New proteins in the food chain: Is there evidence of new sensitization and allergies?

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ILSI HESI Sensitizing Properties of Proteins
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Today - briefly

• Risk of food allergy – foods & proteins
• “Research” Methods of detecting IgE binding
• Examples: de novo sensitization (Ag-specific IgE) and food allergy (actual de novo….impossible to know)
  – Well known - Brazil nut 2S albumin in soybean (GM)
  – Food allergens in peanut (natural)
  – Introduction of kiwi species (natural)
• Investigation of potential food allergy to GM proteins
  – CP4 EPSPS (from bacteria) herbicide tolerant soybean
  – Alpha amylase inhibitor (from common bean) into legumes
Common Health Risks from “Food” …

1. Lack of adequate nutrition: (whole population at risk, > 20% in India…opinion)
2. Over / unbalanced nutrition: (whole population > 20% in US / EU…India, China?…opinion)
3. Food borne pathogens: (whole population at risk)
4. Celiac disease from a few proteins in wheat / barley / rye (< 1.5% of individuals controlled by genetics and environment)
5. Food allergy (<6% of individuals: genetics and environment)
6. Toxic small molecules – a variety of uncommonly consumed plants (whole population)
7. Few toxic proteins from a few known plants (whole population)
8. Anti-nutrients [trypsin inhibitors, amylase inhibitors] (whole population)
9. Chemical pesticides (whole population)
10. Mycotoxins (whole population)
Sensitization and food allergy (and celiac disease) can begin with first exposures at any age or after multiple “safe” exposures.

Common
< 3 years

Moderately common
3 years to 20’s

Rare
But possible
After 50
Those with the “disease” must avoid foods that others commonly eat – small percent are severe

- **Celiac disease**: Once symptomatic…AVOID gluten
  - T cell mediated: wheat and highly related grains
  - ~ 1 to 1.5% of population
  - MHC Class II, DQ 2.5 or DQ 8 (~30% of population)

- **IgE mediated allergy**: Once symptomatic…AVOID the allergen
  - ~3-6% of population
  - Diverse foods and proteins,
  - Few cause severe reactions
Assessing Potential Allergenicity of GMOs....addresses the following in order of risk:

1. Is the source of the new gene clearly allergenic? Do serum testing: Is the protein an allergen?
2. Is the new protein sequence nearly identical to a known allergen so cross-reactivity might occur? Do serum testing: Is the protein cross-reactive?
3. Is the protein a glutenin or gliadin from wheat, barley or rye? Then evaluate potential celiac.
4. Is there an increased risk the new protein will sensitize de novo (stability in pepsin, abundance)???
5. Did insertion of the new gene significantly increase the endogenous allergens ?????
Known (IgE) Allergens in Food Crops

Very few proteins represent major risks

• Peanuts
  – Possibly > 50 deaths per year in the U.S.
  – 4 major allergens, 5 to 7 minor allergens
  – With hundreds of proteins in the nut and 10,000-40,000 total genes

• Soybeans
  – Probably < 1 fatal reaction per year in the U.S.
  – 3 to 5 moderate allergens
  – ~20,000 total genes

• Maize (corn)
  – No published reports of fatal reactions (global)
  – 1 major allergen (LTP), 2 to 5 minor allergens
  – 20,000-40,000 total genes

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Diagnosis of Food Allergies - Complex

1. Careful History of consumption & reactions
2. Food Diary
3. Elimination diet

In Vivo Challenges

- Skin prick tests with standardized extract or prick to prick
- Food challenges are Time consuming
  - Some risk to patient
  - Difficult / expensive

In Vitro (IgE) tests – one tool

- Total IgE is not very informative
- Specific IgE is often helpful to verify the allergenic food and allergen (protein)
  - CAPS (Pharmacia Diagnostics)

RESEARCH METHODS

- ELISA/RAST
- Western blots

In vitro tests are useful in confirming diagnosis, but are not proof of allergy
Serum IgE tests: should be reliable, sensitive and specific...but

The ideal serological IgE immunoassay

True Clinically Allergic Subjects

True Non-Allergic Subjects

# of People

Specific IgE Quantity IU/mL

Cut-off

- +
Potential IgE Antibody Binding Epitopes

Conformational or discontinuous IgE epitope
Often heat labile

Sequential or Linear IgE
Usually heat stable

A Few Specific Asparagine-linked Glycans questionable relevance

NH₂

CHO

ATYNPGFL

CO₂H

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Serum IgE Tests – if necessary

- Must be specific
- Require validation
- Should include positive and negative control allergic sera
- Should include positive and negative control allergenic proteins & extracts
- Differ in detecting binding:
  - Native Ptn
  - Denatured / reduced Ptn
  - Extracts
  - Pure Proteins
Some anti-IgE may give “false” positive signals (most people have IgG to some dietary proteins, most also have some IgE)

SN Pramod, RE Goodman

Bound antibodies (diluted 1:1,000) were detected with ECL- SuperSignal

Goat anti-human IgE (ε chain) – HRP antibody from Sigma # A9667
only 100:1 preference

Monoclonal anti-human IgE (ε chain) – HRP antibody from Southern-biotech #9160-05
~ 10,000:1 preference

Monoclonal anti-human IgG (γ chain) – HRP antibody from Southern-biotech #9042-05
> 100,000 : 1
Specific IgG can be high even in allergic subjects

Immunoblot. Soybean (719) and peanut (730) allergic plasma binding to extracts of peanut (PN), soybean (SB) or markers (M), detected with Sigma anti-IgE, Southern Biotech anti-IgE or Southern Biotech anti-IgG. Exposure times of 1 min. for both anti-IgE and anti-IgG
Blocking solutions SHOULD Reduce Non-Specific Binding

**Human Peanut Allergic Serum**, diluted 1:10 in blocker, incubated With blotted proteins, then anti-IgE using ECL detection

15-second film exposure

Samples

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<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Loading</th>
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<tbody>
<tr>
<td>1</td>
<td>MWM</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Der f2</td>
<td>0.1 µg</td>
</tr>
<tr>
<td>3</td>
<td>Corn</td>
<td>5 µg</td>
</tr>
<tr>
<td>4</td>
<td>Soybean</td>
<td>5 µg</td>
</tr>
<tr>
<td>5</td>
<td>Peanut</td>
<td>1.25 µg</td>
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</table>

Blocking agents

<p>| | | | |</p>
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<tbody>
<tr>
<td>2% BSA</td>
<td>2% NFDM</td>
<td>5% NFDM</td>
<td>0.2% Tween 20</td>
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</table>

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Unpublished study (no-blocker, only 0.2% Tween 20)
IgE binding to a recombinant protein from *E. coli*; serum donors all children with atopic dermatitis: ELISA and Immunoblot
Is this specific or non-specific binding?

P = rabbit IgG; 2 extremely high CAPS to milk-egg-modest soy; 9 high CAPS to milk only; 15 modest CAPS to everything tested
1996 GM Soybean Evaluation – 2S Albumin
Brazil nut, Appropriate Subjects & Tests…results,
STOPPED Development (NE J Med Nordlee et al. 334:688)

**Immunoblot**
Brazil nut allergic sera
- IgE detection

**SPT**
Brazil nut allergic patient

- RAST Inhibition
  Brazil nut protein solid
  - GM soy inhibits
  - Brazil nut inhibits
  - Non-GM soy does not

Figure 1. Results of Radioallergosorbent Assay with Extracts

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IgE specificity: Inhibition IgE western blots for Ara h 2, a major peanut allergen:

Serum from one peanut allergic subject Ara h 2 is a major allergen

Lanes:
1) 10 ug soy extract
2) 2.5 ug peanut extract
3) 0.04 ug Ara h 2
4) 0.4 ug Ara h 2
5) Marker Proteins.

Other published data demonstrates Ara h 2 is a potent food allergen

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Green, Gold, Hardy Kiwifruit IgE binding for risk assessment new species w/green kiwi allergic patient sera….conclusion, cross-reactive, label: Hardy Kiwi juice as “kiwi”

**Immunoblots**

**A. Reduced & Heated**

**B. NOT Reduced NO Heat**

**Direct ELISA**
Monsanto Post-market Study 2002-2005 of GM soybean…because many investigators were performing poorly controlled studies

Serum testing for binding of IgE to CP4 EPSPS and Soybean event 40-3-2 using clinically verified soybean allergic subjects

- ~ $500,000 research dollars to 3 centers
- Additional Monsanto costs ($$$$)
- > 2+ years to complete from samples to results
- Technically challenging
Hoff et al., 2007: Serum testing of GM soy… Mol Nutr Food Res 51:946-955

• **Materials:**
  - pure CP4 EPSPS (from soy and from E. coli)
  - two varieties each of GM and non-GM soy
  - other control pure proteins.

• **Sera:**
  - Switzerland, 10 soy food challenge positive or anaphylactic to soy, 22 other allergic and 5 non-atopic adults
  - Korea, 10 atopic dermatitis (AD) young children, 3 egg positive AD, 13 “non-allergic”

• **Methods,** ELISA, ELISA inhibition, Immunoblot and immunoblot inhibition

• **FINDINGS:** No specific IgE binding to CP4 by 20 clinically diagnosed soybean allergic subjects
Published CP4 EPSPS Immunoblots Hoff et al., 2007

1. Marker
2. Rubisco @ 400 ng
3. E. coli CP4 @ 400 ng
4. E. coli CP4 @ 40 ng
5. Soy CP4 @ 40 ng
6. GM soy a @ 10 ug
7. Non-GM soy b @ 10 ug
8. Non-GM soy c @ 10 ug
9. GM soy d @ 10 ug
10. Marker
11. Rice @ 10 ug
12. E. coli soy P34 @ 400 ng
13. Peanut Ara h 2 @ 400 ng
14. HDM Der f 2 @ 400 ng
15. Egg OVA @ 400 ng

Ponceau S stain

Hyperfilm (25 minutes exposure)

Goat anti-CP4

EU soy patient 1

EU soy patient 8

Repeated, NO BINDING to CP4

2 CP4
3 Rubisco
4 GM soy
5 non-GM
6 Ara h 2
7 OVA
CONCLUSION: No specific IgE binding to CP4 EPSPS by ELISA or Immunoblot
Alpha-amylase inhibitor from common beans—
**Transferred into peas, cowpeas and chickpeas by TJ Higgins,** to inhibit storage beetle pests

Sequence searches using

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aa length</th>
<th>Identity FASTA overall</th>
<th>Highest identity in 80 aa search</th>
<th>Number of matches of &gt;80%</th>
<th>Expectation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-amylase common bean</td>
<td>246</td>
<td>peanut agglutinin 35%</td>
<td>peanut agglutinin 45%</td>
<td>1</td>
<td>Small likelihood of cross-reactions but Goodman lab is testing</td>
</tr>
</tbody>
</table>
Peanut Agglutinin has RARELY been reported as binding IgE — in fact may NOT cause allergy

- Only one published report of IgE binding to PNA from clinically proven peanut allergic subjects
- We tested serum from 34 peanut allergic subjects, found 1 with clear IgE binding to peanut agglutinin (PNA), 5 weak binders
Reactivity to reduced PNA only (6 of 34 peanut allergic plasma)

IgE binding was found to aAl, was it due to cross reactivity to PNA?

All of these peanut allergic subjects bound IgE to aAl and also to Phytohemagglutinin, PHA), suggesting it is due to CCD. Control serum RG75 also binds PNA and aAl…and PHA).

Next we tested for CCD binding using inhibition.

Reduced Blot:
1, peanut (10 µg); 2, peanut agglutinin (5 µg); 3, peanut agglutinin (0.5 µg); 4, Tendergreen aAI (0.5 µg); 5, Transgenic pea (10 µg ); 6, Native pea (10 µg); M, mw marker, BIORAD#161-0374
Potential “False Positives” due to Modifications to Asparagine - Linked Glycans – Bind IgE of some allergic subjects

Plant glycoproteins

Can bind IgE

Insect and Animal glycoproteins – no binding

Can bind IgE
The antigens immobilized on the PVDF membranes are:

1) 0.5 μg Tendergreen αAI
2) 0.5 μg GM green pea αAI
3) 0.5 μg GM chickpea αAI
4) 0.5 μg cowpea αAI
5) 10 μg crude corn
6) 0.5 μg PNA
7) 0.5 Ara h 2

Inhibition data demonstrates IgE binding to CCD
Serum RGLEG 120: IgE Western Blot αAI CCD?

αAI = alpha-amylase inhibitor
T = transgenic
NT = non-transgenic
Var = variety

A) Peanut
B) Red kidney bean = CCD plus protein IgE
C) GM Chickpea
D) Chickpea
E) Cowpea
F) GM Cowpea
G) Pea
H) GM Pea
I) Corn meal
J) Wheat
M) Marker
K) Tendergreen αAI
L) Pea αAI [T]
N) Chickpea αAI [T]
P) Cowpea αAI [T]
R) Pinto bean natural αAI
S) Red kidney bean natural αAI

Allergy: Navy bean [Throat swelling, dry mouth hoarse voice], milk [GI distress], peanut, soy, green pea
Inhibition IgE (RGLEG120) non-Tg pea inhibits IgE binding to αAl as much as Tg pea does. ....= CCD binding

Inhibition with extracts

Antigens used were: 1) 2 µg of purified Tendergreen αAl; 2) 2 µg purified transgenic pea αAl; 3) 10 µg transgenic pea extract; 4) 10 µg, non-transgenic pea extract; M) molecular weight marker, Bio-Rad #161-0374.
Test Bioactivity of IgE (mast cell activation) to aAI and GM peas

• TESTING ALLERGIC SERA – from appropriately allergic serum donors
  – Human basophil histamine release
  – Humanized rat basophil leukemia cells β-hexosaminidase release
RGLEG120 IgE clearly binds to αAI, BUT...there is very little or no activation or β-hexosaminidase release to αAI, to peanut or navy bean until osmotic shock is reached.
B-hexosaminidase release assay from humanized RBLs passively sensitized with highly peanut allergic sera

![Graph showing β-hexosaminidase release assay](image)

- **PN** = peanut...more than 100 fold stronger
- **NB** = Navy bean
- **NTP** = non-transgenic pea
- **TP** = transgenic pea (aAI)
- **AlgE** = anti-IgE control

Legend:
- 1. Peanut
- 2. Soybean
- 3. Corn
- 4. Lima bean
- 5. Cow pea
- M. Marker
The Positive IgE Binding to aAI was NOT biologically relevant in tested sera

• Testing by basophil activation
• So far there is no indication that the IgE binding to aAI CCD is effective (have tested 5 CCD / aAI binding sera or plasma
• Food challenges would be very difficult….no available patients, also ethical questions
CONCLUSIONS

• All consumers are initially naïve & non-allergic
• FEW proteins in a small number of foods cause the rare severe food allergic reactions
• Most (all?) foods may cause allergic reactions in at least one of seven billion people, but again only a few proteins are allergens
• Introducing a new food in the diet is more risky then introducing a new GM protein!
• So far NO approved GM product has been proven to have become an allergen
• One (Ber e 1 in soybean) was stopped in development
• But no food is risk free!

Goodman FARRP – UNL, 2012
Working Through Regulatory Hurdles and Food Safety Issues…No Food is 100% safe…

It is time to face the tough opposition … “the wall of vocal opposition and …minor scientific uncertainty” standing against GM approvals and allow science to prevail!

Scientists should work hard for the best data, then wrestle regulators if necessary for solid decisions…based on real risks

And the testing foundation that allows approval of safe new GM products
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- BASF
- Bayer
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- AllergenOnline.org
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- Bayer
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