*2002 ACAAI Presentation # P10*

# MAJOR ALLERGEN LEVELS vs. STANDARDIZED EXTRACT POTENCIES : COMPARISONS, COMPLICATIONS AND CLINICAL CONSEQUENCES

***Thomas J. Grier, Ph.D., Dawn M. Hazelhurst, B.S., Elizabeth A. Duncan, B.S., K. Diane Gilbert and Robert E. Esch, Ph.D.***

**Research & Development/Quality Control Labs**
**Greer Laboratories, Inc.**
**Lenoir, North Carolina USA**

## *ABSTRACT*

**The standardization of allergen extracts based on the immunochemical properties of specific protein constituents remains a challenging process for researchers, manufacturers and federal regulators. Standardized extract potencies are attributed to prominent individual (major) allergens (cat, short ragweed) or multiple molecular components (dust mites, grasses). Mite, grass and non-standardized cockroach extracts can also be assessed for major allergen concentrations using sensitive ELISA methods. For these products, accurate conversions from current unit dosing (AU or BAU, human IgE reactions) to major allergen content (µg, mouse/rabbit IgG reactions) require a close relationship between individual protein and total extract reactivities, with consistent proportions maintained for all pertinent component structures and epitopes.**

**Data from our laboratory confirm published reports that the major allergen levels of dust mite extracts (Der 1, Der 2) do not correlate consistently with their AU/mL concentrations. Up to 4-fold differences in Der 1 and Der 2 content are observed for 10,000 AU/mL mite extracts possessing comparable human IgE ELISA reactivities. Specificity differences between animal and human antibodies, or between different human serum pools employed for potency testing, contribute significantly to these discrepancies. In addition, Group 1 grass allergens which are unstable to heat based on human IgE reactions are stable or increase in detectability after heat exposures when probed with rabbit IgG antibodies. Cockroach extracts (candidates for standardization) also display noticeable differences between human IgE potencies and major allergen properties. Aqueous German cockroach extracts retain high levels of Bla g 1 and Bla g 2 but virtually no IgE reactivity. Bla g 1 and Bla g 2 epitopes recognized by mouse and rabbit IgG are resistant to the endogenous proteases which degrade IgE-binding structures rapidly in aqueous cockroach extracts. Structural changes in critical cockroach allergens are reduced (but not eliminated) by inclusion of 50% glycerin in these products.**

**These data support the notion that correlations between major allergen and total extract potencies may be inconsistent or low for products characterized by multiple (greater than 5) component activities (mites, grasses, cockroach), and suggest that considerable caution should be employed regarding the clinical use of major allergen levels for these products.**

## *U.S. STANDARDIZED EXTRACTS*

- **Current standardized extracts are formulated at concentrations corresponding to specific biological (allergenic) activities using validated, FDA-approved analytical methods**

| *Extract* | *Units of measure* | *Single or multiple allergens* | *Compendial potency assay* |
|---|---|---|---|
| Cat hair/pelt | BAU/mL | Single (Fel d 1) | Radial immunodiffusion |
| Short ragweed | AgE U/mL | Single (Amb a 1) | Radial immunodiffusion |
| Dust mites | AU/mL | Multiple | ELISA inhibition |
| Grasses | BAU/mL | Multiple | ELISA inhibition |
| Venoms | µg/mL | Multiple | Ninhydrin protein |

- **Single (major) allergen concentrations have also been determined for extracts standardized to multiple protein components (mites, grasses)**
- **Close correlations between single and multiple allergen reactivities for an extract require similar levels or proportions of major and minor IgE-binding proteins and biochemical comparabilities among test methods**

## *POTENCY COMPARISONS*

- **Major allergen concentrations for many extracts are measured using highly-specific, sensitive double-bind (2-site, sandwich) ELISA methods**

| *Assay method* | *Single or multiple allergens* | *Antibodies* | *Sensitivity (ng/mL)* |
|---|---|---|---|
| Radial immunodiffusion | Single | Rabbit IgG | 20-200 |
| ELISA inhibition | Multiple | Human IgE | 2-10 |
| Double-bind ELISA | Single | Mouse IgG / Rabbit IgG | 0.001-0.02 |

- **Double-bind ELISA assays often employ antibody reagents and extract standards which differ from lab-to-lab, and have not been validated for specificity, accuracy and comparability across multiple labs**
- **The relationships among the current validated and non-validated test methods used with standardized or characterized extracts must be established to determine the clinical relevance of major allergen levels in extracts and of maintenance immunotherapy dose recommendations**

## *COMPLICATIONS*

- **Inconsistent criteria for major allergen identification**
  - ***3D structures of IgE/IgG-binding epitopes***
  - ***Prevalence/prominence, conservation/stability***
- **Allergen variability**
  - ***Differing proportions of major & minor allergens***
  - ***Natural genetic or geographic variations***
  - ***Isoallergens, complexation, stability/compatibility***
- **Analytical method/reagent differences**
  - ***Assay sensitivity/precision/comparability across labs***
  - ***Reagent source/specificity/qualification/availability***
  - ***Structural requirements for Ag-Ab interactions***

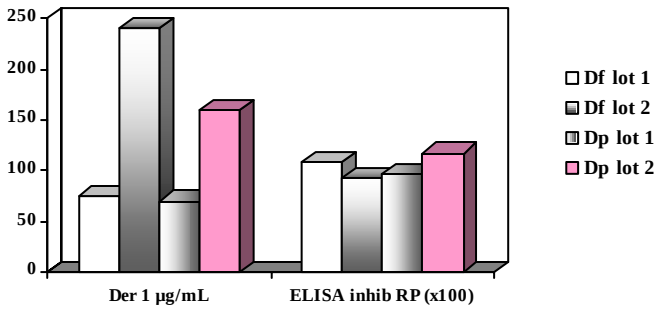
## DUST MITE EXTRACT POTENCIES

- Major allergen and total extract potencies were assessed for 10,000 AU/mL standardized *D. farinae* and *D. pteronyssinus* extracts

| *Assay method* | *Specificity* | *Allergens* | *Antibodies* | *Validated/ FDA approved?* |
|---|---|---|---|---|
| ELISA inhibition | Total extract | 10-15 | Human IgE | Yes |
| Double-bind ELISA | Major Ag (Der f 1, Der p 1) | 1 | Mouse IgG/ Mouse IgG | No |

- Product lots manufactured within a 4 month period (March-June 2001) and tested with the same FDA reference serum (S5-Dpf) displayed consistent total potencies (RP) but variable Der 1 concentrations

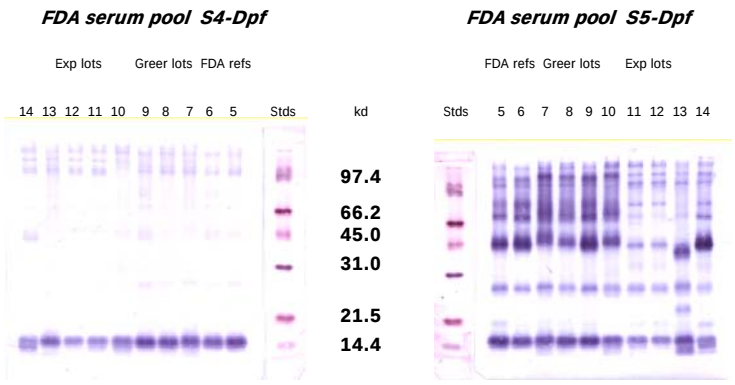
| *Extract/lot* | *Der 1 (µg/ml)* | *Total potency* | *Lot 2/Lot 1* |
|---|---|---|---|
| D. farinae, lots 1-2 | 75-241 | 0.93-1.08 | 3.7 |
| D. pter, lots 1-2 | 70-160 | 0.96-1.17 | 2.0 |




- Human serums used to standardize dust mite extracts may possess distinct and variable IgE specificities to multiple allergenic epitopes, and may not include structures recognized by mouse/rabbit IgG's

IgE immunoblots w/ consecutive serum/extract references:

*D. farinae extracts @ 0.7 µg protein per lane*




## GRASS POLLEN EXTRACT POTENCIES

- Major allergen and total extract potencies were assessed for 100,000 BAU/mL standardized Perennial rye, Meadow fescue and Timothy grass extracts and for 1:10 w/v non-standardized Italian rye extracts

| *Assay method* | *Specificity* | *Allergens* | *Antibodies* | *Validated/ FDA approved?* |
|---|---|---|---|---|
| ELISA inhibition | Total extract | 10-15 | Human IgE | Yes |
| Double-bind ELISA | Major Ag (Group 1, Lol p 1) | 1 | Rabbit IgG/ Rabbit IgG | No |

- Grass extracts at equivalent BAU/mL (total potency) vary considerably in major allergen content. Group 1 and Group 5 allergen levels are low for grasses (Mf) exhibiting high BAU potencies prior to standardization.

| *Extract* | *Group 1 (µg/ml)* | *Group 5 (µg/mL, published data)* |
|---|---|---|
| Timothy | 2000-3000 | 350-1350 |
| Meadow fescue | 400-520 | 75-200 |

- Major allergen (Group 1) detection increases at least 20-fold for grass extracts exposed to high temperature (3 min, 100°C). By comparison, the total potencies of these samples based on IgE binding are reduced or unchanged after heat treatment. Heating aggregates Group 1 (and other) grass proteins, increasing epitope valency and availability for multi-site binding required for double-bind ELISA assay reactivity.

| *Assay method* | *Perennial rye (% of 2-8°C control)* | *Italian rye (% of 2-8°C control)* |
|---|---|---|
| ELISA inhibition | 89-115 | 41-72 |
| Double-bind ELISA (Group 1) | > 20,000 | > 2,000 |

## CANDIDATES FOR STANDARDIZATION

- Several allergens targeted for standardization based on clinical importance and identification/characterization of prominent allergens present new and formidable challenges to current standardization process in U.S.

| *Extract* | *Allergens* | *Proposed units* | *Alternate units* |
|---|---|---|---|
| Dog | 3 | Total extract potency | Major Ag (Can f 1, albumin) |
| Cockroach | 15-20 | Total extract potency | Major Ag (Amer: Per a 1) (Ger: Bla g 1/ 2) |
| Alternaria | 3-5 | Total extract potency | Major Ag (Alt a 1) |

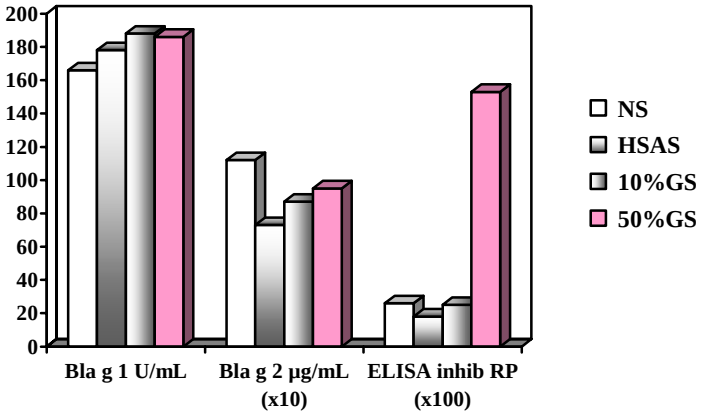
- Dog hair, dander and epithelial extracts contain very different levels of the two major allergens. Can f 1 is prevalent in dander; albumin is exclusive to epithelia.
- Cockroach and Alternaria extracts contain proteases which may compromise product stability/compatibility
- Different strains of *Alternaria alternata* exhibit distinct morphologies and biochemical compositions which may influence the effectiveness of testing and treatment
- The relationships between total extract potencies and major allergen levels are unknown for these products

## *COCKROACH EXTRACT POTENCIES*

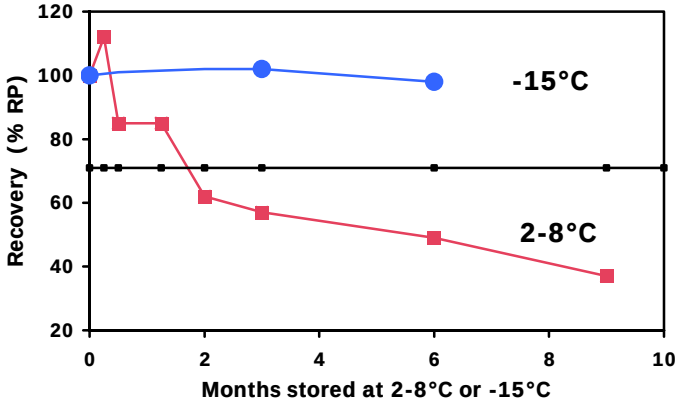
- **Major allergen and total extract potencies have been determined for aqueous, glycerinated and freeze-dried German cockroach extracts**

| *Assay method* | *Specificity* | *Allergens* | *Antibodies* | *FDA references available?* |
|---|---|---|---|---|
| ELISA inhibition | Total extract | 15-20 | Human IgE | Yes |
| Double-bind ELISA | Major Ag (Bla g 1, Bla g 2) | 1 | Mouse IgG/ Rabbit IgG | No |

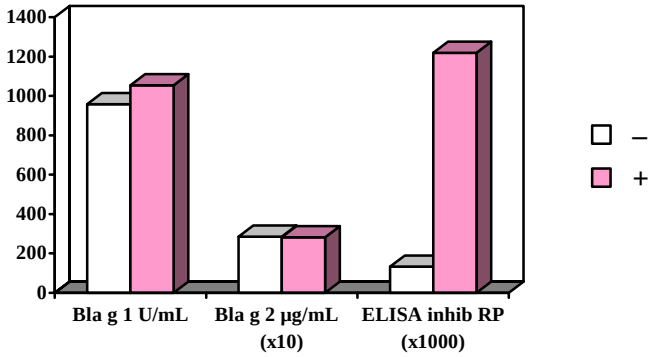
- **German cockroach extracts stored for 1 month at 2-8°C in normal saline (NS), HSA-saline (HSAS), 10 % glycero-saline (10 %GS) and 50 % glycero-saline (50 %GS) exhibited similar Bla g 1 and Bla g 2 levels but retained significant IgE binding in 50 %GS only. Glycerin produces concentration-dependent inhibition of endogenous cockroach proteases which degrade allergenic structures but not antigenic determinants (Bla g 1/Bla g 2 epitopes for mouse/rabbit antibodies) rapidly at 2-8°C.**




- **Cockroach extracts in 50 %GS stored for extended periods at 2-8°C were highly unstable based on IgE binding. After 2 months, extract potencies were below the lower equivalence limit (71% RP) of the ELISA assay. Storage at –15°C (freezer) resulted in near-complete recoveries of essential allergens. No changes in Bla g 1 or Bla g 2 activities were observed for products stored for 18 months at 2-8°C.**




- **Extraction of German cockroach in the presence (+) or absence (-) of protease inhibitors confirmed the protease resistance of Bla g 1 and Bla g 2 as well as the instability of cockroach allergens to extraction and storage conditions which promote enzyme-catalyzed protein degradation.**

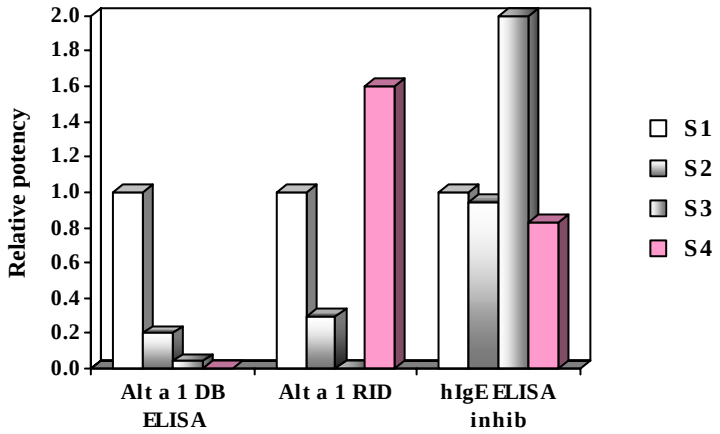



## *ALTERNARIA EXTRACT POTENCIES*

- **Major allergen and total extract potencies have been determined for aqueous, glycerinated and freeze-dried Alternaria extracts**

| *Assay method* | *Specificity* | *Allergens* | *Antibodies* | *FDA references available?* |
|---|---|---|---|---|
| ELISA inhibition | Total extract | 3-5 | Human IgE | No |
| Radial immunodiffusion | Major Ag (Alt a 1) | 1 | Rabbit IgG | No |
| Double-bind ELISA | Major Ag (Alt a 1) | 1 | Rab. IgG/ Rabbit IgG | No |

- **Alternaria extracts prepared from 4 different strains (S1-S4) exhibited total potencies or major allergen levels which varied noticeably from strain-to-strain and, more importantly, from assay-to-assay. As a result, the immunochemical reactivities of these products are dependent on the test methods employed for their analysis in addition to their biochemical compositions.**



## *MAJOR ALLERGEN SUMMARY*

| *Extract (analyte)* | *Prevalence* | *Assay characteristics* | *Clinical significance* |
|---|---|---|---|
| Cat (Fel d 1)<br>Short ragweed (Amb a 1) | > 80% | Standardized to single allergens<br>FDA reference Ag/sera<br>Validated for product lot testing<br>Consistent results from lab-lab | High |
| Dust mites (Der 1/2)<br>Grass pollens (Group 1/5)<br>Dog (Can f 1, alb)<br>Cockroach (Bla g 1/2)<br>Alternaria (Alt a 1) | 30-70% | Standardized to multiple allergens (mite, grass) or non-standardized (dog, cockroach, Alternaria)<br>DB ELISA references, reagents and results vary from lab-lab<br>[Major Ag] do not correlate closely with AU or BAU levels<br>Different Ag epitopes may be involved in binding intns with human IgE and mouse/rabbit IgG antibodies | Questionable |

## *IMMUNOTHERAPY ISSUES*

- **Maintenance immunotherapy doses defined in micro-grams (µg) of major allergen have been recommended recently in lectures and publications**
- **The inconsistent relationships between major allergen levels, specific allergen test methods and IgE-binding extract potencies for products containing multiple prominent allergens raises strong concerns regarding the clinical suitability, efficacy and safety of major allergen dosing**
- **In addition, IT doses recommended for these products (in µg major allergen and corresponding AU or BAU for Greer extracts) are based on limited studies (often single dose trials) and may vary significantly from maintenance doses currently used by board-certified allergists compiled and reported in a recent survey (JACI 106, 41-45, 2000)**
- **The wide dose ranges reported by allergists for each product (up to 2,000-fold vs. 4-fold recommended) may reflect differences in patient sensitivity as well as individual physician/practice preferences**

| *Extract* | *Recommended IT doses in µg of major allergen and corresponding units for Greer products* | *Reported doses and median levels* |
|---|---|---|
| Cat hair/pelt | 9-18 µg Fel d 1<br>1,500-3,000 BAU | 20-3,000 BAU<br>*Median: 400 BAU*<br>*~ ¼ of lower limit* |
| Short ragweed | 6-24 µg AgE | 3-45 µg AgE<br>*Median: 27 µg*<br>*Sl. above upper limit* |
| Dust mites | 4-12 µg Der 1<br>250-750 AU | 1-2,000 BAU<br>*Median: 250 BAU*<br>*= lower limit* |
| Grass pollens | 12-24 µg Group 1/5<br>1,200-2,400 BAU | 50-20,000 BAU<br>*Median: 2,500 BAU*<br>*Sl. above upper limit* |