

Clinical safety of Food Allergy Herbal Formula-2 (FAHF-2) and inhibitory effect on basophils from patients with food allergy: Extended phase I study

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Background: Food allergy is a common and increasing health concern in westernized countries. No effective treatment is available, and accidental ingestion can be life-threatening. Food Allergy Herbal Formula-2 (FAHF-2) blocks peanut-induced anaphylaxis in a murine model of peanut-induced anaphylaxis. It was found to be safe and well tolerated in an acute phase I study of patients with food allergy.

Objective: We sought to assess the safety of FAHF-2 in an extended phase I clinical trial and determine the potential effects on peripheral blood basophils from patients with food allergy.

Methods: Patients in an open-label study received 3.3 g (6 tablets) of FAHF-2 three times a day for 6 months. Vital signs, physical examination results, laboratory data, pulmonary function test results, and electrocardiographic data were acquired at baseline and at 2-month intervals. During the course of the study, basophil activation and basophil and eosinophil numbers were evaluated by using CCR3/CD63 staining and flow cytometry.

Results: Of 18 patients enrolled, 14 completed the study. No significant drug-associated differences in laboratory parameters, pulmonary function study results, or electrocardiographic findings before and after treatment were found. There was a significant reduction ($P < .010$) in basophil CD63 expression in response to *ex vivo* stimulation at month 6. There was also a trend toward a reduction in eosinophil and basophil numbers after treatment.

Conclusion: FAHF-2 was safe and well tolerated and had an inhibitory effects on basophil numbers in an extended phase I clinical study. A controlled phase II study is warranted. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Food allergy, Food Allergy Herbal Formula-2, basophil activation

Food-induced allergic reactions affect approximately 5% of young children and 3% to 4% of adults.¹ To date, the treatment for food allergy involves avoidance of the triggering food.² However, accidental ingestions of the food substances to which subjects are allergic can happen and can lead to potentially life-threatening anaphylaxis. In addition, food allergies also negatively affect the quality of life of the patient. Development of safe and effective interventions for food allergy is a priority.

Traditional Chinese medicine (TCM) has a long history of human use in China and other Asian countries, such as Korea and Japan, for treating and preventing disease and is part of mainstream medicine in these countries. TCM is beginning to play a role in the United States. Acupuncture needles have been approved by the US Food and Drug Administration (FDA) as a medical device. Traditional Chinese herbal medicines are viewed as dietary supplements, and the cost of these preparations is not covered by insurance. However, this situation might change in the future if herbal medicines are shown to be effective. The US FDA provides guidance for investigating botanical drug products, including complex formulas containing several herbs, focusing on efficacy, safety, and consistency.³ Several publications, including ours, indicate that TCM has potential for treating asthma⁴⁻⁹ and food allergy,¹⁰⁻¹³ managing allergic rhinitis,¹⁴ and improving the quality of life of patients with atopic dermatitis.¹⁵ Therefore some TCM remedies might become botanical prescription drugs through clinical investigation.

Food Allergy Herbal Formula-2 (FAHF-2), derived from Wu Mei Wan, which is used to treat parasitic infection and food allergy-like symptoms¹⁶ is an extract of 9 herbs. We previously showed that FAHF-2 completely protects against peanut-induced anaphylactic symptoms in a murine model of peanut allergy^{10,11} and that this protection persists for at least 6 months after therapy after a single 7-week course of treatment.¹² Clinical protection was associated with a reduction in T_H2 cytokine and serum IgE levels and increased levels of IFN- γ and IgG_{2a}. Furthermore, we recently showed that FAHF-2 reduced basophil and mast cell numbers and mast cell activation independent from FAHF-2-mediated suppression of IgE and enhancement of IFN- γ .^{12,13} Thus FAHF-2 has immunotherapeutic effects on both T/B lymphocytes

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Abbreviations used

AE:	Adverse event
BAT:	Basophil activation test
FAHF-2:	Food Allergy Herbal Formula-2
FDA:	US Food and Drug Administration
fMLP:	N-formyl-methionyl-leucyl-phenylalanine
OIT:	Oral immunotherapy
SHIP-2:	Src homology domain 2-containing inositol phosphatase 2
SPT:	Skin prick test
Syk:	Spleen tyrosine kinase
TCM:	Traditional Chinese medicine
TN:	Tree nut

and effector cells, such as mast cells and basophils. Previous animal studies showed that FAHF-2 has a high "margin of safety" because mice fed 24 times the effective dose of FAHF-2 showed no mortality or morbidity.¹¹ This preclinical study provided the rationale for a clinical trial of FAHF-2 aimed at developing a botanical drug for treating food allergies.

We received approval on an investigational new drug for FAHF-2 from the US FDA and completed an acute, 1-week, randomized, double-blind, placebo-controlled, dose-escalation phase I trial in subjects with peanut, tree nut (TN), or both; fish; and shellfish allergies. In that acute phase I study, 18 patients received one of 3 doses of FAHF-2 or placebo: 2.2 g (4 tablets), 3.3 g (6 tablets), or 6.6 g (12 tablets) 3 times a day for 7 days. The results showed that FAHF-2 was safe and well tolerated over a 1-week course of treatment and that *in vitro* treatment of patients' PBMCs with FAHF-2 reduced IL-5 secretion and increased IFN- γ and IL-10 secretion.¹⁷

Basophils comprise less than 1% of leukocytes but are critical to allergic reactions, as are tissue mast cells. CCR3 is a well-established basophil marker, as determined by means of flow cytometry.¹⁸ CD63 expression, a marker of basophil activation,¹⁹ correlates with release of histamine from intracytoplasmic granules and is triggered by IgE and allergen cross-linking of Fc ϵ RI receptors. The basophil activation test (BAT) is a flow cytometry-based assay that detects basophil CD63 expression, requires the use of very small quantities of blood, and does not require isolation of cells. The real-time flow cytometry readout makes this assay a convenient tool for longitudinal clinical studies.²⁰⁻²³ A recent study by Jones et al²⁰ found that basophil activation was significantly reduced by 4 to 6 months of oral immunotherapy (OIT) and that inhibition of basophils but not IgE correlated with clinical protection.

Because peanut, TN, fish, and shellfish allergies are typically lifelong, chronic treatment is essential. Long-term safety of human FAHF-2 consumption has not been assessed.

To further ensure the safety and tolerability of this formulation before phase II study, we conducted an open-label, single-dose, 6-month extension of the phase I study. Although the primary outcome of this study was to evaluate the 6-month safety and tolerability of FAHF-2 based on substantial animal study results and the fact that this is a 6-month study, we hypothesized that FAHF-2 might have induced some immunotherapeutic effects on basophils.

METHODS**Study patients**

Patients with food allergy aged 12 through 45 years with a convincing history of allergy to peanut, TN, fish, or shellfish, as documented by a positive

skin test response (mean wheal diameter >3 mm greater than the mean of saline control), food allergen-specific IgE level (peanut-, TN-, fish-, or shellfish-specific IgE >0.7 kU_A/L), or both were eligible for the study. Female patients of childbearing potential were included but had to be sexually inactive or using effective birth control measures, as deemed appropriate by the investigator, for the duration of the study.

Exclusion criteria included acute infection; history of systemic disease; abnormal hepatic, bone marrow, or renal function; clinically significant abnormal electrocardiographic results; current uncontrolled moderate-to-severe asthma with FEV₁ less than 80% of predicted value; drug or alcohol abuse; pregnancy or lactation; and participation in another research protocol within the previous 30 days.

This study was approved by the Mount Sinai Medical School Institutional Review Board. Written informed consent was obtained before enrollment.

Study design

The initial evaluation consisted of a thorough medical history and physical examination, vital signs, skin prick and food-specific IgE testing, baseline pulmonary function tests, electrocardiography, urinalysis, and routine laboratory blood tests (complete blood count, serum chemistries, renal function, liver function tests, and pregnancy tests for female participants).

After initial screening, subjects were started on FAHF-2 (3.3 g, 6 tablets) 3 times a day for 6 months. Subjects continued food allergen avoidance for the duration of the study and were asked to refrain from other herbal medication use. Subjects were seen every 8 weeks and telephoned every 2 weeks by study investigators to reinforce and confirm medication compliance and assess potential adverse events (AEs). Subjects were instructed to complete a symptom diary during participation in the trial. At each study visit, the interim medical history was reviewed, and physical examination, spirometry, electrocardiography, and laboratory studies were repeated.

Study medication

FAHF-2 was in the form of tablets (0.5 g/tablet) produced by Xiyuan Chinese Medicine Research and Pharmaceutical Manufacturer, China. The same batch of FAHF-2 that was used in the acute phase I study¹⁷ was used for this open-label study. The quality of raw herbs, manufacturing process, and quality control of the final FAHF-2 products were established according to FDA guidance under the botanical drug title (Chemical, Manufacturing, and Control Data [21 CFR 312.23{a} {7}]), as published previously.¹⁷

We generated the HPLC fingerprint of FAHF-2 as a means of standardizing the FAHF-2 product¹⁷ and to monitor its consistency and shelf-life. Fig E1 (in this article's Online Repository at www.jacionline.org) shows the consistency of HPLC fingerprints obtained in July 2007 (acute phase I study period; see Fig E1, A) and July 2008 (current study period; see Fig E1, B) carried out on the same batch of tablets. The HPLC methods were described previously.¹⁷

Study procedures

Skin prick testing. Before enrollment and at the end of the 6-month treatment phase, titrated skin prick tests (SPTs) were performed in duplicate with serial 10-fold dilutions (1:20 to 1:200,000) of stock peanut or individual TN, fish, and/or shellfish extract (Greer Laboratories, Lenoir, NC). Negative (phenol-saline solution) and positive (1 mg/mL histamine base) controls were also included. SPTs were performed by pricking through a drop of extract with a bifurcated needle. A mean wheal diameter of greater than 3 mm than that elicited by the negative control was considered a positive response.

Allergen-specific IgE measurements. At each study visit, specific IgE to peanut, TN, fish, and/or shellfish was detected with the ImmunoCAP system (Phadia, Uppsala, Sweden).

Safety monitoring. Patients were monitored for potential AEs throughout the study based on our AE criteria table.¹⁷ AE criteria were adapted from World Health Organization Recommendations for Grading of Acute

and Subacute Toxicity²⁴ with the following modifications: grade 1 AEs under the World Health Organization grading were considered grade 3 AEs in our study, making our criteria for AEs much more stringent. This grading system was approved by the FDA for our phase I clinical trial. According to the study protocol, any AE of grade 3 in an FAHF-2-treated subject would result in immediate discontinuation of the study medication in that subject.

BATs

BATs were performed with the Flow 2 CAST kit (Alpco Diagnostics, Windham, NH). BATs were performed at 0 months (before treatment) and at 3 time points (2, 4, and 6 months of treatment). Crude peanut, pecan, hazelnut, and cashew extracts prepared by using a previously described protocol²⁵ were used as specific antigens for *ex vivo* stimulation. Because the study subjects were allergic to peanut, TNs, fish, and/or shellfish, the allergen was chosen based on the following criteria. If a subject was allergic to peanut and TNs and possibly other foods, the cells were stimulated only with peanut allergen extract. If a subject was allergic to TNs and other foods but not peanut, the cells were stimulated with 1 specific TN allergen. In this BAT assay a stimulation buffer containing IL-3 was used as the negative control. Antigen and a highly specific mAb recognizing the high-affinity IgE binding receptor (FcεRI) was used as a positive control, and N-formyl-methionyl-leucyl-phenylalanine (fMLP) was used as a nonspecific positive control, according to the manufacturer's instructions. Briefly, 100 μL of stimulation buffer containing IL-3, heparin, and calcium was added to 6 aliquots of 50 μL of heparinized blood. Fifty microliters of either stimulation buffer, anti-FcεRI, fMLP, or allergen (200 ng/mL, 200 pg/mL, and 0.2 pg/mL) was added to each aliquot. Twenty microliters of staining reagent containing anti-CCR3–fluorescein isothiocyanate– and anti-CD63–phycoerythrin–labeled mAbs was added. The tubes were then incubated at 37°C in a water bath in the dark for 15 minutes. The red blood cells were lysed, and cells were resuspended in wash buffer and acquired on a LSR-II flow cytometer (BD Biosciences, San Jose, Calif). Basophils and eosinophils were gated as CCR3⁺ cells and segregated on the basis of side scatter. CCR3⁺ cells with low side scatter were considered basophils, and among these, the CD63⁺ cells were termed activated basophils. Fifty thousand to 100,000 leukocytes were acquired, and 300 or more basophils were used for analysis by using FlowJo software, version 5.4 (Tree Star, Inc, Ashland, Ore).

Statistical analysis

Of the patients who completed this 6-month, long-term, open-label extension safety trial, 86% were the same subjects enrolled in the initial acute phase I trial. All subjects received FAHF-2. Formal statistical methods were not used to calculate the sample size. The number of subjects was chosen to minimize risk by not exposing a large number of subjects to treatment but still providing a sample size sufficient to gain impressions of chronic safety and possible preliminary immunologic effects.

The *ex vivo* immunologic data were analyzed as follows. A mixed-model analysis was performed to evaluate the effect of time on the outcomes examined in the context of allergen-specific basophil activity (3 doses of allergen stimulation, stimulation buffer, anti-FcεRI, and fMLP). For the initial analysis, we assumed the correlation between times was the same (compound symmetry) because this leads to a more robust analysis in which outliers cannot have an undue effect. If this analysis showed any trend toward significance, we carefully evaluated more complex models, in particular the unstructured model, which makes no assumptions concerning the pattern of correlations. If there was a difference among the 4 time points, pairwise comparisons were performed to compare months 2, 4, and 6 with baseline. A Bonferroni correction was used for multiple testing. The Wilcoxon signed-rank test (non-parametric) was performed to evaluate the difference between percentages of basophils and eosinophils before and after 6 months of FAHF-2 treatment. A *P* value of less than .05 was considered statistically significant. These statistical analyses were performed with SAS statistical software version 9.2 (SAS Institute, Inc, Cary, NC); in particular, PROC GENMOD was used for the repeated-measures analysis, and PROC UNIVARIATE was used for the changes in basophil and eosinophil numbers.

RESULTS

Subjects' characteristics

Eighteen subjects were enrolled in this study. Four patients withdrew: 1 because of pregnancy, 2 for difficulty with compliance related to the time commitment and high number of tablets required daily, and 1 with transient abdominal complaints without vomiting or diarrhea. Fourteen patients completed the 6-month treatment. Baseline characteristics of study subjects are shown in Table I. The median age of the patients was 16 years (range, 12-27 years), and 67% were male. Peanut allergy often coexists with other food allergies; 77% of patients with peanut allergy were also allergic to at least 1 TN with or without shellfish/fish allergies, 17% were allergic to a TN only, and 11% were allergic to TN and shellfish/fish. No patient was allergic only to peanut. In this cohort 95% of the study subjects exhibited other allergic diseases, including asthma (11%); allergic rhinitis (6%); asthma and allergic rhinitis (22%); asthma and atopic dermatitis (22%); allergic rhinitis and atopic dermatitis (6%); asthma, allergic rhinitis, and atopic dermatitis (28%); and no other allergies (5%). The median allergen-specific IgE level was 25 kU_A/L (interquartile range, 4.43-52.2; 100 kU_A/L was the upper limit), and the median total IgE level was 360 kIU/L (interquartile range, 168-831 kIU/L).

Laboratory test results

There were no changes in hematology or chemistry laboratory values, pulmonary function study results, or electrocardiographic findings obtained at baseline, at 2-month intervals, or after completing 6 months of FAHF-2 treatment (Table II). There were no changes in SPT results at baseline or after 6 months of FAHF-2 treatment.

Clinical AEs

One patient had an AE. This subject had a history of eosinophilic esophagitis diagnosed 2 years before but was not believed to have active disease and was not receiving treatment at the time of enrollment. After 5½ weeks on FAHF-2 treatment, she contacted the study coordinator to report that she had what she believed to be a recurrence of her eosinophilic esophagitis. Symptoms included intermittent, moderate-to-severe, noncrampy abdominal pain; food impaction; and nausea. She was instructed to discontinue FAHF-2 until she was evaluated by her gastroenterologist. Her gastroenterologist performed an upper endoscopy. On the basis of the endoscopic results, which showed eosinophilic infiltration in the esophagus, the subject was started on swallowed fluticasone and Prevacid (lansoprazole; Takeda Pharmaceuticals North America, Inc, Deerfield, Ill). Radiography revealed a significant amount of stool throughout the colon, and she was given an enema and later started on Miralax (polyethylene glycol; Merck & Co, Inc, Whitehouse Station, NJ) for constipation. The abdominal pain did not recur. She was restarted on FAHF-2 two months later and completed the study with no other abdominal complaints.

Reduced allergen-specific basophil activity in response to *ex vivo* antigen challenge after FAHF-2 treatment

Basophil activity assays of peripheral blood samples from 11 patients were completed at all time points. Basophils were identified on the basis of CCR3⁺ staining and distinguished from

TABLE I. Demographics of the patients enrolled in the 6-month extended phase I clinical study

Patient no.	Age (y)	Sex	Food allergies	Other allergies	Allergen-specific IgE (kU _A /L) (all peanut, where indicated)	Total IgE (kIU/L)	Time since last reaction to allergen
1	22	M	PN, TN, sesame	Asthma	>100	1440	21 y
2	15	M	PN, TN, sesame, SF, milk	Asthma, AD	7.59	1455	Never ingested
3	18	M	PN, TN, milk, egg, legumes	Asthma, AR	4.37	284	14 y
4	18	F	PN, TN, fish	Asthma, AR	>100	529	17 y
5	19	F	PN, TN, sesame	Asthma, AR	18.4	600	6 mo
6	16	F	PN, TN, sesame	Asthma, AD	76.7	254	1 y
7	23	M	TN	None	9.35 (hazelnut)	106	Never ingested
8	16	M	PN, TN, soy, legumes	Asthma, AR, AD	52.2	168	10 y
9	12	M	PN, TN	AR, AD	24.9	142	7 y
10	15	M	PN, TN, fish, SF	Asthma, AR, AD	3.79	2877	14 y
11	16	M	PN, TN, seeds, egg	Asthma, AD	4.43	1067	15 y
12	16	M	PN, TN, seeds, egg	Asthma, AR, AD	28	254	14 y
13	17	F	TN, seeds, fish, SF, egg	Asthma, AR, AD	3.03 (pecan)	681	1 y
14	12	M	TN	Asthma, AR, AD	41.4 (cashew)	831	1 y
15	12	M	TN	Asthma, AR	3.40 (pecan)	56	4 y
16	17	F	TN, SF	AR	25.0 (pecan)	418	12 y
17	12	M	PN, TN	Asthma	71.1	320	10 y
18	27	F	PN, TN, fish, SF	Asthma, AD	36.2	68	3 mo

AD, Atopic dermatitis; AR, allergic rhinitis; PN, peanut; SF, shellfish.

TABLE II. Summary of laboratory results for subjects completing the extended phase I trial (6 months of treatment with FAHF-2)

Component	FAHF-2 (N=14)		Reference range
	Pretreatment	Posttreatment	
Glucose (mg/dL)	72 (16)	69 (24.8)	60-120
Sodium (meq/L)	140 (2)	140 (1.4)	135-145
Potassium (meq/L)	3.9 (0.3)	3.9 (0.2)	3.5-5
Chloride (meq/L)	101 (2)	101 (2)	96-108
CO ₂ (meq/L)	27.7 (2.0)	26.6 (2.2)	22.0-32
Urea (mg/dL)	14 (2.5)	13 (3.0)	11-25
Creatinine (mg/dL)	0.8 (0.2)	0.8 (0.2)	0.4-1.2
SGPT (U/L)	16 (3.6)	20 (8.1)	01-53
SGOT (U/L)	22 (4.7)	25 (5.2)	01-50
WBC ($\times 10^3/\mu\text{L}$)	6.5 (1.7)	7.2 (1.7)	4.5-11
Hemoglobin (g/dL)	14.0 (1.5)	14.2 (1.3)	13.9-16.3
Platelet ($\times 10^3/\mu\text{L}$)	245 (49)	213 (77)	150-450

Data are presented as mean (SD).

CO₂, Carbon dioxide; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; WBC, white blood cell.

eosinophils by means of low side scatter (see Fig E2 in this article's Online Repository at www.jacionline.org). Activated basophils were defined as CD63⁺CCR3⁺ cell percentages. After correcting for multiple testing, there was a significant reduction ($P < .010$) in CD63⁺ basophil percentages in blood from patients after 6 months of FAHF-2 treatment after 200 pg/mL allergen stimulation (Fig 1, B) compared with baseline levels. Although there was a P value of less than .02 for the nonadjusted difference at month 4, after adjustment with the Bonferroni test for multiple testing, there was a significant reduction ($P < .05$) only for the compound symmetry model. The data after the low (0.2 pg/mL; Fig 1, C) and high (200 ng/mL; Fig 1, A) doses of allergen stimulation tended to be bunched near 0% or 100%, respectively, and were not conducive to a parametric statistical analysis (highly nonnormal with outliers that would remain even after transformation).

On the basis of the information from Alpco Diagnostics, the maker of the Flow 2 CAST kit, and the literature,²⁶⁻²⁸ basophils from some subjects, termed nonresponders, in BAT assays do not react to the positive control stimulation (<5% activation). The opposite phenomenon is basophil hyperreleasability, in which subjects' basophils respond to negative control stimulation (>5% activation). In this study we found high baseline percentages of CD63⁺ cells in IL-3-containing stimulation buffer (median, 13% CD63⁺ basophils [0% to 60%]). There were no nonresponders. Basophils from all subjects responded to anti-FcεRI and fMLP positive stimulation, and these responses were not different at baseline or after treatment (Fig 2). Blood samples from healthy subjects ($n = 8$) contained less than 2% CD63⁺ basophils when stimulated with allergen or IL-3 containing stimulation buffer and responded to anti-FcεRI and fMLP positive stimulation (data not shown).

Reduction in basophil and eosinophil percentages after FAHF-2 treatment

On the basis of the gating strategy according to the manufacturer's instructions (Flow 2 CAST kit, Alpco Diagnostics), we also analyzed changes in peripheral blood basophil and eosinophil percentages between baseline and after 6 months of FAHF-2 treatment. There was a trend associated with borderline statistical significance of reduction in basophil ($P = .067$; Fig 3, A) and eosinophil ($P = .08$; Fig 3, B) percentages after 6 months of FAHF-2 treatment. Basophil percentages decreased from a median of 0.68% to 0.39% (Fig 3, A), and eosinophil percentages decreased from 5.3% to 3% (Fig 3, B).

DISCUSSION

Complementary and alternative medicine treatments are increasingly used in Western countries because of their reputed effectiveness, low cost, and favorable safety profiles and because

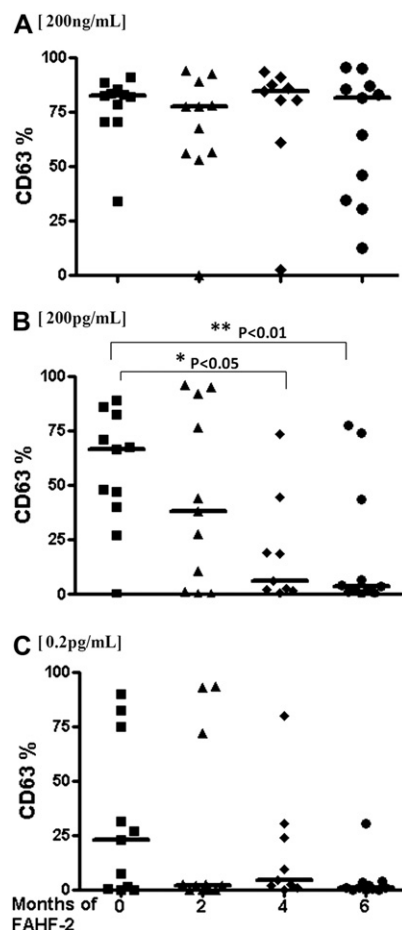


FIG 1. Suppression of allergen-stimulated basophil activation by FAHF-2 in a 6-month phase I clinical study. Patients' blood before treatment (0 months) and at consecutive 2-month time points during a 6-month clinical phase I study for FAHF-2 was stimulated with increasing doses of allergen in the presence of stimulation buffer. Percentages of CD63 cells for 200 ng/mL (A), 200 pg/mL (B), and 0.2 pg/mL (C) of allergen are shown. * $P < .05$ and ** $P < .01$. Symbols represent individual patients, and bars are medians from each group.

conventional therapies are not completely satisfactory for allergy. Studies have suggested the utility of Chinese herbal medicine for the treatment of a variety of allergic diseases, including allergic rhinitis and asthma.^{8,9,29} We developed a 9-herb formula that is highly effective at protecting mice with peanut allergy from peanut-induced anaphylaxis and has a high safety profile and long duration of action. Our group is the first to obtain a US FDA investigational new drug approval to investigate a botanical product to treat food allergy, and we recently published the results of the acute phase I safety trial, indicating that short-term use of FAHF-2 is safe and well tolerated.¹⁷ In this communication we demonstrate that all laboratory parameters, pulmonary function studies, and electrocardiograms of patients treated with FAHF-2 for 6 months were within the normal range. No patient reported a food-induced allergic reaction during the course of the trial. These results demonstrate that long-term treatment with FAHF-2 was safe and well tolerated by patients with food allergy and that FAHF-2 itself does not cause any adverse reactions. This was the expected outcome based on the long-term history of human use of the constituent herbs and substantial preclinical

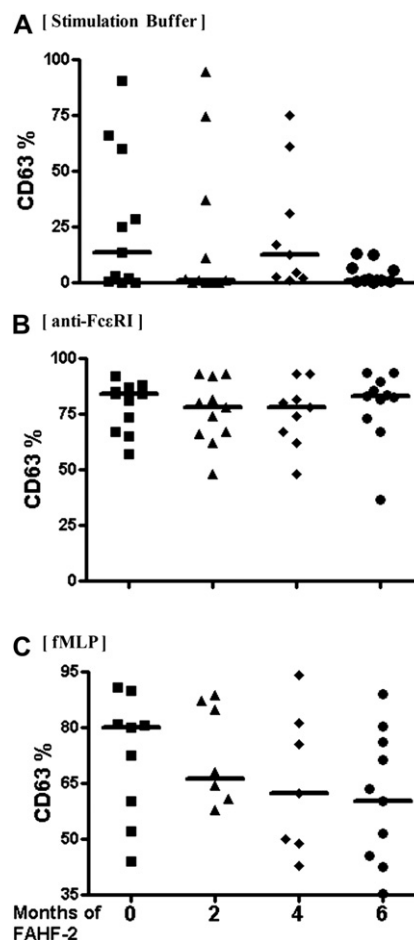


FIG 2. Effect of FAHF-2 on IL-3-, anti-FcεRI-, and fMLP-stimulated basophils in a 6-month phase I clinical trial. Patients' blood before treatment (0 months) and at consecutive 2-month time points during a 6-month clinical phase I study for FAHF-2 was stimulated in the presence of stimulation buffer alone (A), stimulation buffer plus anti-FcεRI (B), and stimulation buffer plus fMLP (C).

animal studies. These extended safety results are also consistent with the acute phase I study.

BATs have been used along with other conventional allergy tests in the diagnosis of peanut allergy.^{20,27,30} Jones et al²⁰ found that basophil activity was significantly reduced by 4 to 6 months of OIT.²⁰ FAHF-2 treatment of mice with peanut allergy produced significant suppression of mast cell and basophil numbers and activation.¹³ This finding prompted us to investigate FAHF-2 treatment effects on basophil activity in this 6-month safety trial by using CD63 as a basophil activation marker. We measured basophil activation in response to varying doses of antigen after 6 months of FAHF-2 treatment compared with that seen at baseline. Basophil activation in response to 200 pg/mL allergen was significantly reduced after 4 and 6 months of treatment, and a reduced trend was observed at the 0.2 pg/mL allergen dose. In this study the optimal concentration of antigen stimulation was 200 pg/mL. At higher (200 ng/mL) and lower (0.2 pg/mL) doses, the data on basophil activation tended to be bunched near 0% or 100%, respectively. In the study by Jones et al,²⁰ quantities of allergen used for the basophil activation assay were between 0.1 and 10 $\mu\text{g/mL}$. A recent study by Kim et al³¹ used 1 ng/mL to 1 $\mu\text{g/mL}$ allergen for BATs. The differences in concentrations

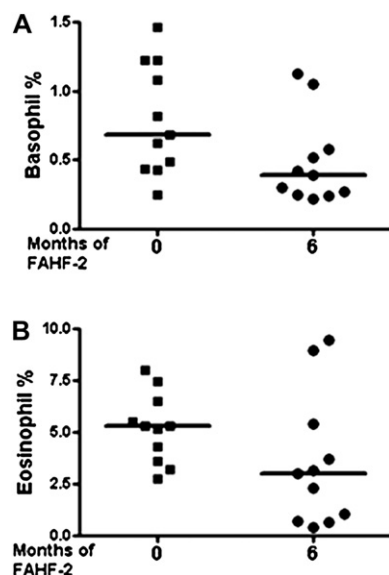


FIG 3. Effect of FAHF-2 treatment on basophil and eosinophil percentages. Patients' blood at baseline and after 6 months of a clinical phase I study for FAHF-2 was stimulated in the presence of stimulation buffer alone or stimulation buffer with anti-FcεRI, fMLP, and allergen dilutions. Basophil (A) and eosinophil (B) percentages are shown. Symbols indicate an individual patient, and bars show median values. A *P* value of less than .05 was considered significant.

of peanut allergen used for testing basophil activation in different studies might be due to differences in the sensitivity of cells from different populations, different *in vitro* stimulation times, or differences between the commercial kits used. Therefore it is important to optimize the concentrations of antigens used for basophil activation assay. The study did not include an observational untreated group with food allergy in which basophil activation was monitored for 6 months. However, Jones et al²⁰ reported that mere avoidance of peanut for 6 months does not result in reduced basophil activation responses. Furthermore, most of the patients in our study had avoided the food substances to which they are allergic for several years before enrolling in the study (Table I) and still had a high baseline level of basophil activation responses on *ex vivo* allergen stimulation. This suggests that the observed reduction in the basophil activation response was due to FAHF-2 treatment.

We also found basophil hyperreleasability (high spontaneous basophil histamine release to negative control stimulation in those subjects). Although this study was not designed as a controlled study, to confirm the accuracy of BATs, we performed BATs on samples from healthy subjects. Less than 2% basophils were CD63⁺ in response to allergen stimulation. There was no basophil activation after negative control stimulation (stimulation buffer), but there were similar responses to positive stimulation controls (anti-FcεRI and fMLP), suggesting basophil hyperreleasability was specific to basophils from patients with food allergy, as has been reported previously.^{28,32-34} Vonakis et al²⁸ demonstrated that cultured peripheral blood basophils from patients with chronic idiopathic urticaria spontaneously degranulated and that basophils from responders among these patients contained significantly increased spleen tyrosine kinase (Syk) and decreased Src homology domain 2-containing inositol phosphatase 2 (SHIP-2) levels compared with those seen in nonresponders and healthy

subjects. In addition, Kepley et al³⁵ detected less Syk in the basophils of nonresponders. Thus the balance between Syk and SHIP-2 levels plays an important role in spontaneous histamine release. Song et al¹³ showed that the persistent protection of FAHF-2 in a murine model of peanut allergy is associated with reduced basophil and mast cell numbers, as well as prevention of IgE-mediated mast cell activation. Three compounds from a purified fraction of FAHF-2 inhibited RBL-2H3 cell degranulation through suppression of Syk phosphorylation. Therefore the decrease in basophil activation after stimulation buffer only at 6 months might be because of suppression of Syk levels by FAHF-2. However, this possibility requires further investigation at the molecular level.

Ducrest et al³⁶ reported blood basophil percentages using CCR3 and CD123 as basophil markers. A more recent study demonstrated robust expression of CCR3 to be a valid single basophil selection marker in flow cytometry.¹⁸ The present study analyzed basophil percentages by using only the CCR3 marker and discrimination from eosinophils on the basis of side scatter, as per the manufacturer's instructions (Alpco Diagnostics). We found a trend in reduction in basophil percentages in FAHF-2-treated subjects (*P* = .067). There was also a trend toward a reduction in eosinophil percentages (*P* = .08). Patients with food allergy often have other allergic conditions, such as asthma, allergic rhinitis, and atopic eczema. In this study 95% of subjects also have other allergic conditions. Eosinophils play an important pathological role in these disorders. FAHF-2 might provide additional immunologic benefit for patients with food allergy with other coexisting allergic conditions. A controlled study is warranted to investigate this possibility.

In our previous acute phase I study, we tested 3 doses of FAHF-2.¹⁷ Although no dose-limiting side effects were reported in the phase I study, several participants in the high-dose group believed that 12 tablets 3 times daily posed a significant burden. Therefore we used the medium dose in this 6-month open-label study, which was equivalent to the half dose used in our previous murine model.¹¹ We did not find a significant reduction in specific IgE levels (baseline, 21.65 kU_A/L; after treatment, 20 kU_A/L [interquartile range, 4.7-36.8 kU_A/L]) or significant increase in specific IgG₄ levels despite the reduction in basophil activation. Jones et al²⁰ found that 4 to 6 months of peanut OIT reduced basophil activity, whereas IgE levels were increased at 6 to 8 months. Reductions in IgE levels were found after 12 to 18 months, perhaps because of the difficulty in altering memory B cells from patients with peanut allergy. These data, along with those from previously published reports, suggest an IgE-independent suppression of basophil activation, perhaps because of reduced Syk expression,^{20,35} and that a higher dose and longer duration of treatment should be considered for the phase II study design to achieve greater efficacy.

In conclusion, FAHF-2 was safe and well tolerated by patients with food allergy in a 6-month extended phase I clinical study. The results are consistent with a previous acute phase I study. FAHF-2 reduced allergen-stimulated basophil activation, hyperreleasability, and percentages of circulating basophils. The suppression of basophil activation and FAHF-2's *in vitro* effects on PBMCs in a previous study (ie, increased IL-10 and IFN-γ and reduced IL-5 levels¹⁷ in mice with peanut allergy^{11,12}) suggest that FAHF-2 exhibits beneficial immunologic effects. A double-blind, placebo-controlled, phase II efficacy trial is planned. Studies are also underway to investigate the mechanisms of action of FAHF-2 on basophil responses of patients with food allergy.

We thank Luda Bardina for her help with allergen extract preparation.

Clinical implications: FAHF-2 was safe and well tolerated and inhibited basophil numbers and activation in a 6-month clinical trial for patients with food allergy. FAHF-2 might provide a safe immunotherapeutic option for patients with food allergy.

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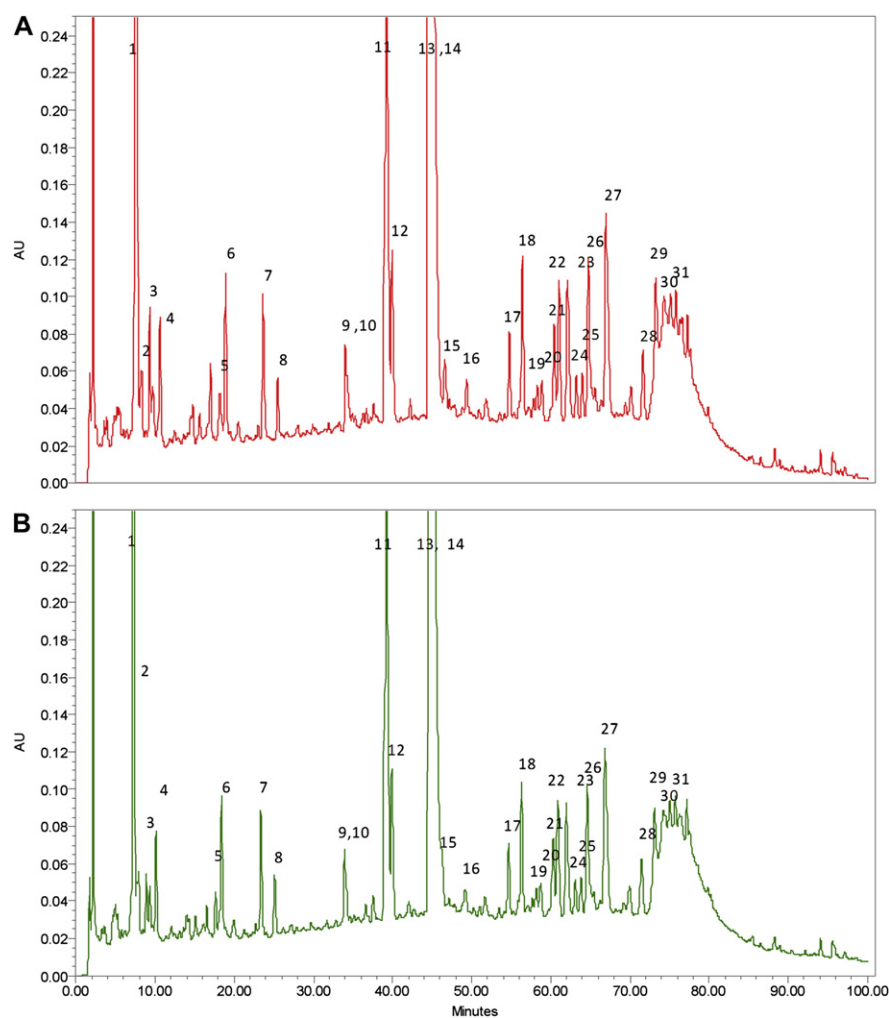


FIG E1. HPLC fingerprints. FAHF-2 tablets obtained from the same batch were tested at time points before the studies. **A**, HPLC result of an FAHF-2 tablet generated in July 2007. **B**, HPLC result of an FAHF-2 tablet generated in July 2008.

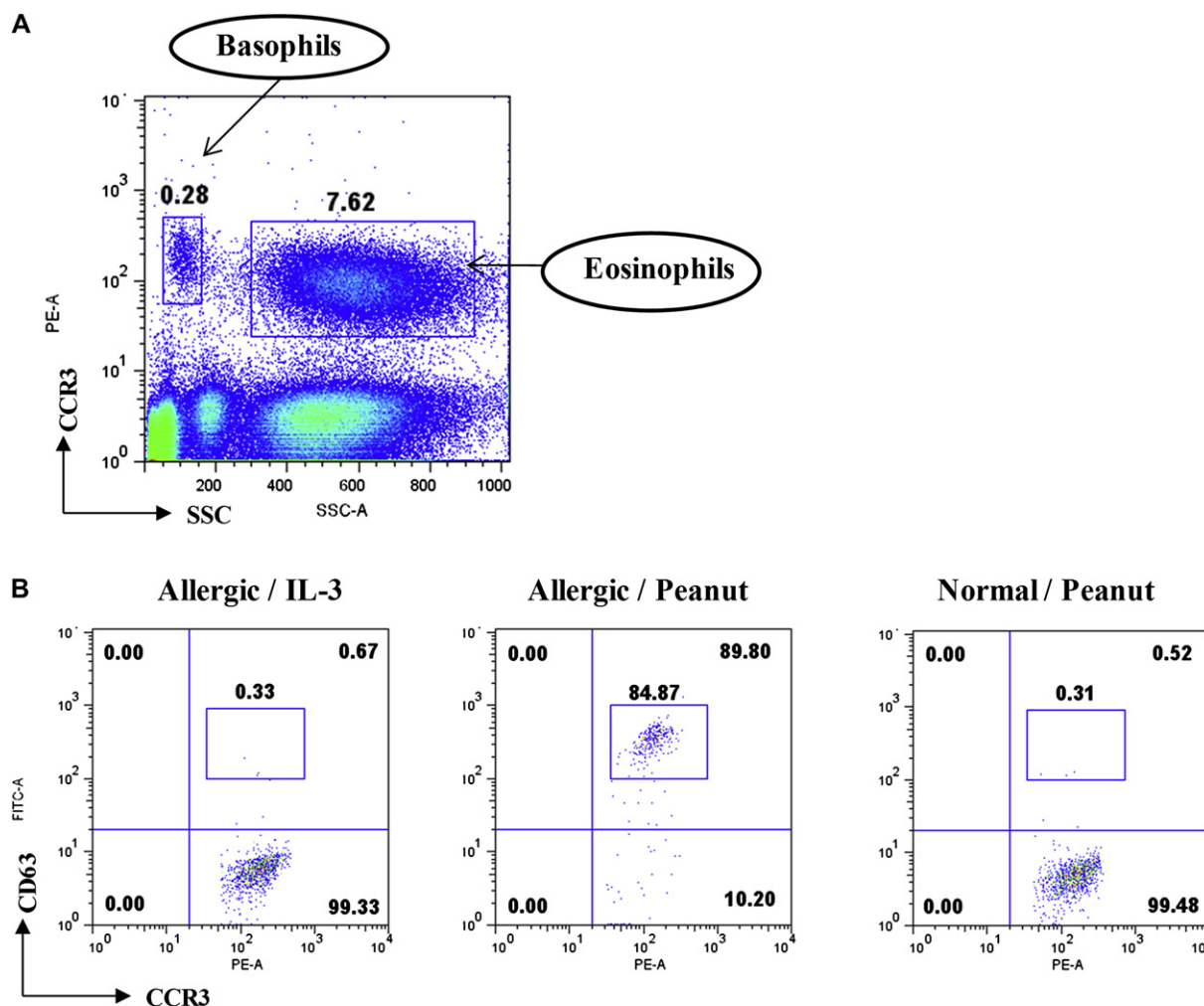


FIG E2. Gating strategy for the BAT. Aliquots of patients' blood were stimulated with either IL-3 containing stimulation buffer alone (negative control) or stimulation buffer with anti-FcεRI, fMLP, or antigen in the presence of anti-CCR3-fluorescein isothiocyanate- and anti-CD63-phycoerythrin-labeled mAbs. **A**, Basophils were gated as CCR3⁺ cells and distinguished from eosinophils on the basis of low side scatter. CD63⁺ cell percentages were identified as CCR3⁺ basophils expressing the activation marker CD63. **B**, Representative dot plots of allergic patients' blood sample stimulated with stimulation buffer alone or stimulation buffer plus peanut (200 ng/mL) and a healthy subject's blood stimulated with stimulation buffer plus peanut antigen (200 ng/mL).