, Cox HE. Nutritional problems related to food allergy in childhood. *Pediatr Allergy Immunol*. 2008;19:188–195.
- [7] Ostblom E, Egmar AC, Gardulf A, et al. The impact of food hypersensitivity reported in 9-year-old children by their parents on health-related quality of life. *Allergy*. 2008;63:211–218.
- [8] Sicherer SH, Noone SA, Munoz-Furlong A. The impact of childhood food allergy on quality of life. *Ann Allergy Asthma Immunol*. 2001;87:461–464.
- [9] Leung TF, Yung E, Wong YS, et al. Quality-of-life assessment in Chinese families with food-allergic children. *Clin Exp Allergy*. 2009;39:890–896.
- [10] Leung DY, Sampson HA, Yunginger JW, et al. Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med*. 2003;348:986–993.
- [11] Sampson HA. A phase II, randomized, double-blind, parallel-group, placebo-controlled, oral food challenge trial of XOLAIR® (omalizumab) in peanut allergy (TOPS). *J Allergy Clin Immunol* 2007;119:S117.
- [12] Li XM, Schofield BH, Huang CK, et al. A murine model of IgE mediated cow milk hypersensitivity. *J Allergy Clin Immunol*. 1999;103:206–214.
- [13] Helm RM. Food allergy animal models: an overview. *Ann N Y Acad Sci*. 2002;964:139–150.
- [14] Bashir ME, Andersen P, Fuss IJ, et al. An enteric helminth infection protects against an allergic response to dietary antigen. *J Immunol*. 2002;169:3284–3292.
- [15] Bashir ME, Louie S, Shi HN, et al. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J Immunol*. 2004;172:6978–6987.
- [16] Li XM. Beyond allergen avoidance: update on developing therapies for peanut allergy. *Curr Opin Allergy Clin Immunol*. 2005;5:287–292.
- [17] Qu C, Srivastava K, Ko J, et al. Induction of tolerance after establishment of peanut allergy by the food allergy herbal formula-2 is associated with up-regulation of interferon-gamma. *Clin Exp Allergy*. 2007;37:846–855.
- [18] Srivastava KD, Kattan JD, Zou ZM, et al. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *J Allergy Clin Immunol*. 2005;115:171–178.
- [19] Srivastava KD, Qu C, Zhang T, et al. Food Allergy Herbal Formula-2 silences peanut-induced anaphylaxis for a prolonged posttreatment period via IFN-gamma-producing CD8+ T cells. *J Allergy Clin Immunol*. 2009;123:443–451.
- [20] Song Y, Qu C, Srivastava K, et al. Food allergy herbal formula 2 protection against peanut anaphylactic reaction is via inhibition of mast cells and basophils. *J Allergy Clin Immunol*. 2010;126:1208–1217.
- [21] Wang J, Patil S, Yang N, et al. Safety, tolerability, and immunologic effects of a food allergy herbal formula (FAHF-2) in food allergic individuals: a randomized, double-blinded, placebo-controlled, dose escalation phase I study. *Ann Allergy Asthma Immunol*. 2010;105:75–84.
- [22] Patil SP, Wang J, Song Y, et al. Clinical safety of Food Allergy Herbal Formula-2 (FAHF-2) and inhibitory effect on basophils from patients with food allergy: extended phase I study. *J Allergy Clin Immunol*. 2011.
- [23] Institute of Laboratory Animal Resources Commission of Life Sciences NRC. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press; 1996.
- [24] Talbot RW, Foster JR, Hermon-Taylor J, et al. Induced mucosal penetration and transfer to portal blood of luminal horseradish peroxidase after exposure of mucosa of guinea pig small intestine to ethanol and lysolecithin. *Dig Dis Sci*. 1984;29:1015–1022.
- [25] Xiu SY. *The Experimental Method of Pharmacology*. Beijing: The People's Public Health Publisher; 1986.
- [26] Li XM, Serebrisky D, Lee SY, et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol*. 2000;106:150–158.
- [27] WHO/IUIS Allergen Nomenclature Sub-committee. Allergen nomenclature. Available at www.allergen.org/Allergen.aspx. Accessed March 19, 2012.
- [28] Hirose J, Doi Y, Kitabatake N, et al. Ovalbumin-related gene Y protein bears carbohydrate chains of the ovomucoid type 1. *Biosci Biotechnol Biochem*. 2006;70:144–151.
- [29] Bugajska-Schretter A, Elfman L, Fuchs T, et al. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca²⁺ depletion. *J Allergy Clin Immunol*. 1998;101:67–74.
- [30] Finkelman FD, Katona IM, Urban JFJ, et al. IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol*. 1988;141:2335–2341.
- [31] de Vries JE. The role of IL-13 and its receptor in allergy and inflammatory responses. *J Allergy Clin Immunol*. 1998;102:165–169.
- [32] Kapoor S, Roberts G, Bynoe Y, et al. Influence of a multidisciplinary paediatric allergy clinic on parental knowledge and rate of subsequent allergic reactions. *Allergy*. 2004;59:185–191.
- [33] Baldo BA, Baker RS. Inhalant allergies to fungi: reactions to bakers' yeast (*Saccharomyces cerevisiae*) and identification of bakers' yeast enolase as an important allergen. *Int Arch Allergy Appl Immunol*. 1988;86:201–208.
- [34] Achatz G, Oberkofler H, Lechenauer E, et al. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol Immunol*. 1995;32:213–227.
- [35] Wagner S, Breiteneder H, Simon-Nobbe B, et al. Hev b 9, an enolase and a new cross-reactive allergen from hevea latex and molds: purification, characterization, cloning and expression. *Eur J Biochem*. 2000;267:7006–7014.
- [36] Rosmilah M, Shahnaz M, Masita A, et al. Identification of major allergens of two species of local snappers: *Lutjanus argentimaculatus* (merah/red snapper) and *Lutjanus johnii* (jenahak/golden snapper). *Trop Biomed*. 2005;22:171–177.
- [37] Das A, Chakraborti P, Chatterjee U, et al. Identification of allergens in Indian fishes: hilsa and pomfret exemplified by ELISA and immunoblotting. *Indian J Exp Biol*. 2005;43:1170–1175.
- [38] Yamada S, Nolte H, Zychlinsky E. Identification and characterization of allergens in two species of tuna fish. *Ann Allergy Asthma Immunol*. 1999;82:395–400.
- [39] Arias K, Chu DK, Flader K, et al. Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. *J Allergy Clin Immunol*. 2011;127:1552–1561.
- [40] Srivastava K, Yang N, Chen Y, et al. Efficacy, safety and immunological actions of butanol-extracted Food Allergy Herbal Formula-2 on peanut anaphylaxis. *Clin Exp Allergy*. 2010.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.anai.2012.03.008.

eMethods

Food extract preparation

Food extracts were prepared as follows. Freshly ground, whole roasted PN (White Rose brand, Avenel, New Jersey) was homogenized in PBS (Mediatech, Manassas, Virginia) as described previously.¹ Fresh cod filets were purchased locally and immediately homogenized in PBS. Egg white powder (Debel Food Products, Elizabethport, New Jersey) was dissolved in PBS. Protein concentrations of each food in the sensitization regimen was calculated using protein content values listed in the USDA Nutrient Data Laboratory database (www.nal.usda.gov/fnic/foodcomp/search)².

SDS-PAGE analysis of peanut, codfish, and egg proteins

Food proteins were prepared for SDS-PAGE as follows. Peanut protein extract was prepared from commercially roasted peanuts as previously described.³ Crude codfish extract was prepared by homogenizing commercially available cod filet in PBS containing a protease-inhibitor cocktail without EDTA (Roche, Indianapolis, Indiana), followed by centrifugation at 25,000g for 30 minutes at 4°C and measuring protein content of the supernatant. Chicken egg white protein solution was prepared from standard dried egg white powder (Debel Food Products) according to manufacturer directions. Proteins were separated using Nu-PAGE 4–12% Bis-Tris gels (Invitrogen, Grand Island, New York), according to the manufacturer protocol. Ten micrograms protein was loaded into each well. The resolved proteins were subsequently transferred to Immobilon-P membrane (Millipore, Billerica, Massachusetts) and then stained with SimplyBlue (Invitrogen) for total protein analysis. SeeBlue Pre-stained Molecular Markers (Invitrogen) were used for identification of molecular weight of protein bands labeled with mouse sera IgE.

IgE immunoblot

Sera from MFA mice were diluted 1:20 in PBS-Tween containing 2% BSA and incubated with membranes bearing transferred food

proteins for 1 hour with gentle agitation at room temperature. After rinsing with PBS, sheep anti-mouse IgE (Binding Site, San Diego, California) diluted to 1 µg/mL in PBS-Tween and 2% bovine serum albumin (BSA) was applied. After agitation for 1 hour at room temperature and washing with PBS, immunoblots were incubated with donkey anti-sheep HRP (Binding Site) at a concentration of 0.1 µg/mL diluted in PBS-Tween and 2% BSA. The binding of mouse IgE to corresponding protein bands was revealed by using Immobilon Western Chemiluminescent HRP substrate (Millipore) and Kodak BioMax MS imaging film (Sigma-Aldrich, St. Louis, Missouri).

Measurement of food-specific IgE and IgG2a

Ninety-six-well microtiter plates were coated with protein extracts of PN, codfish, or egg. Some wells were coated with rat anti-mouse IgE or DNP-HSA for generation of reference curve. Plates were held overnight at 4°C. After washing 3 times, plates were blocked for 2 hours at room temperature with 2% BSA-PBS for PN and egg and 5% Tween-PBS for codfish immunoglobulin detection. After 3 washes, plates were incubated with samples and standards (purified mouse IgE for IgE measurements and mouse anti-DNP IgG2a for IgG2a measurements) overnight at 4°C. Biotinylated anti-IgE or IgG2a detection antibodies were added to the plate followed by avidin-peroxidase and ABTS substrate. For codfish-specific immunoglobulins, 5% Tween-PBS was used as blocking buffer and 0.5% Tween-PBS was used as reagent diluent.

eReferences

- [1] Li XM, Serebrisky D, Lee SY, et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol*. 2000;106:150–158.
- [2] USDA National Nutrient Database for Standard Reference. Available at www.nal.usda.gov/fnic/foodcomp/search. Accessed March 19, 2012.
- [3] Bannon GA, Cockrell G, Connaughton C, et al. Engineering, characterization and in vitro efficacy of the major peanut allergens for use in immunotherapy. *Int Arch Allergy Immunol*. 2001;124:70–72.