STABILITY, COMPATIBILITY AND CROSS-REACTIVITY OF ALLERGENS: IMMUNOCHEMICAL REACTIVITIES AND PRACTICAL CONSIDERATIONS

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ABSTRACT

The effectiveness of testing and treatment procedures in the allergy clinic may be influenced significantly by the consistency and predictability of allergenic product compositions and potencies. To address this issue, the immunochemical characteristics of these products (thermal stabilities, compatibilities in treatment vaccines, cross-reactivities with related or unrelated allergens) have been examined using analytical methods specific for a diverse group of component proteins, including major allergens.

ELISA and immunoblot analyses of product stabilities in several diluents (HSA, 10% glycerin) at strengths as low as 1:10,000 w/v indicated that cockroach and short ragweed proteins were unstable to both high temperature (45°C, 113°F) and low temperature (-15°C, 5°F) incubations. Alternaria, ryegrass, fire ant and elm proteins exhibited low reactivities under some conditions. Dog epithelial proteins displayed no apparent changes in structure and potency. For most products, dilutions in 10% glycerin possessed similar heat stabilities and up to 100-fold increases in freeze-thaw stability compared to analogous dilutions in HSA.

Compatibility studies with vaccine formulations containing up to 3 of 6 common allergens (Alternaria, cat, cockroach, dog, dust mite and meadow fescue) confirmed the degradative effects of fungal and insect products on grass allergen reactivities but also demonstrated that many other combinations may coexist with no significant changes in component structures.

Allergenic cross-reactivities among grass pollens examined by competitive-binding immunoassays supported the close taxonomic relationships reported for these allergens. Within the Pooidae subfamily, grasses from different tribes (timothy/orchard, red top/sweet vernal) or from within the same tribe (timothy/red top, kentucky blue/meadow fescue) exhibited distinct IgE inhibitions with some sensitive subjects but not others.

The results of these studies may be useful to clinicians seeking to optimize the design, preparation and storage of their allergen dilutions and vaccines.

EXPERIMENTAL APPROACH

- Develop and validate sensitive immunoassays (ELISA, immunoblot) specific for a select group of extract protein/epitope structures
- Analyze immunochemical (IgE, IgG) integrity of extracts and major allergens during shipping, storage or intended use conditions
- Correlate direct and competition ELISA results (discontinuous epitopes, conformations) with corresponding immunoblot profiles (continuous epitopes, linear protein sequences)
- Determine sources of instability or incompatibility, and the predictability of cross-reactions based on taxonomic relationships
- Assess clinical utility of laboratory methods by comparisons with results from skin prick testing of sensitive subjects
- Identify materials and conditions producing optimal clinical and biochemical characteristics

MATERIALS AND METHODS

- Extract concentrates/diluents from Greer inventories Rabbit antiserums (IgG), human allergic sera (IgE)
- Double-bind (sandwich) and inhibition ELISA analyses Direct/ inhibition SDS-PAGE immunoblot procedures Skin prick/ puncture tests using GreerPick device
- Individual protein (major allergen) analytes Alternaria Alt a 1, Cat albumin, Dog albumin/Can f 1, Fire ant Sol i 3, Grass Group 1, Short ragweed AgE
- Multiple protein (extract) analytes Alternaria, Cockroach, Dust mite D. farinae, Elm, Short ragwee, Temperate and subtropical grasses
- Skin prick/ puncture testing Barbara Magera, M.D., Charleston, South Carolina Bonita Wilson, M.D., Morganton, North Carolina
STABILITY INVESTIGATIONS

- Test extracts/analytes
  Alternaria, Alternaria Alt a 1, Cockroach, Dog albumin, Dog Can f 1, Elm, Fire ant Sol i 3, Grass Group 1 antigens, Meadow fescue, Short ragweed, Short ragweed Antigen E

- Extract strengths
  1:10, 1:100, 1:1,000 and 1:10,000 w/v

- Diluents
  HSA-Saline or 10% Glycerosaline

- Methods of analysis
  Double-bind ELISA and SDS-PAGE immunoblotting

- Incubation conditions
  Control temp. 3 days at 4°C (39°F)
  High temp. 3 days at 23°C (70°F), 34°C (93°F) or 45°C (113°F)
  Low temp. 1-2 Freeze-thaw cycles (-15°C/4°C)

STABILITY COMPARISONS/CRITERIA

- Double-bind ELISA
  Test extracts incubated under native, non-denaturing conditions
  Sensitive primarily to conformational (discontinuous, 3D) epitopes
  Confirmed using native vs. heat-denatured allergen samples
  Stability defined as retention of >50% of 4°C reactivities

- SDS-PAGE Immunoblotting
  Test extracts denatured by heat and detergent treatments
  Disrupts conformational binding sites, retains linear sequence epitopes
  Stability defined as recovery of all major IgE+ or IgG+ bands (vs. 4°C)

- Results
  Freeze/thaw stability
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stable</th>
<th>Unstable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria Alt a 1</td>
<td>Dog albumin</td>
<td>Dog Can f 1</td>
</tr>
<tr>
<td>Short ragweed AgE</td>
<td>Cockroach, Italian ryegrass, Grass Group 1 Ags</td>
<td>Short ragweed</td>
</tr>
</tbody>
</table>

  Thermal stability
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stable</th>
<th>Unstable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria Alt a 1</td>
<td>Dog albumin</td>
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<td>Short ragweed AgE</td>
<td>Cockroach, Italian ryegrass, Grass Group 1 Ags</td>
<td>Short ragweed</td>
</tr>
</tbody>
</table>

  Dilutions stabilized by HSA:
  Alternaria, Alternaria Alt a 1, Elm, Fire ant Sol i 3

  Dilutions stabilized by 10% Glycerin:
  Alternaria, Alternaria Alt a 1, Elm, Fire ant Sol i 3, Cockroach, Italian rye, Grass Group 1 antigens, Short ragweed, Short ragweed Antigen E

  Similar thermal stabilities, improved freeze-thaw stabilities

Thermal Stabilities of 1:1,000 w/v Extracts

ELISA Results

Alternaria, Alt a 1, Stable

Short ragweed, AgE, Unstable
**Thermal Stabilities**

**Immunoblot results**

**Alternaria, Alt a 1, Stable**

<table>
<thead>
<tr>
<th>w/v</th>
<th>1:10</th>
<th>1:100</th>
<th>1:1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>4</td>
<td>23</td>
<td>34</td>
</tr>
</tbody>
</table>

**Short ragweed, AgE, Unstable**

<table>
<thead>
<tr>
<th>w/v</th>
<th>1:10</th>
<th>1:100</th>
<th>1:1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>4</td>
<td>23</td>
<td>34</td>
</tr>
</tbody>
</table>

**COMPATIBILITY STUDIES**

- **Test extracts/analytes**
  - Alternaria/Alt a 1 (A), Cat albumin (C), Cockroach (R), Dog albumin (D), Dust mite D. farinae (M), Meadow fescue (F)

- **Extract combinations and strengths**
  - 1-3 extracts per sample
  - 10 mL volumes in 10-20 SEVs
  - Each component present at 1:10 dilution of glycerinated concentrate strength

- **Diluent**
  - Normal saline

- **Methods of analysis**
  - Double-bind ELISA and SDS-PAGE immunoblotting
  - Skin prick testing (Magera clinic)

- **Storage and sampling conditions**
  - Storage time/temperature: 3 months at 4°C (39°F)
  - Test intervals: 0, 1, 2 and 3 months

**COMPATIBILITY SAMPLES/CRITERIA**

- **Test samples (mixtures) and controls (individual extracts (shadowed))**

<table>
<thead>
<tr>
<th>#</th>
<th>Extract</th>
<th>Alternaria (A)</th>
<th>Cat (C)</th>
<th>Dog (D)</th>
<th>M. fescue (F)</th>
<th>Mite (M)</th>
<th>Cockroach (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>A</td>
<td>ACM</td>
<td>AD</td>
<td>AF</td>
<td>AM</td>
<td></td>
<td>RF</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>AC</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>AD</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>AF</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>ACM</td>
<td>CM</td>
<td>FM</td>
<td>RFM</td>
<td>RM</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>AC</td>
<td>ACM</td>
<td>DM</td>
<td>RF</td>
<td>RM</td>
<td></td>
</tr>
</tbody>
</table>

- **Extract compatibility criteria**
  - **ELISA**: Retention of >50% of initial/control reactivity
  - **SPT**: Retention of >50% of control wheal diameters
  - **Blots**: Recovery of all major IgE/G+ bands (vs. control)

- **Focus**
  - Specific detection of individual extracts w/in mixtures
  - Instability due to endo- vs. exo-genous (mix) components
  - Influence of high [protease] of mold & insect extracts
  - Correlations between ELISA (3°), blot (1°), SPT results

- **ELISA/immunoblot results per test extract/analyte**
  - High compatibility (mixtures) or stability (controls) samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Comparability of ELISA and blot results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria (A)</td>
<td>Strong</td>
</tr>
<tr>
<td>Cat (C)</td>
<td>Moderate</td>
</tr>
<tr>
<td>Dog (D)</td>
<td>Strong</td>
</tr>
<tr>
<td>M. fescue (F)</td>
<td>Moderate</td>
</tr>
<tr>
<td>Cockroach (R)</td>
<td>Strong</td>
</tr>
</tbody>
</table>

- **Samples displaying moderate compatibility/stability**

<table>
<thead>
<tr>
<th>Alternate (A)</th>
<th>Cat (C)</th>
<th>Dog (D)</th>
<th>M. fescue (F)</th>
<th>Mite (M)</th>
<th>Cockroach (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACM</td>
<td>AD</td>
<td>ACM</td>
<td>AD</td>
<td>DM</td>
<td></td>
</tr>
<tr>
<td>AF</td>
<td>RF</td>
<td>RM</td>
<td>RPM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Mixtures exhibiting low (poor) compatibility**

<table>
<thead>
<tr>
<th>Alternate (A)</th>
<th>Cat (C)</th>
<th>Dog (D)</th>
<th>M. fescue (F)</th>
<th>Mite (M)</th>
<th>Cockroach (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>RF</td>
<td>RPM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **ELISA/immunoblot correlations**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Comparability of ELISA and blot results</th>
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<tbody>
<tr>
<td>Alternaria (A)</td>
<td>Strong</td>
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</tr>
<tr>
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<td>Strong</td>
</tr>
<tr>
<td>M. fescue (F)</td>
<td>Moderate</td>
</tr>
<tr>
<td>Cockroach (R)</td>
<td>Strong</td>
</tr>
</tbody>
</table>

- **Skin test results**

<table>
<thead>
<tr>
<th># of subjects tested with 19 test samples/controls</th>
<th>%</th>
<th># of subjects with dominant skin reactions to one control allergen</th>
<th>13</th>
<th># of subjects with dominant skin reactions to mite allergens</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td># of subjects with dominant skin reactions to fescue allergens</td>
<td>6</td>
<td># of mite reactions reduced to &lt; 50% of controls by cockroach</td>
<td>3/7</td>
<td># of mite reactions reduced to &lt; 50% of controls by Alternaria</td>
<td>7/7</td>
</tr>
<tr>
<td># of fescue reactions reduced to &lt; 50% of controls by cockroach</td>
<td>5/6</td>
<td># of fescue reactions reduced to &lt; 50% of controls by Alternaria</td>
<td>4/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ELISA Compatibility Results

**Alternaria**

![Graph showing % Recovery over time for Alternaria at 2-8°C (months)]

**Cockroach**

![Graph showing % Recovery over time for Cockroach at 2-8°C (months)]

**Meadow fescue**

![Graph showing % Recovery over time for Meadow fescue at 2-8°C (months)]

Immunoblot Compatibility Results

**Alternaria, Rabbit anti-Alt a 1 serum**

![Immunoblot image for Alternaria, Rabbit anti-Alt a 1 serum]

**Cockroach, Rabbit anti-German cockroach serum**

![Immunoblot image for Cockroach, Rabbit anti-German cockroach serum]

**Meadow fescue, Grass-positive human serum pool**

![Immunoblot image for Meadow fescue, Grass-positive human serum pool]
**CROSS-REACTIVITY RELATIONSHIPS**

- **Focus**
  Clinical/ biochemical cross-reactivities vs. predictions from taxonomy
  Consistency of cross-reactions across subjects, extracts and analyses
  Redundancies in common grasses
  Optimization of Rx/ Dx allergens

- **Materials and methods**
  Extracts Standardized and non-std grass pollens
  Glyc. concentrates (1:20 w/v, 10-100K BAU/mL)
  Subjects Grass-sensitive Greer employees
  Analyses ELISA and SDS-PAGE immunoblotting
  Skin prick testing (Wilson clinic)
  Analysis modes Direct-bind
  Reciprocal (cross-wise) inhibitions

- **Grass species**
  Temperate Kentucky blue (Kb), Meadow fescue (Mf), Orchard (Or), Perennial rye (Pr), Red top (Rt), Sweet vernal (Sv), Timothy (Ti)
  Subtropical Bermuda (Be), Bahia* (Ba), Johnson* (Jo)
  * Non-standardized

- **Taxonomic relationships: Grasses in Gramineae family**

- **Reactivity scales for ELISA, blot and skin test results**

- **Subject reactivity profiles**

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**Compositional similarities of IgE determinants in grass pollen extracts**

**Similar:** Parallel ELISA inhibition reactivities for inhibitors vs. solid phase allergens

**Distinct:** Non-parallel ELISA inhibition dose-response curves (paired t-test)

<table>
<thead>
<tr>
<th>Solid phase</th>
<th>Highest potency per mg protein (% of total)</th>
<th># of subjects with cross-reactive vs. distinct grass reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kb</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Mf</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Or</td>
<td>4</td>
<td>4 4 5 2 4 4</td>
</tr>
<tr>
<td>Pr</td>
<td>16</td>
<td>3 3 2 2 1 1 1 0 1</td>
</tr>
<tr>
<td>Rt</td>
<td>3</td>
<td>4 4 5 6 4 7</td>
</tr>
<tr>
<td>Sv</td>
<td>3</td>
<td>0 1 0 0 0 0</td>
</tr>
<tr>
<td>Ti</td>
<td>26</td>
<td>1 1 1 2 2 2 0</td>
</tr>
<tr>
<td>Be</td>
<td>0</td>
<td>2 2 3 0 3 1 1 1 0</td>
</tr>
</tbody>
</table>

**ELISA Inhibition Patterns**

**Parallel vs. non-parallel responses**

Timothy vs. Red top, Subjects G060, G067

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**Red top vs. Timothy, Subjects G008, G060**

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**Taxonomic relationships: Grasses in Gramineae family**

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Tribe</th>
<th>Genus</th>
<th>Common name</th>
<th>Predicted similarity to Timothy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooidae</td>
<td>Phleum</td>
<td>Timothy</td>
<td>Red top</td>
<td>High</td>
</tr>
<tr>
<td>Poaceae</td>
<td>Festuca</td>
<td>Kentucky</td>
<td>Blue</td>
<td>Moderate</td>
</tr>
<tr>
<td>Chloridoideae</td>
<td>Anthoxanthum</td>
<td>Sweet</td>
<td>Vernal</td>
<td>Low</td>
</tr>
<tr>
<td>Pooideae</td>
<td>Cynodon</td>
<td>Bermuda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panicoideae</td>
<td>Paspalum</td>
<td>Bahia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andropogoneae</td>
<td>Sorghum</td>
<td>Johnson</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Reactivity scales for ELISA, blot and skin test results**

Analysis | Unit of measure | Grade 0 | Grade 1 | Grade 2 | Grade 3 |
----------|----------------|---------|---------|---------|---------|
ELISA     | Delta Abs 305'  | > 0.500 | 0.250-0.499| 0.050-0.249| < 0.050 |
Blot      | Band intensities | High    | Moderate | Low     | None    |
Skin test | Sum of erythema  | > 100 mm| 50-95 mm| 1-49 mm | 0 mm    |

**Subject reactivity profiles**

**High**
G008
**Moderate**
G020
G033
**Low**
G009
G092

[Diagram of inhibition patterns and reactivity profiles]
CONCLUSIONS

- Cockroach (high protease content) and short ragweed (low protease) extracts are unstable to low or high temperature exposures

Extracts diluted with HSA or 10% glycerin often possess equivalent or higher stabilities compared to 1:10 w/v concentrates

10% glycerin dilutions provide similar thermal stabilities and improved freeze-thaw stabilities compared to analogous HSA dilutions

- Grass allergens are degraded after mixing with Alternaria or cockroach extracts (active proteases) but not with dust mite (inactive proteases)

Alternaria and cockroach antigens are protected by proteins from other extracts, consistent with the non-specific nature of mold/insect proteases

- Grass pollen sensitivities and allergenic cross-reactivities correlate closely between in vivo and in vitro test methods but are not always predicted by botanical or taxonomic relationships among grass species

PRACTICAL CONSIDERATIONS

- Repreare unstable extract dilutions (1:100-1:10,000 w/v) every 2-3 months (buffered saline or normal saline diluent) or every 6 months (10% glycerin or HSA diluent)

Use glycerinated extracts whenever possible (glycerin protects unstable allergens from denaturation and inhibits protease activity)

- Keep molds and insects separate from pollens in Rx mixtures

OK to combine dust mites with pollens, cat/dog with molds

- Timothy allergens cover most (if not all) Pooidae grasses effectively and may also represent Bahia and Johnson allergens

Bermuda allergens are distinct from those of other grasses

IgE specificities (proteins) similar, epitopes may vary from patient-to-patient