Effects of summer mailing on in vivo and in vitro relative potencies of standardized timothy grass extract

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Background: Allergen extracts can degrade when exposed to temperatures significantly beyond the optimum storage recommendation of 4°C. Many allergen extracts are mailed to their final destinations throughout the year with exposure to varied environmental conditions.

Objective: To evaluate the effect of summer mailing on the in vitro and in vivo potency of timothy grass extract.

Methods: Standardized timothy grass extracts, 10,000 and 100,000 BAU/mL, were mailed round-trip between San Antonio, Texas, and Phoenix, Arizona, during August 2007. In-transit temperatures were recorded using a portable temperature logger. After mailing of the extracts, we performed quantitative in vitro enzyme-linked immunosorbent assay inhibition and in vivo ID_{50}EAL (Intradermal Dilution for 50-mm Sum of Erythema Determines Bioequivalent Allergy Units) analysis.

Results: Measured extract exposure temperatures were greater than 20°C for 11 days and 30°C for 6 hours during standard mailing in weather temperatures exceeding 38°C. Enzyme-linked immunosorbent assay inhibition results for the 100,000- and 10,000-BAU/mL control samples were 97,900 and 10,580 BAU/mL, respectively, and for the mailed extracts were 96,800 and 7,830 BAU/mL, respectively. These measurements fell within the current Food and Drug Administration lot release limits (67%–149%) and stability limits (50%–200%) relative to the standardized reference. The ID_{50}EAL determinations of the control vs mailed extracts were 12.98 vs 12.28, 12.66 vs 12.32, and 11.97 vs 11.70 for the 3 patients. These differences were not statistically significant.

Conclusions: Mailing of timothy grass extract produced no significant reductions in in vitro relative potencies or in vivo skin test reactivity in 3 sensitive patients.

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INTRODUCTION

Allergen extracts are used for the diagnostic evaluation and therapeutic treatment of allergic disease. These compounds are composed of allergenic proteins mixed with diluents according to extract manufacturers' formulations. Clinically significant allergenic proteins most commonly originate from plant pollens, dust mites, animal dander, and insects. These proteins, when inhaled by or injected into an allergic-susceptible individual, bind to specific IgE and initiate the allergic response.[1] The interaction of allergenic proteins and specific IgE can be observed and measured by means of skin testing.[2] These proteins are also responsible for the protective effect of immunotherapy, which acts through immunologic pathways to block the action of specific IgE and to ameliorate symptoms of allergic disease.[3]

The potency and stability of allergen extracts are critical factors for diagnostic and therapeutic efficacy in the management of allergic disease. Extract stability is described as the persistence of adequate quantities of relevant antigens in an allergen vaccine from the time of initial assay to the time of clinical use.[4] Factors that affect allergen extract stability include the initial extract concentration, storage temperature, stabilizers and antimicrobial agents added to the extract, the volume of the storage vial, and the interaction of allergens in an extract mix.[5]

In vitro studies[6–8] of allergen extracts, mixed with and without 50% glycerin, have demonstrated decreased stability with exposure to elevated temperatures. Therefore, storage recommendations for extracts specify refrigeration temperatures to help ensure maintenance of potency.[5] Controlled refrigeration is standard in clinical settings, but during mailing and shipping of extracts, this temperature control is not easily maintained. Mailing of extracts between manufacturers, mixing laboratories, and final clinical destinations occurs year-round and is associated with exposure to a wide range of environmental temperatures and conditions. Despite the common practice of mailing extracts, there is no peer-reviewed published data regarding potency changes as a result of elevated temperatures encountered during mailing. The pur-

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poses of this study were to identify the environmental temperatures that allergen extracts encounter during summer mailing and to evaluate the stability of extracts after summer mailing using in vitro and in vivo analyses.

METHODS

Allergen Extracts

This was a Wilford Hall Medical Center institutional review board–approved protocol in which written informed consent was obtained from all the study participants. A standardized 100,000-BAU/mL timothy grass (*Phleum pretense*) extract in 50% glycerin was obtained from Hollister-Stier Laboratories LLC (Spokane, Washington). The extract arrived at the study site in a 50-mL vial packaged with a cold gel pack in a Styrofoam box. Dilutions were prepared of two 10-mL vials of 100,000 BAU/mL and two 10-mL vials of 10,000 BAU/mL. The 10,000-BAU/mL extract was mixed with a diluent containing 0.9% sodium chloride, 0.03% human serum albumin, and 0.4% phenol. One each of the 10,000- and 100,000-BAU/mL vials stored at 4°C (40°F) for the study duration served as controls (Fig 1).

Mailing

Summer mailing of the duplicate 10,000- and 100,000-BAU/mL study vials was completed round-trip between San Antonio, Texas, and Phoenix, Arizona, during August 2007. The vials were packed in a Styrofoam container with a portable temperature logger (Veriteq Instruments Inc, Richmond, British Columbia, Canada). The temperature logger was programmed to read the environmental temperature every 5 minutes. The container was mailed in a padded envelope through standard US Postal Service mail from the Wilford Hall Medical Center Allergy Clinic in San Antonio to the Luke Air Force Base Allergy Clinic in Phoenix and then back to the Wilford Hall Medical Center Allergy Clinic (Fig 1). On completion of mailing, the study vials were returned to storage at 4°C (40°F) with the control vials. The extracts were not shipped with any external cooling product.

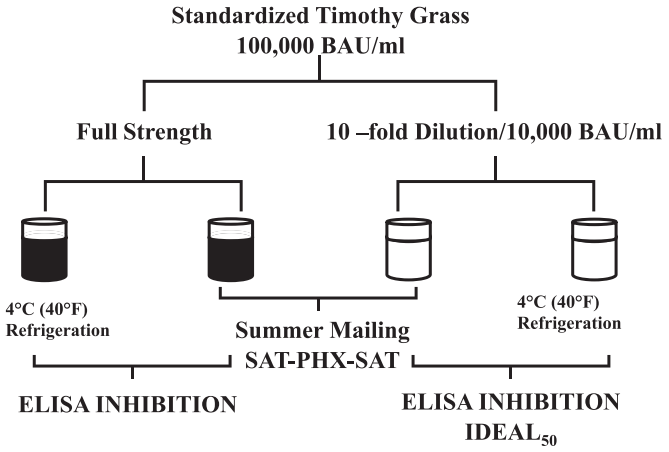

Figure 1. Study diagram for the evaluation of the effects of summer mailing on the potency of standardized timothy grass extract. ELISA indicates enzyme-linked immunosorbent assay; ID_{50}EAL, Intradermal Dilution for 50-mm Sum of Erythema Determines Bioequivalent Allergy Units; PHX, Phoenix; and SAT, San Antonio.

Enzyme-Linked Immunosorbent Assay Inhibition

Enzyme-linked immunosorbent assay (ELISA) inhibition is recognized by the US Food and Drug Administration (FDA) for extract lot release and stability testing of grass pollen.[9] This in vitro measure of potency determination is used by allergen extract manufacturers to ensure lot-to-lot consistency for standardized extracts. The ELISA inhibition method assigns a bioequivalent allergy unit per milliliter value determined by comparison with a single reference standard developed by the FDA. The use of a central reference ensures a single source for standardization of allergen extracts in the United States and increases consistency between manufacturers.[10]

The ELISA inhibition was completed on the control and mailed vials of the 10,000- and 100,000-BAU/mL dilutions at Greer Laboratories (Lenoir, North Carolina) (Fig 1). Extract vials were mailed together during February 2008 to avoid any additional variation in heat exposure between the vials. Immulon microplates (Dynax Technologies Inc, Chantilly, Virginia) were coated with 300 μL per well of 1:2,000 dilution of 100,000-BAU/mL timothy grass reference extract in carbonate coating buffer (pH 9.6) for 16 hours at 2°C to 8°C. After washing with phosphate-buffered saline containing 0.05% Tween 20, plates were incubated with human serum pools (1:50 vol/vol dilutions) containing serial dilutions of reference controls and test mixtures for 4.5 hours at 20°C to 25°C. Bound IgE was then detected by means of successive incubations with biotinylated anti–human IgE (Kirkegaard and Perry Laboratories Inc, Gaithersburg, Maryland), avidin–alkaline phosphatase (Zymed, South San Francisco, California), and para-nitrophenyl phosphate (Amresco Inc, Solon, Ohio). Absorbance values were determined at 405 nm using a microplate reader (ELx808; BioTek Instruments Inc, Winooski, Vermont). Using dose-response curves, the IgE-binding potencies of test mixtures relative to the corresponding extract references were determined using parallel-line bioassay.

The ID_{50}EAL Method

The ID_{50}EAL (Intradermal Dilution of 50-mm Sum of Erythema Determines Bioequivalent Allergy Units) method is an in vivo determination of potency that is used to assign bioequivalent allergy units. The FDA uses the ID_{50}EAL to establish the reference standard to which all other standardized extracts in the United States are compared. The ID_{50}EAL can also be used to compare the allergenicity of extracts from different sources.[11]

The ID_{50}EAL was completed using the control and mailed vials of the 10,000-BAU/mL extract concentration (Fig 1). Twenty-one serial 3-fold dilutions were mixed from the control and mailed vials using the diluent 0.9% sodium chloride,

0.03% human serum albumin, and 0.4% phenol. Skin testing was performed by injecting 0.05 mL of the serial study extracts, using a 27-gauge needle, into the intradermal space as described by Turkeltaub and Rastogi.[12] After 15 minutes, the erythema of the longest and orthogonal diameters was measured and was used to determine the sum of erythema for each testing site. The best-fit regression line was determined for each participant from the serial measurements of sum erythema. Using the best-fit regression line, the dilution that results in 50-mm sum erythema was calculated, corresponding to the ID_{50}EAL. The ID_{50}EAL results of the test participants were averaged. The 2-tailed *t* test was used to determine significant differences between the mean results.

RESULTS

Exposure Temperatures

The mailing time from San Antonio to Phoenix and back to San Antonio was 12 days round-trip (August 15–27, 2007). The recorded temperatures in San Antonio during this period were 23°C (74°F) to 36°C (96°F).[13] The recorded temperatures in Phoenix ranged from 24°C (75°F) to 44°C (111°F), which included 2 days of record-setting temperatures.[14]

The portable temperature logger measured temperatures between 4°C (40°F) and 36°C (96°F) during mailing. There were 11 continuous days of temperatures greater than the standard room temperature of 20°C (68°F). Temperatures higher than 30°C (86°F) were measured for 6 continuous hours and more than 35°C (95°F) for 95 minutes.

ELISA Inhibition

The 100,000-BAU/mL control and mailed vials measured 97,900 and 96,800 BAU/mL, respectively (Fig 2A). The 10,000-BAU/mL control and mailed vials measured 10,580 and 7,830 BAU/mL, respectively (Fig 2B). The potencies of the mailed vials of both concentrations were lower than the measured potencies of the control vials. However, all the vials were within the FDA lot release limit of 67% to 149% of the reference standard.

ID_{50}EAL

The ID_{50}EAL in vivo measure of potency was performed on 3 human volunteers sensitive to timothy grass allergen extract. A lower ID_{50}EAL value correlates to a lower dilution number, which indicates that a more concentrated extract is required to induce a 50-mm sum of erythema. For the 3 participants, the ID_{50}EAL values of the control vials were 12.98, 12.66, and 11.97 and of the mailed vials were 12.29, 12.32, and 11.70.

The mean ID_{50}EAL of the participants was 12.54 for the control vials and 12.1 for the mailed vials. These results indicate that a lower dilution number in the mailed extract, corresponding to a higher concentration, was necessary to produce 50 mm of erythema with intradermal skin testing. This difference of 0.44 dilutions was not significant ($P = 0.3$).

The slope of the best-fit regression line is also determined when calculating the ID_{50}EAL from the intradermal skin test results. The slopes of the curves corresponding to the control and mailed extracts were 22 and 16, 20 and 19, and 16 and 10, respectively, for the 3 participants.

DISCUSSION

Summer mailing of allergen extracts in the southern United States did not result in significant loss of potency. During mailing, the extracts underwent prolonged exposure to temperatures greater than the recommended 4°C (40°F), with a total of 11 days higher than room temperature. The in vitro ELISA inhibition of the mailed extract showed degradation, but the potency remained within the FDA lot release limits. In vivo potency, measured by means of the ID_{50}EAL, was not statistically significantly different between the control and mailed vials. To our knowledge, this is the first study to use in vivo measurements to evaluate potency changes in mailed extracts.

The skin prick test used by physicians to determine a patient's allergenicity to a specific allergen is a measure of in vivo reactivity, and it is this evaluation that is of paramount

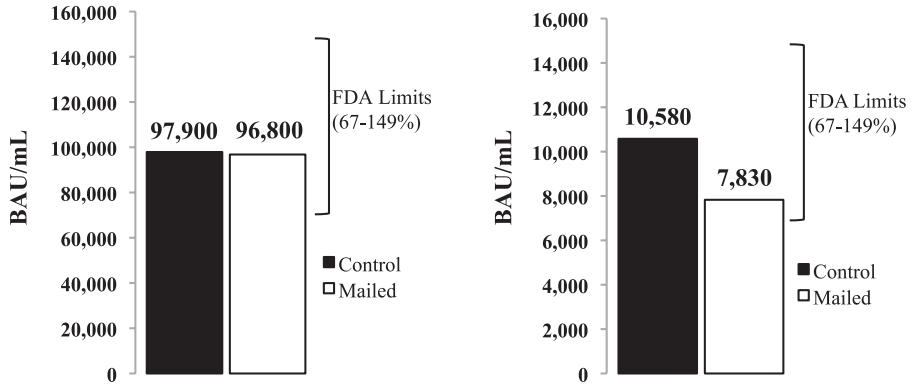

Figure 2. Enzyme-linked immunosorbent assay inhibition results of control and mailed vials of standardized timothy grass extract of 100,000 BAU/mL (A) and 10,000 BAU/mL (B). Food and Drug Administration (FDA) lot release limits are indicated by brackets.

importance to the clinical allergist. Previous studies have shown discrepancies in measures of potency between ELISA inhibition in vitro testing and in vivo skin testing. Niemeijer et al[8] reported stable skin test reactivity to dust mite and timothy pollen extracts stored at elevated temperatures for 24 months, but the same extracts showed a 50% loss of in vitro potency. Franklin et al[15] showed that ragweed-sensitive patients maintained skin test reactivity to a ragweed study extract despite a 60% loss of in vitro activity after 18 months of 4°C (40°F) storage. These studies demonstrate that even when potency measures decrease with in vitro assessments, the individual patient response may not be significantly affected. The ID_{50}EAL determination in this study provides reassurance for the physician that similarly mailed extracts used for skin testing will likely retain a reliable level of in vivo potency.

The experimental procedures for this study used real-world mailing exposure rather than simulated laboratory conditions to evaluate the stability effects associated with mailing allergen extracts. By recording real-time temperature data, we provided an accurate report of the mailing environment during shipping procedures. In addition, this study was strengthened by the record-setting high temperatures in the southwestern United States during the shipping dates. These stability results represent not only effects associated with summer mailing but also mailing during record-setting high temperatures that would be expected to be near the outer limits of exposure for this geographical area. The logger temperatures were elevated above the optimal conditions for allergen extracts as would be expected, but reassuringly they were not so exceedingly high to suggest a mailing crate sitting on hot asphalt in the afternoon sun. The extract vials in this study were sent through the US Postal Service standard mail. Other mailing companies and shipping procedures can be used to transport allergen extracts and may be associated with exposures that vary from the temperatures recorded in this study and could have different effects on extract stability. Although this study design represents the standard mailing procedures we use for our mixing laboratory, it may not universally represent the mailing procedures of all shipping companies.

This study used 2 different extract concentrations: 100,000 and 10,000 BAU/mL. The 100,000-BAU/mL concentration is the standard timothy grass extract corresponding to the stock vial and is the concentration used by this practice for skin prick testing. The 10,000-BAU/mL extract represents a maintenance immunotherapy concentration corresponding to the 1:1 vol/vol red vial of an immunotherapy treatment kit. The 100,000-BAU/mL extract maintained 97% of its original in vitro potency compared with the 78% maintained by the 10-fold dilution (10,000 BAU/mL) after summer mailing. It is widely reported[5,7,11,16] that more dilute extracts show greater loss of potency across time, as was encountered with this study. These findings bring into question the potency that more diluted vials would retain after mailing. Would additional dilutions fail to meet the FDA lot release limits? Immunotherapy extract kits are generally ordered in 4- to 5-vial sets with the most diluted vial containing a 1:1,000 to 1:10,000 concentration of each extract, equivalent to 1 to 10 BAU/mL, compared with the maintenance vial. How well would the allergens of these further diluted vials maintain stability after heat exposure encountered during summer mailing?

The in vivo and in vitro measurements of potency used in this experiment are intended to identify varying levels of IgE binding. Allergenic proteins can have multiple binding sites for specific IgE. For example, there are 12 allergenic proteins characterized for timothy grass pollen.[17,18] These binding proteins can be heat labile, which would decrease IgE binding affinity after exposure to elevated temperatures, and heat stable. The allergenic proteins that bind specific IgE are responsible for activating the immediate, type 1 hypersensitivity, allergic response. As allergists, we measure this response to determine individual patient reactivity during allergen skin testing. Therefore, significant potency loss determined by in vitro or in vivo measures could be associated with a decline in diagnostic reliability of allergen extracts as a result of degradation of IgE-binding proteins.

Conversely, reduced potency measured through the use of ELISA inhibition and ID_{50}EAL may not affect the treatment response to immunotherapy. T-cell epitopes, not IgE-binding sites, have increasingly been shown to stimulate the immunologic changes associated with the therapeutic effects of immunotherapy.[19,20] These 10– to 30–amino acid–length peptides act through regulatory T cells to downregulate the allergic response without inducing the strong adverse effects that can occur with IgE binding to the larger allergenic protein. Therefore, although potency declines may be associated with a reduced skin test response in susceptible patients, the loss of potency as measured by IgE binding may not negatively affect the therapeutic benefits of immunotherapy.

In summary, summer mailing of timothy grass extract does not result in significant loss of in vitro relative potency or in vivo potency. Degradation occurred in the 10,000- and 100,000-BAU/mL concentrations, with the more dilute extract showing greater potency loss; however, the final relative potency of all the extracts was within the lot release limits. Additional studies would be beneficial to determine whether extract stability is maintained after summer mailing of more dilute extract concentrations and in mixtures of multiple pollen extracts. Although it is important to note that at this time these study results cannot be generalized to all allergen extracts, they may provide reassurance to extract mixing laboratories and clinical allergists that summer mailing of timothy grass extract during warm temperatures does not significantly affect clinical potency.

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REFERENCES

1. Wallace D, Dykewicz M, Bernstein D, et al. The diagnosis and management of rhinitis: an updated practice parameter. *J Allergy Clin Immunol.* 2008;122:S32–S35.
2. Demoly P, Bousquet J, Romano A. In vivo methods for study of allergy. In: Adkinson NF, Bochner BS, Busse WW, et al, eds. *Middleton's Allergy: Principles and Practice.* 7th ed. Philadelphia, PA: Mosby; 2009:1267–1279.
3. Nelson H. Immunotherapy for inhalant allergens. In: Adkinson NF, Bochner BS, Busse WW, et al, eds. *Middleton's Allergy: Principles and Practice.* 7th ed. Philadelphia, PA: Mosby; 2009:1657–1677.
4. Soldatova LN, Paupore EJ, Burk SH, Pastor RW, Slater JE. The stability of house dust mite allergens in glycerinated extracts. *J Allergy Clin Immunol.* 2000;105:482–488.
5. Cox L, Li J, Nelson H, Lockey R. Allergen immunotherapy: a practice parameter second update. *J Allergy Clin Immunol.* 2007;120(suppl): S25–S85.
6. Anderson MC, Baer H. Antigenic and allergenic changes during storage of a pollen extract. *J Allergy Clin Immunol.* 1982;69:3–10.
7. Nelson HS. Effect of preservatives and conditions of storage on the potency of allergy extracts. *J Allergy Clin Immunol.* 1981;67:64–69.
8. Niemeijer NR, Kauffman HF, van Hove W, Dubois AE, de Monchy JG. Effect of dilution, temperature, and preservatives on the long-term stability of standardized inhalant allergen extracts. *Ann Allergy Asthma Immunol.* 1996;76:535–540.
9. Grier TJ. Laboratory methods for allergen extract analysis and quality control. *Clin Rev Allergy Immunol.* 2001;21:111–140.
10. Becker WM, Vogel L, Vieths S. Standardization of allergen extracts for immunotherapy: where do we stand? *Curr Opin Allergy Clin Immunol.* 2006;6:470–475.
11. Slater JE, Esch RE, Lockey RF. Preparation and standardization of allergen extracts. In: Adkinson NF, Bochner BS, Busse WW, et al, eds. *Middleton's Allergy: Principles and Practice.* 7th ed. Philadelphia, PA: Mosby; 2009:557–568.
12. Turkeltaub PC, Rastogi SC. Methods of the Allergenics Products Testing Laboratory: Quantitative Intradermal Procedure for Evaluation of Subject Sensitivity to Standardized Allergenic Extracts and for Assignment of Bioequivalent Allergy Units to Reference Preparations Using the ID_{50}EAL Method (Intradermal Dilution for 50 mm Sum of Erythema Determines Bioequivalent Allergy Units). Docket No. 94N-0012. October 1993. http://www.fda.gov/ohrms/dockets/98fr/94n-0012-bkg0001-01-vol1.pdf.
13. AccuWeather.com. San Antonio, TX, past month's weather: August 2007. http://www.accuweather.com. Accessed August 29, 2007.
14. AccuWeather.com. Phoenix, AZ, past month's weather: August 2007. http://www.accuweather.com. August 29, 2007.
15. Franklin RM, Baer H, Hooton ML, Brown H. The stability of short ragweed pollen extract as measured by skin test and antigen E. *J Allergy Clin Immunol.* 1976;58:51–59.
16. Plunkett G. Stability of allergen extracts used in skin testing and immunotherapy. *Curr Opin Otolaryngol Head Neck Surg.* 2008;16:285–291.
17. Petersen A, Suck R, Hagen S, Cromwell O, Fiebig H, Becker WM. Group 13 grass allergens: structural variability between different grass species and analysis of proteolytic stability. *J Allergy Clin Immunol.* 2001;107:856–862.
18. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol.* 2003;130:87–107.
19. Litwin A, Pesce AJ, Fischer T, Michael M, Michael JG. Regulation of the human immune response to ragweed pollen by immunotherapy: a controlled trial comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. *Clin Exp Allergy.* 1991;21:457–465.
20. Burton MD, Blaher B, Suphioglu C, O'Hehir RE, Carbone FR, Rolland JM. T-cell receptor contact and MHC binding residues of a major rye grass pollen allergen T-cell epitope. *J Allergy Clin Immunol.* 1999;103: 255–261.

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