Compatibility of imported fire ant whole body extract with cat, ragweed, *Dermatophagoides pteronyssinus*, and timothy grass allergens

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Background: Recommendations regarding the administration of imported fire ant whole body extract (IFA WBE) combined with aeroallergens or environmental allergens in a single immunotherapy injection are lacking.

Objective: To evaluate the degradative effect of IFA WBE on cat, ragweed, *Dermatophagoides pteronyssinus*, and timothy grass allergens.

Methods: Imported fire ant whole body extract was combined with extracts of cat, ragweed, *D pteronyssinus*, and timothy grass. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on each sample after storage for 0, 1, 3, and 6 months at 4°C. In addition, cat and ragweed combinations were evaluated by radial immunodiffusion (RID); *D pteronyssinus* by enzyme-linked immunosorbent assay (ELISA) inhibition; and timothy grass by ELISA inhibition and Western blot.

Results: Imported fire ant whole body extract combined with timothy grass demonstrated degradation of timothy grass allergens by SDS-PAGE, ELISA inhibition, and Western blot results. Cat and ragweed allergens were stable after mixing with IFA WBE, based on SDS-PAGE and RID analyses. Stability of *D pteronyssinus* allergens with IFA WBE was evident from SDS-PAGE and ELISA inhibition data.

Conclusions: Imported fire ant whole body extract combined with timothy grass resulted in significant and rapid timothy protein degradation. Imported fire ant whole body extract mixed with cat, ragweed, or *D pteronyssinus* revealed aeroallergen stability, yielding the possibility of combining these extracts in a single immunotherapy injection. Compatibilities of IFA WBE with other common aeroallergens remain undetermined and thus are not recommended for single-injection immunotherapy formulations.

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INTRODUCTION

The 2007 allergen immunotherapy practice parameter recommends administering allergens with high proteolytic activity, such as cockroach and molds, as a separate injection from other allergens.[1] This recommendation is based on multiple studies that demonstrate protein degradation and therefore presumed loss of potency when these extracts are combined.[2–6] The practice parameter also recommends that flying Hymenoptera such as wasp or honeybee venom be given apart from yellow jacket and hornet venom.[1] However, no specific comment pertaining to the administration of imported fire ant whole body extract (IFA WBE) in the same immunotherapy injection with other Hymenoptera or pollens was provided. Imported fire ants became established in the United States (*Solenopsis richteri* in 1918 and *Solenopsis invicta* around 1940) through the port of Mobile, Alabama.[7] These 2 species have since spread throughout much of the southern United States and have the potential to increase their spread from Delaware to Washington, being limited by climate and moisture.[8] Imported fire ants are aggressive when disturbed, and outdoor activities routinely place an individual at risk for anaphylaxis if stung. Estimations of systemic reactions from IFA range from 0.6% to 1%.[9,10] For those determined to be IFA allergic, IFA WBE immunotherapy has been demonstrated to be efficacious by retrospective evaluation of systemic reactions to field stings while receiving IFA WBE immunotherapy and prospectively by rush immunotherapy.[11,12] However, there has been no double-blind placebo-controlled study published in the literature verifying efficacy.

Initially, this study examined the potency effect of IFA WBE on select aeroallergens and environmental allergens, simulating maintenance immunotherapy at 0, 1, 3, and 6 months. IFA WBE was combined with extracts of cat, ragweed, *Dermatophagoides pteronyssinus*, and timothy grass and analyzed qualitatively and quantitatively. Subsequent studies with additional formulations will confirm the degree to which our results apply to other, related mixtures. This

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research was approved by the local institutional review board as an exempt protocol.

METHODS

Extracts

The extracts used in this study included IFA WBE, 1:10 wt/vol (a mixture of *S invicta* and *S richteri*); cat, 10,000 BAU/mL; ragweed (giant, short, western), 1:20 wt/vol; *D pteronyssinus*, 10,000 AU/mL; and timothy grass, 100,000 BAU/mL. These extracts were used clinically at the investigation site, Wilford Hall Medical Center (WHMC), Lackland Air Force Base, San Antonio, Texas, at the time of this study for both skin testing and immunotherapy and were purchased from Hollister-Stier (Spokane, Washington). Each extract contained 50% glycerin as a preservative.

Extract Combinations

One milliliter of IFA WBE was mixed with 1 mL of each of cat, ragweed, *D pteronyssinus*, and timothy grass. Each combination was then added to 8 mL of diluent (0.9% sodium chloride + 0.4% phenol + 0.03% human serum albumin; Greer Laboratories, Lenoir, North Carolina) to total a 10 mL vial. Controls were prepared by combining 1 mL of each of IFA WBE, cat, ragweed, *D pteronyssinus*, and timothy grass with 9 mL of diluent. Four vials of each extract and control combinations were made for storage at 4°C for 0, 1, 3, and 6 months. Duplicates of each combination were prepared and shipped to Greer Laboratories for analysis.

Human and Rabbit Sera

Western blot analysis used pooled frozen human serum containing specific IgE to timothy grass (class 2 to 3 Immuno CAP). The sera collected at WHMC was obtained from a previously approved WHMC human institutional review board study that collected serum from informed, consenting participants who gave permission to store their serum for future use. For the enzyme-linked immunosorbent assay (ELISA) inhibition performed at Greer Laboratories, freeze-dried human serum pools containing specific IgE to timothy grass and dust mite allergens were obtained from the Food and Drug Administration (FDA) (grass-positive serum lot S5-grass and mite-positive serum lot S5-Dpf) or prepared at Greer Laboratories (mite-positive serum lot ZE-P3). Sheep antisera directed against cat (Fel d 1; lot S-2b) and short ragweed (Amb a 1; lot S-8) were also procured from the FDA for radial immunodiffusion (RID) analyses.

Extract Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on all extract combinations and controls at both WHMC and Greer Laboratories. The details of this procedure have previously been described.[13] Briefly, extract combinations, controls, diluent, and low-molecular weight controls (Mark 12 unstained standard; Invitrogen, Carlsbad, California) were performed on 10% to 20% gradient gels (PAGEr Gold Precast Gel; Lonza Rockland Inc, Rockland, Maine) for 90 minutes at 125 V on a Hoefer Mighty Small SE-250 electrophoresis unit (San Francisco, California) and subsequently silver-stained for visualization (Invitrogen).

ELISA inhibition is recognized by the FDA for extract lot release and stability testing of dust mite and grass pollen[14–16] and was used in the evaluation of *D pteronyssinus*/IFA WBE and timothy grass/IFA WBE combinations. Microtiter plates (Costar; Corning Inc, Corning, New York) were coated with saturating levels of dust mite or meadow fescue reference extracts in carbonate buffer (pH 9.6) for 15 to 20 hours at 2°C to 8°C. After washing with phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20, plates were incubated with human serum pools (1:30 to 1:50 vol/vol dilutions) containing serial dilutions of controls (reference) and test mixtures for 4 to 6 hours at 20°C to 25°C. Bound IgE was then detected by means of successive incubations with biotinylated anti-human IgE (KPL, Gaithersburg, Maryland), avidin-alkaline phosphatase (Zymed, South San Francisco, California), and para-nitrophenyl phosphate (Amresco, Solon, Ohio). Absorbance values were determined at 405 nm in a microplate reader (ELx808; BioTek Instruments Inc, Winooski, Vermont). Using dose-response curves established by linear regression, the IgE-binding potencies of test mixtures relative to the corresponding single-extract references were determined using parallel-line bioassay. Statistical tests (paired *t* tests) were performed to confirm parallelism and validity of test and reference curves.

Radial immunodiffusion assays recognized by the FDA for extract lot release and stability testing for the prominent allergenic proteins of cat (Fel d 1) and short ragweed (Amb a 1) were performed on the cat/IFA WBE and ragweed/IFA WBE combinations. Radial immunodiffusion was conducted using 1% agar gels containing specific sheep antisera cast onto support films (GelBond; Cambrex BioScience, Rockland, Maine) and allergen standards established by the FDA (C10-Cat; 4.6 to 13.5 Fel d 1 U/mL; c14-RAS:5 to 30 μg Amb a 1 U/mL).[15,17,18]

Western blot was performed on the timothy grass and IFA combinations at WHMC and Greer Laboratories. The details of this procedure have been previously described.[13] In brief, following protein separation by molecular weights by SDS-PAGE as above, these proteins were transferred to nitrocellulose paper. Pooled sera, containing IgE antibodies from known timothy grass–allergic patients, was added to the nitrocellulose paper followed by the addition of mouse monoclonal anti-human IgE (Sigma, St Louis, Missouri) and tagged with goat anti-mouse IgG Alk Phos (Chemicon, Temecula, California). To visualize banding, an AP conjugate substrate kit (Bio-Rad Laboratories, Hercules, California) was used.

RESULTS

SDS-PAGE

SDS-PAGE to cat/IFA, ragweed/IFA, and *D pteronyssinus*/IFA combinations revealed consistent band intensities after

storage for 0 to 6 months. Unique bands from IFA and cat, ragweed, and *D pteronyssinus* controls were demonstrated in each of the correlated combinations (data not shown). A unique band at approximately 25 kDa in the IFA control remained present in the combinations, qualitatively suggesting that IFA was not degraded. This band was not noted in the timothy control or in the HSA lane. Also, a band present in the timothy grass control at approximately 8 kDa not noted in the HSA or IFA control was barely visible in the timothy grass/IFA combination at time 0 and was not visible at either 3 or 6 months (Fig 1).

Radial Immunodiffusion

Radial immunodiffusion was used to determine the potency effect of cat (Fel d 1) and short ragweed (Amb a 1; data not shown). All extracts (both combination and controls) were compared with the time 0 control. The results ranged from 77% to 102% for Fel d 1 and 97% to 111% for Amb a 1. These results were within acceptable range for product comparability, defined as being 75% to 125% of control or reference extract.

ELISA Inhibition

ELISA inhibition was applied to *D pteronyssinus*/IFA and timothy grass/IFA combinations and performed at Greer Laboratories. ELISA inhibition to the *D pteronyssinus*/IFA combination demonstrated *D pteronyssinus* relative potencies of 90% to 116% of the time 0 control, which is within the acceptable range for product comparability (75% to 125% of control extract; data not shown). ELISA inhibition to the timothy grass/IFA combination demonstrated rapid and significant decreases in timothy protein (Fig 2). The time 0 timothy/IFA combination analyzed immediately after Greer Laboratories received the sample revealed a relative potency of 13% for timothy grass. The relative potency for timothy grass remained significantly reduced in the combinations during the 1-, 3-, and 6-month evaluations.

Storage time at 2-8°C (months)	Test sample	Relative potency					
		# of valid tests	Mean Log_3RP	SD	Mean RP	BAU/mL	% of time 0 control
0	Control	2	-2.004	0.030	0.111	111	100
	Mix	2	-3.869	0.042	0.014	14	13
1	Control	2	-2.236	0.002	0.086	86	77
	Mix	2	-4.657	0.344	0.006	6	5
3	Control	2	-2.227	0.062	0.087	87	78
	Mix	2	-4.965	0.005	0.004	4	4
6	Control	2	-2.121	0.235	0.097	97	87
	Mix	2	-4.675	0.036	0.006	6	5

Figure 2. Enzyme-linked immunosorbent assay inhibition analysis of timothy extracts after mixing with imported fire ants and storing for up to 6 months at 2°C to 8°C, measuring the relative potency of the combinations when compared with timothy control at time 0. Control, timothy control; Mix, timothy/imported fire ant whole body extract combination mixes; RP, relative potency; BAU, bioequivalent allergy units.

Western Blot

A Western blot to the timothy grass/IFA combination was performed to further evaluate the information revealed from SDS-PAGE and ELISA inhibition. Pooled sera from known timothy grass–allergic patients (specific timothy IgE ranging from moderate to high [class 2 to 3] Immuno CAP) was added to nitrocellulose paper after transferring the SDS-PAGE. IgE binding was with consistent intensity in the timothy grass controls but was diminished in the timothy/IFA combinations (Fig 3). These results qualitatively confirm the

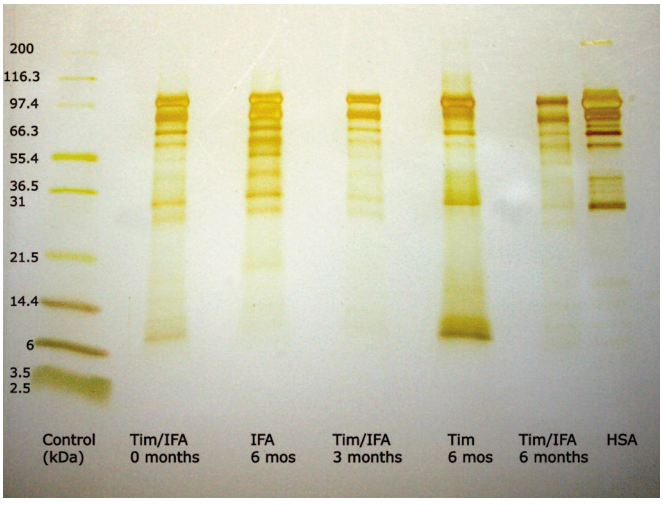

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis results from the timothy (Tim) control and imported fire ant whole body extract (IFA WBE) control at 6 months; the Tim/IFA combinations at 0, 3, and 6 months; and the human serum albumin (HSA) control.

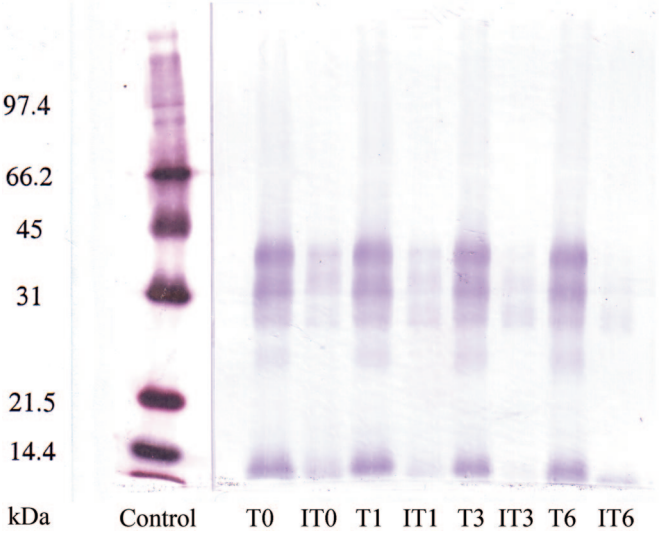

Figure 3. Western blot of timothy grass (T) at 0, 1, 3, and 6 months (T0, T1, T3, and T6) and timothy grass combined with imported fire ant whole body extract (IT) at time 0, 1, 3, and 6 months (IT0, IT1, IT3, and IT6).

results from the timothy grass/IFA combination observed by SDS-PAGE and ELISA inhibition.

DISCUSSION

Allergen immunotherapy is a therapeutic option for patients with clinical relevance to, and specific IgE against, Hymenoptera, pollen, animal, cockroach, mold, and dust mite proteins. Particularly, Hymenoptera hypersensitivity can result in increased risk of future systemic reactions if untreated, and therefore those at risk are strongly encouraged to receive immunotherapy.[11] Whereas flying Hymenoptera contact generally results in few stings, IFA exposure can result in multiple stings, and they are difficult to avoid and eradicate. Tracy et al[19] evaluated 107 students visiting San Antonio, Texas, who had a limited history of IFA exposure. During their 3-week visit, 55 (51%) reported field stings. There were no systemic reactions in this group, but conversion to positive serologic or prick skin test results was noted in 8 (14%) of those who reported being stung. IFAs are firmly established in the southern United States, will continue to cause systemic reactions in susceptible individuals, and have the potential to spread from Delaware to Washington.[8] Although double-blind, placebo-controlled studies have not been published, retrospective and prospective studies demonstrate efficacy in IFA WBE immunotherapy.[11,12] In an 18-month period at our facility (October 2005 to April 2007), 485 patients received immunotherapy, with 60 (12%) of those receiving IFA WBE immunotherapy. Of IFA WBE immunotherapy patients, 41 (68%) were exclusively receiving IFA WBE and 11 (18%) were receiving a combination of IFA WBE and aeroallergen immunotherapy (unpublished data). The data suggest the need to address the feasibility of combination immunotherapy of IFA WBE with aeroallergen. The 2007 allergen immunotherapy practice parameter recommends not mixing venoms together[1] but does not specifically comment on combining venoms or IFA WBE with pollens or other allergens.

Previous studies have demonstrated that interactions between allergen extracts that have high proteolytic enzyme activities, such as mold and cockroach, can diminish the potency of other aeroallergens and may lead to reduced efficacy.[2–6] One study demonstrated resistance of short ragweed to proteolytic effects of fungi and cockroach,[2] but further studies did not reach this conclusion.[4] Hence, consideration should be given to keep mold and cockroach separate from pollen extracts. Dust mites also contain proteolytic activity but at levels considerably lower than fungal and insect extracts, resulting in clinical efficacy when dust mite is combined with other aeroallergens.[3,5]

The present study is only the second to investigate the potency effects of IFA WBE with select aeroallergens. Meier et al[20] investigated the potency effects from combining 1 mL of IFA WBE with 1 mL of the Texas winter-pollinating aeroallergen *Juniperus ashei*, commonly referred to as mountain cedar. These investigations demonstrated that this combination, analyzed qualitatively by SDS-PAGE, did not result in degradation of IFA WBE or *J ashei*. However, no comment about allergenicity could be made because quantitative measures were not evaluated. This study revealed that qualitatively and quantitatively, the combination of IFA WBE with each of cat, ragweed, and *D pteronyssinus* demonstrated stability. The combination of IFA WBE and timothy grass, however, qualitatively and quantitatively revealed significant and rapid degradation of the timothy grass protein by SDS-PAGE, ELISA inhibition, and Western blot. Although not specifically evaluated further, IFA was qualitatively stable in combination with timothy grass because bands from IFA were noted in the IFA/timothy grass combination for up to 6 months.

Several clinical applications can be made from the results of this research. Providers now have an early indication that cat, ragweed, and *D pteronyssinus* extracts are stable in the presence of IFA extract. With additional work to confirm efficacy and potency of these combinations, it is possible that patients could clinically benefit from combination immunotherapy, resulting in decreased cost, injections, and discomfort. The potential for enhanced compliance may also be afforded. Before clinically implementing these strategies, additional work would be required with prospective studies investigating stability and clinical efficacy of IFA with the aeroallergens identified.

Alternatively, the data could serve to reinforce the use of IFA WBE immunotherapy as a separate injection from all pollens. Although only the timothy grass protein resulted in degradation when combined with IFA WBE in this study, this unequivocally demonstrated that degradation can occur between IFA WBE and aeroallergens. No comment can be made regarding the potency effect of IFA WBE when mixed with the variety of other pollens and molds.

There are limitations to this study. The potency effect of IFA WBE on cat, ragweed, *D pteronyssinus*, and timothy grass was evaluated, but the quantitative effect of these proteins on IFA WBE was not. IFA stability was, however, qualitatively determined to be stable by SDS-PAGE. In addition, only maintenance extract vials were evaluated. Therefore, the potency effect on serial dilutions as would be necessary when initiating immunotherapy would need to be addressed before being put into clinical practice. Finally, although the timothy/IFA combination resulted in degradation of the timothy grass protein, it is unknown if this is of clinical significance, and in vivo evaluation is imperative to follow up the results of this study.

Allergen immunotherapy has been proven efficacious in the management of a variety of IgE-based allergic conditions, and allergists are charged with providing appropriate and effective means for treatment. This study demonstrates that IFA WBE has degrading properties on some aeroallergens (timothy grass) but not others (cat, ragweed, *D pteronyssinus*), consistent with a recent report on extract compatibilities with cockroach extracts.[4] Although we present promising initial findings in the area of IFA WBE with select aeroallergen extracts, these study results must be repeated and verified in clinical practice using relevant extract concentra-

tions and diluents, yield positive immunotherapy outcomes to support the mixing practices inferred from the study data, and establish the optimal injection regimen without compromising the integrity of the allergens in these mixtures. Until further clinical data are applied, aeroallergens and environmental allergens still should be administered as a separate immunotherapy injection from IFA WBE.

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