Evaluating the potential allergenicity of GMOs intended for food use

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GMSA Foods
Post-Market-Monitoring
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Today

- Allergy….what is it?
- What are risks?
- Methods for the allergenicity assessment
- Examples assessment
  - αAI Legumes (vs Cry 1)
  - Endogenous allergenicity assessment soy
- Briefly: Is PMM possibly relevant?

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Food/Feed Safety Assessment

- ✓ Historically we have learned through experience what foods are "safe" to eat, must process or must avoid
- ✓ Wheat causes celiac disease in some people
- ✓ Legumes (beans/peas) must be cooked (lectins, trypsin inhibitors)
- ✓ Most foods are allergenic for a few people
- ✓ Assessment methods must be scientifically sound and the standard for acceptance must be relative:

GM. . . "as safe as" . . . conventional

Integrated Approach for GM Crop Safety PRE-MARKET

- Gene(s)
  - Source(s)
  - Predicted protein
  - Insert / copy number / gene integrity
- Protein(s)
  - History of consumption
  - Function / specificity / mode-of-action
  - Level of expression
  - **Toxicology**
  - Allergenicity

- Crop Characteristics
  - Morphology
  - Yield
- Environmental safety NTO
- Food / Feed Composition
  - Proximate analysis
  - Key nutrients
  - Key anti-nutrients
  - Feeding studies
    - » Nutrition / Performance

Food Allergens….

- "Allergens" are proteins that are not hazardous for most people
- Only hazardous for those
  - with specific allergy (IgE mediated)
  - enteropathy (e.g. celiac disease, due to gluten proteins from wheat/barley or rye)
- Proteins introduced into GMOs are assessed for potential risks of allergy based on scientific knowledge and testing on a case-by-case basis

Natural history of food allergy

- Reproducible reactions: same person, same food, same, similar or related symptoms
- May progress over time from dermatitis or hives to vomiting & wheeze to Asthma and Anaphylaxis
- While….~ 85% of individuals with allergies to cows milk, egg, wheat, soy become tolerant by 3 to 5 years of age
- Allergy to peanut, nuts, seafood is typically permanent
- Celiac disease (CD) is caused by a few specific proteins in wheat, barley or rye grain and is life-long after the onset

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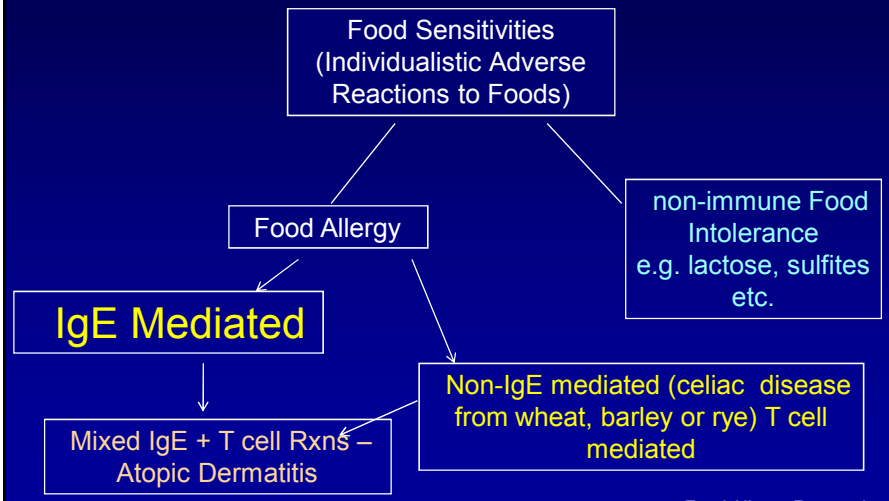
Food Allergy is an adverse immune reaction to normally safe dietary proteins



Food Allergy Research and Resource Program 2008

What is IgE mediated food allergy?

Food allergy causes more than just a runny nose or urticaria !

Sometimes mixed IgE, T- cell and eosinophil reactions

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Celiac Disease (wheat, barley, rye and maybe oats)

Celiac Disease (Gluten-sensitive enteropathy) effects nearly 1% of people in most countries

- In children:
  - Weight loss, malnutrition, diarrhea, abdominal pain
- In adults, average 10 years of nonspecific symptoms:
  - Diarrhea, abdominal pain
  - GERD
  - Malnutrition, osteoporosis, neuropathology, infertility, T-lymphoma

Pathogenesis: an immune-mediated enteropathy triggered by gluten peptides in genetically predisposed patients (HLA DQ2 or DQ8)

- T cell mediated pathology
- Lymphocytic infiltration of small bowel
- Villus atrophy

Sensitization and food allergy (and celiac disease) can begin at any age or after multiple "safe" exposures

Common	Moderately common	Rare
< 3 years	3 years to 20's	But possible After 50

- Or when we eat new foods…an American in Greece, India or China
- An Indian in the US

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Food Allergy Prevalence

(apparently increasing, estimates from US population of 300 million)

- ~ 30% of people have allergies to inhaled allergens
- IgE mediated allergies (Type I) is the most common - allergy
- Occurrence of food allergy in the US and Europe
  - 2-4% of adults
  - 4-8% of young children
  - Severe reactions are relatively rare (U.S. *estimates:* 120,000 Emergency Room visits, < 200 fatal reactions / year)
- Eight foods account for ~ 90% of food allergies & require labels:

Peanuts, Milk, Eggs, Fish, Crustaceans, some tree nuts, (Wheat), (Soybeans)

The EU adds celery root; mustard and sesame seeds; lupine, molluscan shellfish

India, may consider adding: black gram, pigeon pea, mung bean, lentil, Bengal gram

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Common Allergenic Foods: Few people are allergic to any one food

Consideration of Risk

Sampson JACI (2004) 113:805

Table 1. Prevalence of food allergies in the US		
Food	young children	adults
Milk	2.5%	0.3%
Egg	1.3%	0.2%
Peanut	0.8%	0.6%
Tree nuts	0.2%	0.5%
Fish	0.1%	0.4%
Shellfish	0.1%	2.0%
Total all foods	6%	3.7%

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Known Allergenic Proteins in Food Crops

Very few proteins represent major risks

- Peanuts
  - Probably > 50 deaths per year in the U.S.
  - 3 to 5 major allergens, 5 to 7 minor allergens
  - 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), 4 to 5 minor allergens
  - 20,000-40,000 total genes

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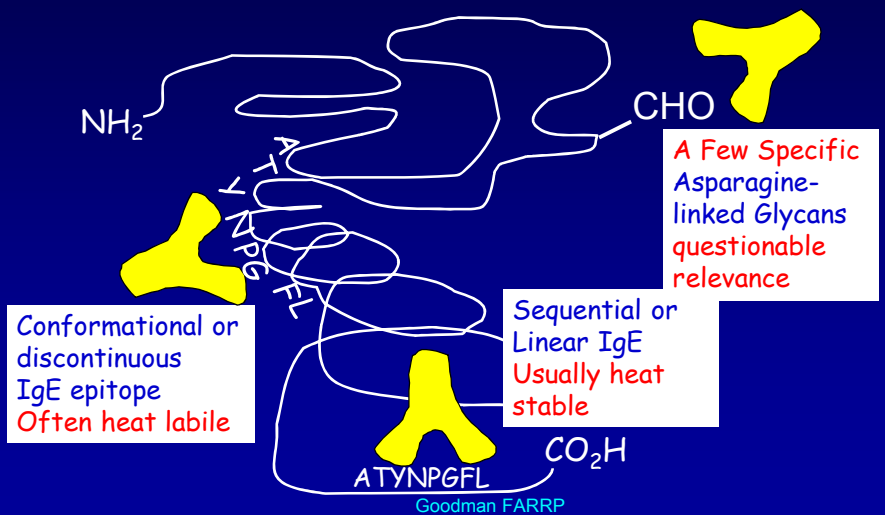
Sensitization…development of antigen-epitope-specific IgE (e.g. peanut allergen Ara h 1) - requires multiple exposures



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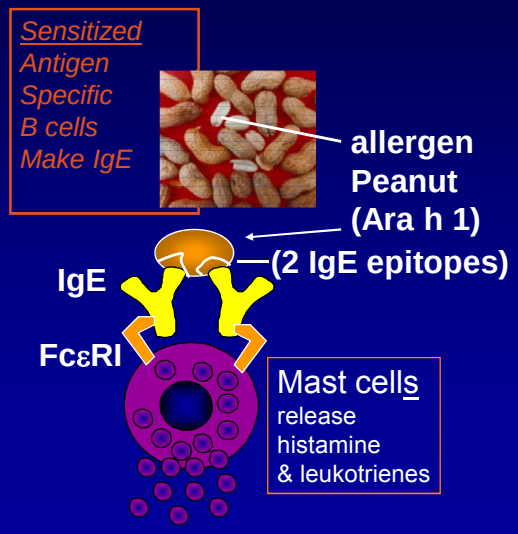
Protein-specific IgE is the key mediator in Food Allergy

IgE Mediated Symptoms

10 to 20 minutes after eating:

- hives
- angioedema
- asthma
- diarrhea/vomiting
- atopic dermatitis
- anaphylaxis



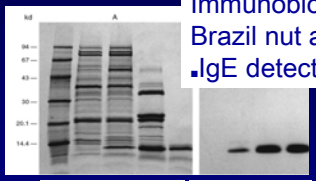
Protecting the Allergic & Celiac Consumers

- They MUST avoid the protein(s) that cause their disease
  - avoiding whole specific foods
  - food ingredients that contain the protein
- Potential problems
  - Prepared food (restaurants, friends)
  - Packaged foods, drinks and snacks
  - New sources that are evaluated to reduce risks
    - Genetically Modified Crops
    - Novel food ingredients

1994 GM Soybean – with Brazil nut 2S Albumin – was NEVER ON MARKET because….

Brazil nut: Appropriate Subjects & Tests (NE J Med 1996 Nordlee et al. 334:688)

Immunoblot
Brazil nut allergic sera
- IgE detection



Mk Soy GM BN Ber e 1 | Soy GM BN Ber e 1

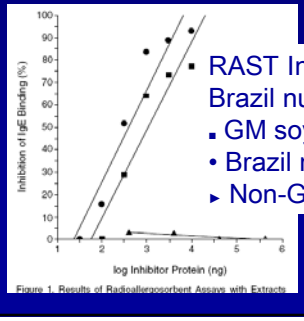


Figure 1. Results of Radioallergosorbent Assays with Extracts

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

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SPT Biological Proof
Brazil nut allergic patient

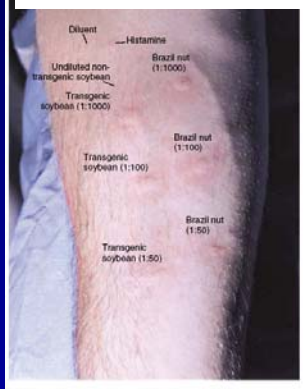

Figure 3. Reactivity on Skin-Prick Testing to Extracts of Transgenic Soybean, Nontransgenic Soybean, and Brazil Nut in a Subject Allergic to Brazil Nuts.
The dilutions are given in parentheses.

Assessing the Potential Allergenicity

from CODEX: (Risk ranking by Goodman)

1. Does the gene encode a protein that is known to be an allergen (or induce celiac disease)? Based on allergenic history of the source & bioinformatics, serum IgE tests (or PBMC challenge for Celiac Disease)
2. Is the sequence of the protein sufficiently similar to an allergen (or celiac causing gluten) to expect allergic cross-reactions (or celiac induction)? Then serum IgE tests (or PBMC challenge for CD) would normally be required
3. Is the protein likely to sensitize and become an allergen? (e.g. stable in pepsin, abundance in GM – food, and stable to heating)
4. Did insertion of the gene increase endogenous allergenicity?…Should only be considered for commonly allergenic crops (not even soybean), and probably only if transcription factors are inserted….

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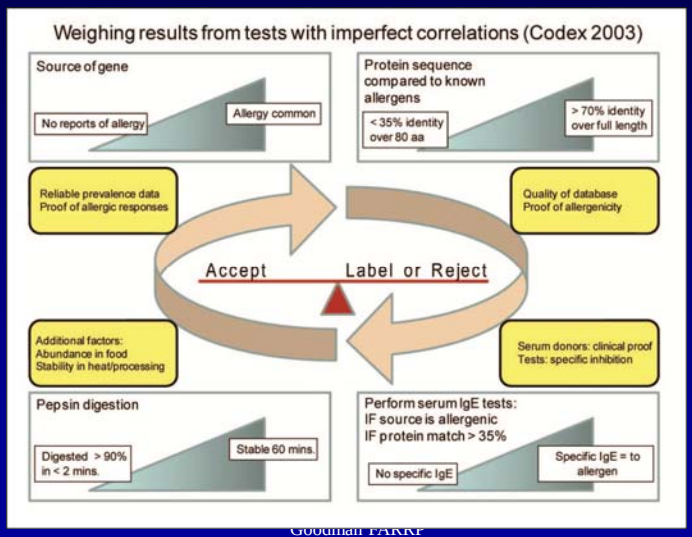
Interpretation of Codex: Goodman et al., Nature Biotech Jan. 2008
Assessing the Potential Allergenicity of GM Crops – What Makes Sense?
Answers are often in shades of grey…require interpretation



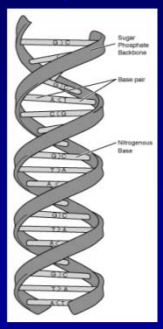
SOURCE of GENE

- If the gene is from a major allergen
  - Food: peanut, tree nut, fish, shrimp, maybe soybeans or wheat
  - Airway: birch, ragweed, house dust mite
  - Contact: latex

THEN DO SPECIFIC SERUM TESTS for IgE binding – using donors allergic to source

PROTEIN BIOSYNTHESIS
Gene Sequence > Protein Structure > Function

Existing gene or
Introduced DNA (*cry 1 A gene*)



mRNA → protein ⋯⋯→ Endogenous protein or Introduced protein such as Cry 1A

translation

transcription

Amino acids

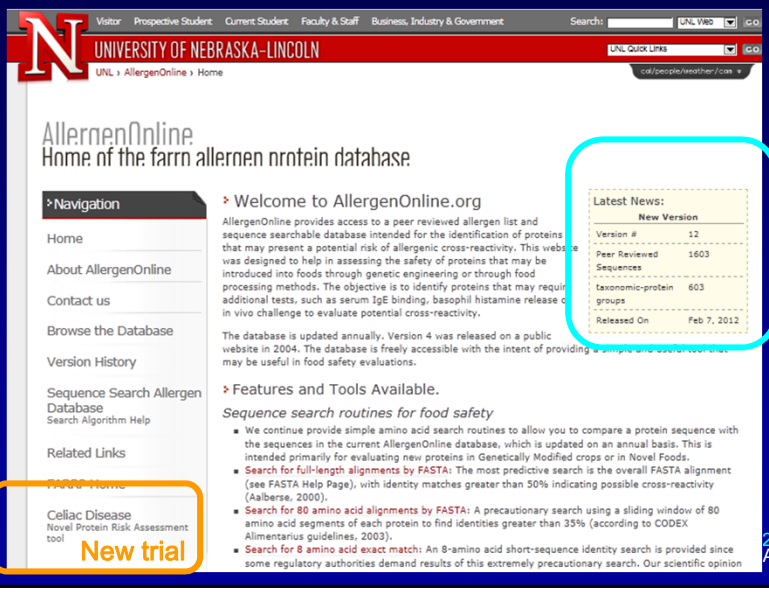
Bioinformatics – amino acid sequence comparison for allergenicity

- Questions to answer:
  - Is the protein already known to be allergenic?
  - Is the protein likely to cause cross-reactions (high sequence identity match)?
- Critical Factors
  - Databases (http://www.AllergenOnline.org at UNL)
  - Sequence comparison methods
  - Criteria for "significance"
  - Results often need expert interpretation – allergens and matches are NOT equal

Decision (Are human serum test or challenges necessary?) **–Yes or No**

Allergenonline Homepage version 12 (Feb 2012)

http://www.allergenonline.org



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Major Allergenic Sources in AllergenOnline: a tool for assessing the safety of novel ingredients and GMOs

- Foods:
  - Peanut, tree nut, cow milk, chicken egg, crustaceans (shrimp, lobster), maybe wheat (?), soybean (?)
  - Sesame, celery root, mustard, kiwi
  - INDIA? Possibly blackgram, chickpea or other legumes
- Airway
  - Pollen: weeds (parietaria, ragweed), grasses (timothy, ryegrass), trees (birch)
  - Molds (Alternaria, Aspergillus, Davidiella sp.)
  - Insect / mite inhalants (dust mites, cockroach)
  - Latex (contact)
- Venoms and salivary proteins
  - Bee, wasp and ant sting venoms
  - Mosquito and tick salivary proteins

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Known Allergenic Proteins in Food Crops

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Amino Acid Sequence Comparison to Allergens: see http:// www.allergenonline.org

1. Full-length FASTA vs. AllergenOnline (>50% identity or E score < 1 e -7 = Most predictive of overall structure and likelihood of allergic cross-reactivity)
2. FASTA scanning 80 aa window (79 aa overlap), (>35% identity = some possibility of cross-reactivity)
3. If matches in steps 1 or 2, do serum IgE tests if possible (How common is allergy to matched protein? Must be able to find appropriately allergic donors, which is also relevant to risk assessment)

EXAMPLES OF CROSS_REACTIVE MATCHES
Peanut Ara h 1 Search AllergenOnline deciding which proteins to test!

Table 1a) Sequence matches to peanut Ara h 1 GI:1168390
Yellow = Direct evidence IgE binding, probable clinical cross-reactivity
Magenta = Indirect evidence IgE binding, possible clinical cross-reactivity
Green = Indirect evidence of reduced IgE binding (probably > $1/100^{th}$), no clear evidence of clinical cross-reactivity
Blue = No evidence (known) of shared IgE binding, no evidence of clinical cross reactivity

Matched Allergen	Organism	Overall FASTA3			80mer (>35%)	8mer (Identical)
Name (GI#)	Genus species	*E* value	Overlap (aa)	Identity (%)	Best % ID	Matches (#)
Ara h 1 (1168390)	Arachis hypogaea	3.1e-197	614	100	100	607
Pis s 1 (42414627)	Pisum sativum	3.9e-46	424	51.4	65	4
Len c 1 (29539109)	Lens culinaris	1.1e-68	424	53.3	63.9	4
Glycinin CG4 (256427)	Glycine max	5.9e-27	457	51.2	63.7	2
Lupinus congl (149208401)	Lupinus angustifolius	7e-57	534	49.1	62.5	1
Jug r 1 (6580762)	Juglans regia	1e-20	625	35	55.0	0
Ana c 1 (21666498)	Anacardium occidentale	3.1e-15	599	28.9	47.5	0
Cor vicilin (19338630)	Corylus avellana	1.8e-20	495	34.1	46.2	0
Ses vicilin (13183177)	Sesamum indicum	2.6e-21	561	33.2	43.9	0

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Allergenicity Assessment of Insect Resistant GM Cowpeas

- Cry 1Ab (Bt) cowpea: lepidopteran pest control
  - Genes from bacteria, *Bacillus thuringiensis,* an organic pesticide
  - Cry 1 GM crops with various forms, have been engineered into: brinjal (eggplant), Brassica sp., cotton, maize and rice
  - Full food approvals current for similar products (corn or maize)
  - Unlikely to have regulatory issues…similar to MON810 maize
- Alpha amylase inhibitor (aAI) cowpea: coleopteran pest control (Bruchid seed storage beetle)
  - Gene from common beans, *Phaseolus vulgaris* – allergy rare
  - aAI has never been submitted for food approvals
  - Excellent history of safe use (HOSU) of common beans…*Phaseolus vulgaris*
  - Significant regulatory hurdles, but probably safe…HOSU

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Cry 1 Cowpea Allergenicity Assessment is Straight-forward

- History of approvals for many Cry 1 events
- Source (*Bacillus thuringiensis*) is not allergenic
- Sequence does not match any known allergen
- Therefore no need for serum testing
- Should rapidly digest in pepsin
- Low abundance
- Many other supportive tests in similar varieties

Scientifically Justified αAI Allergenicity Assessment

Alpha-amylase inhibitor (from common bean) in cowpea.

- Gene is NOT from a commonly allergenic source
- Protein has multiple Asparagine-linked glycans, you cannot use *E. coli* generated test protein, some question of glycan effects
- Protein sequence comparison to known allergens….>35% identity match to peanut agglutinin, a minor peanut allergen
- SERUM IgE TESTING: Goodman laboratory currently testing potential IgE binding & potential cross-reactivity to peanut agglutinin….and to evaluate IgE binding to glycans
- Protein digestion by pepsin at pH 1.2 (stable)
- Protein abundant (~2 to 4% of protein) in cowpeas (abundant)

Over-riding FACT….HISTORY OF SAFE CONSUMPTION

Common beans (Navy, kidney, pinto & green beans express high levels of aAI and RARELY CAUSE ALLERGY!

Alpha-amylase inhibitor from *Phaseolus vulgaris*
Only one match of questionable relevance

Protein	Aa length	Identity FASTA overall	Highest identity in 80 aa search	Number of matches of >80%	Expectation
Alpha-amylase common bean	246	peanut agglutinin 35%	peanut agglutinin 45%	1	Highly unlikely to cross-react, but Goodman lab is testing

Peanut Agglutinin has RARELY been reported as an allergen—in fact may NOT cause allergy

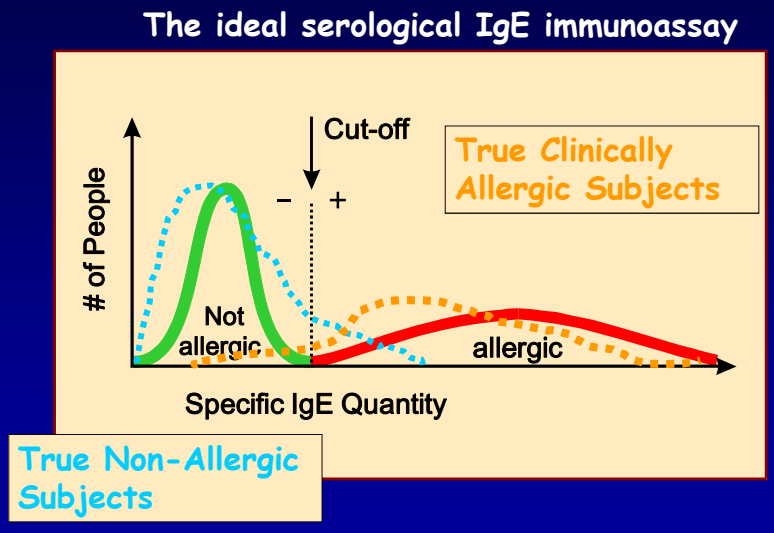
- There is only one published report of IgE binding to PNA from clinically proven peanut allergic subjects
- We tested serum from 34 peanut allergic subjects, found 1 with clear IgE binding to agglutinin, 5 weak binders
- We have performed extensive serum IgE tests….there is NO cross-reactivity!

Serum IgE Tests – Background

(need is based on source of gene or bioinformatics sequence matches)

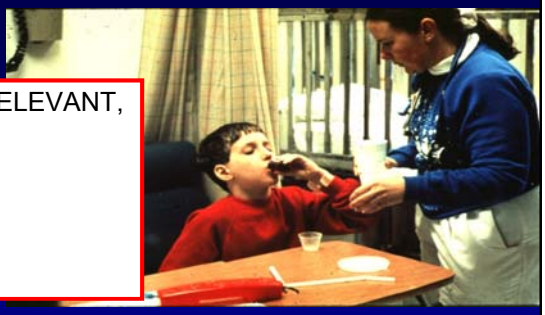
- Must be specific
- Require validation
- Must include positive and negative control allergic sera
- Must include positive and negative control allergenic proteins & extracts

Serum IgE tests: must be reliable, sensitive and specific



GM Safety Testing: Serum IgE Donors

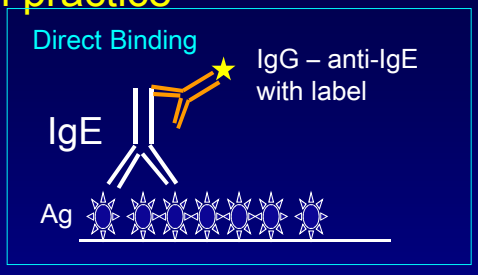
Serum donors must have RELEVANT, PROVEN ALLERGIES

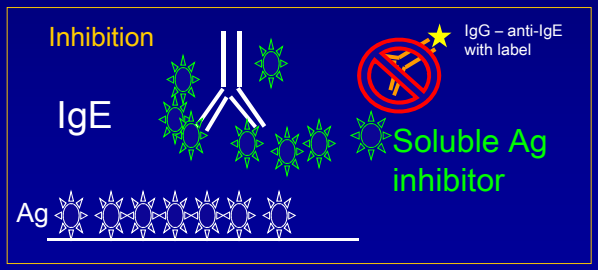


- Objective symptoms
- Consenting donors and controls
- Skin Prick Test + to known allergen
- Significant levels of allergen-specific IgE (e.g. commercial test)

IgE Test Methods: Sound simple….BUT not in practice

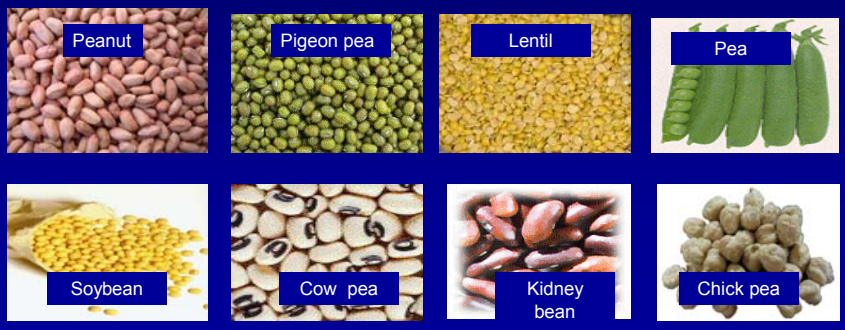
- Dot blot ~ microarray
- Immunoblot
  - Reducing
  - Non-reducing
  - Native
  - 2-Dimensional
- ELISA
- RAST
- EAST
- Inhibition





Allergic cross-reactivity study among legumes – Goodman, et al., US, EU, India (Study funded by the US Environmental Protection Agency)

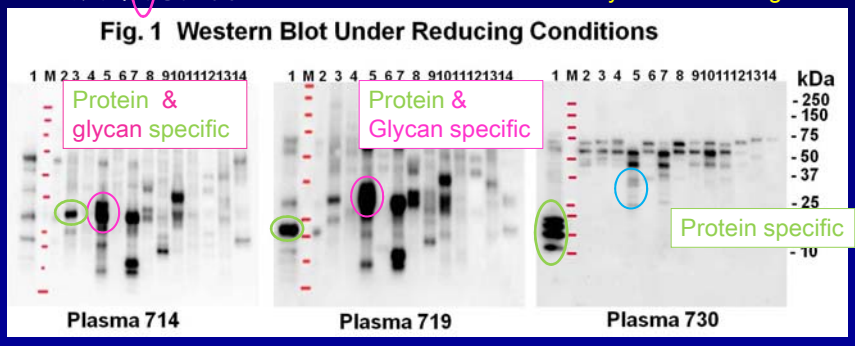
- Leguminosae (Fabaceae) is a large food family including bean, peas, pulses.
- Major source of affordable proteins for the Indian population
- India is the largest producer of Legumes (26% of world production)



Direct IgE Western blot apparent co-sensitization or cross-reactivity for legumes?

Protein Extracts
1: Peanut
M: Marker
2: Lupin
3: Soy
4: Green peas
5: Navy bean
6: Soja noir
7: Red Kidney bean
8: Blackgram
9:Pigeon pea
10:Lima bean
11:Cowpea
12:Fava bean
13: Rice
14: Walnut

Glycoproteins in Navy bean bind IgE from some legume allergic subjects, but it is unlikely to cause allergic rxns



Soybean Allergic

Soybean & peanut Allergic

Peanut Allergic

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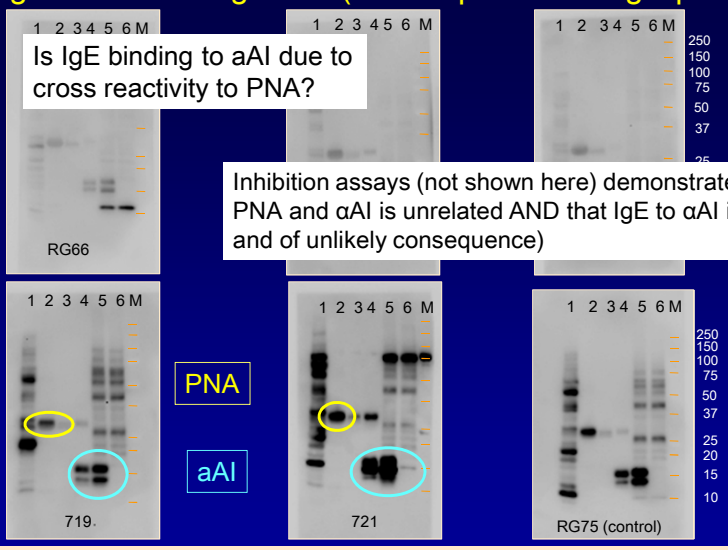
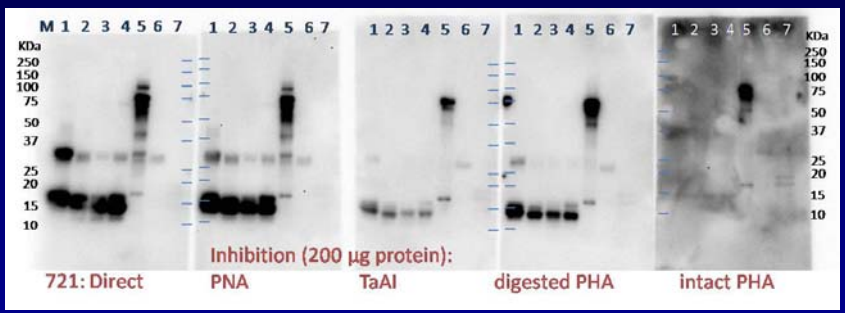
IgE direct binding: PNA (6 of 34 peanut allergic plasma) αAI



Reduced Blot:

1, peanut (10 µg); **2**, peanut agglutinin (5 µg); **3**, peanut agglutinin (0.5 µg); **4**, Tendergreen αAI (0.5 µg); **5**, Transgenic pea (10 µg); **6**, Native pea (10 µg); M, mw marker, BIORAD#161-0374)

αAI and PNA Direct and Inhibition IgE binding: serum 721

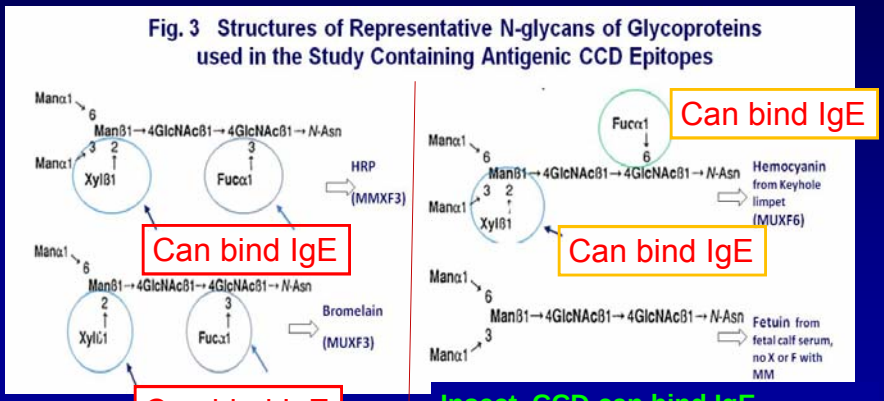


The antigens immobilized on the PVDF membranes are:

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 ug PNA
7) 0.5 Ara h 2

Data demonstrates IgE binding to CCD - irrelevant[40]

Potential IgE binding to Asparagine - Linked Glycans (~1200 structures– Some bind IgE of some allergic subjects…are they allergenic?



Plant glycoproteins

Insect CCD can bind IgE
Animal glycoproteins – usually no IgE binding…

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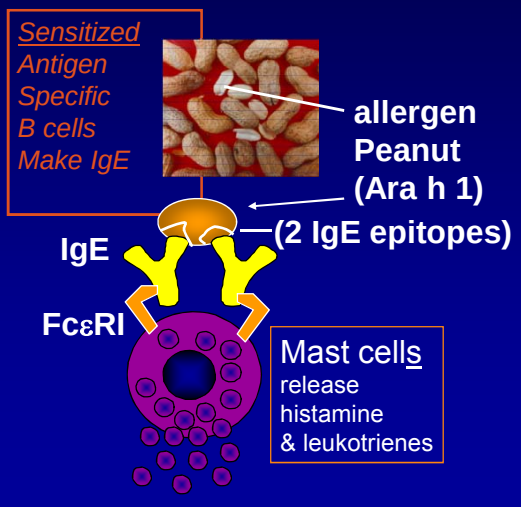
Follow-up TESTING TO CONFIRM PROBABLE RELEVANCE OF INVITRO BINDING Basophil activation or histamine release

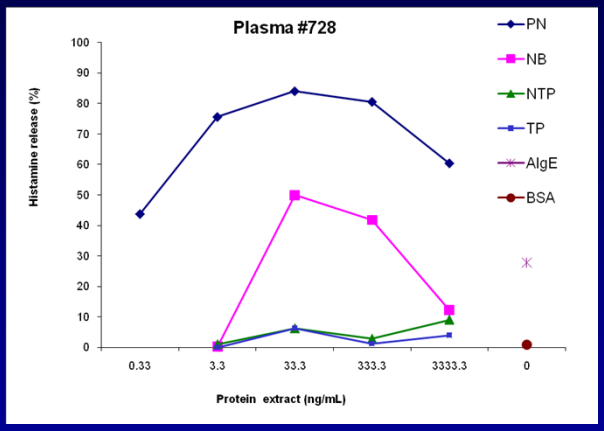
IgE Mediated Symptoms

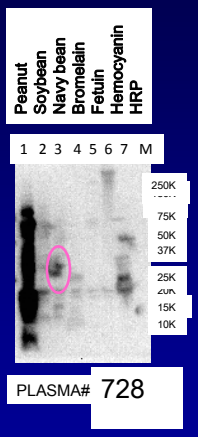
10 to 20 minutes after eating:

- hives
- angioedema
- asthma
- diarrhea/vomiting
- atopic dermatitis
- anaphylaxis



Histamine release assay from stripped human basophils passively sensitized with highly peanut allergic sera #728





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

Peanut CAPS 76 kU/L
Bean CAPS < 1 kU/L

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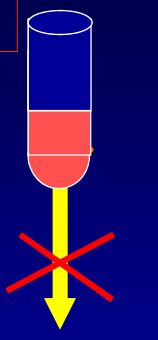
Pepsin Digestion

pH 1.2
Pepsin



- Assay conditions tested
  - K. Thomas et al., Regulatory Toxicology and Pharmacology 39(2004) 87-98
  - Optimized further by Ofori-Anti AO, et al., 2008. Reg Toxicol Pharmacol 52:94-103

Provides a correlation for major food allergens.

This test is not meant to "mimic" real digestion

Goodman FARRP

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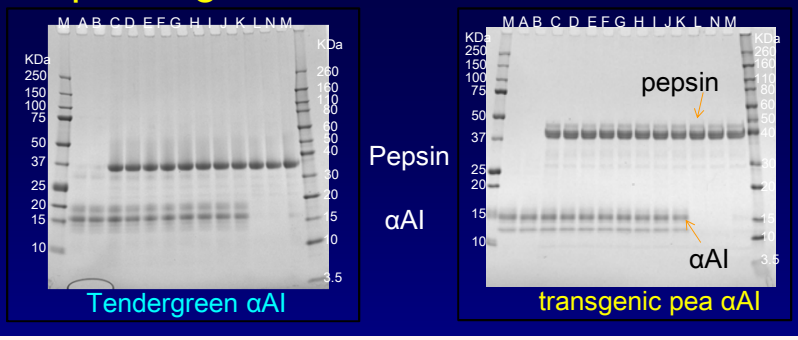
Pepsin digestion of αAI – no difference



A, B: Protein aAI only (1.47 µg), time 0, 60 min. **C**: pepsin + protein, time 0 min. **D**: pepsin + protein, time 0.5 min. **E**: pepsin + protein, time 2 min. **F**: pepsin + protein, time 5 min. **G**: pepsin + protein, time 10 min. **H**: pepsin + protein, time 20 min. **I**: pepsin + protein, time 30 min. **J**: pepsin + protein, time 60 min. **K, L**: pepsin only control (0.147 µg), time 0 min. **M**: molecular weight marker

Four varieties of *Phaseolus vulgaris* αAI (Tendergreen, pinto bean, red kidney bean, navy bean)
Three types of transgenic αAI (GM pea, cowpea, chickpea) were all stable to pepsin.
αAI in whole GM pea and GM chickpea was stable as well.

Alpha-amylase Inhibitor transformed into cowpeas

The weight of evidence indicates that transgenic αAI does not pose a risk of allergy

- Very strong history of safe use…as long as it is cooked. Cowpeas are always cooked.
- Bioinformatics low identity match to peanut agglutinin, led to serum IgE testing.
  - No evidence of cross-reactivity
  - Clear evidence of IgE binding to some Asn-linked CCD, but basophil activation demonstrated lack of relevance.
- Stability in pepsin….again, long history of safe use…

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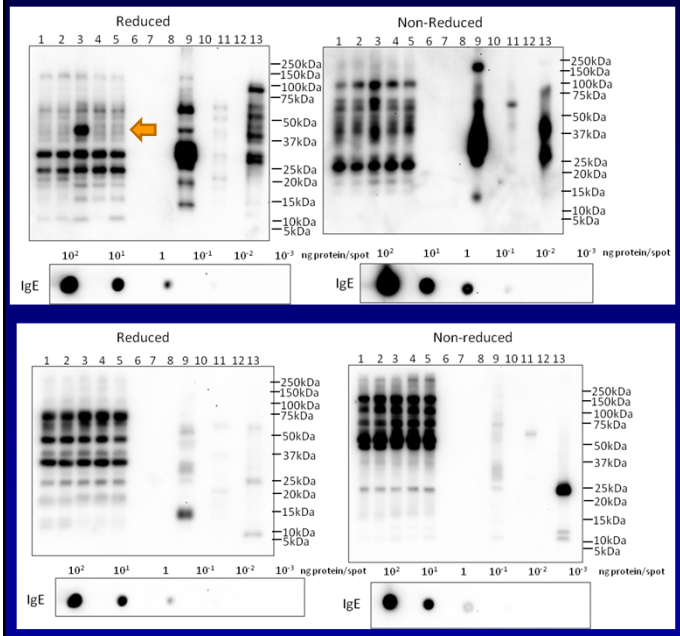
Possible Unintended Effects – Can inserting the gene increase allergenicity?

- Possible, but unlikely
  - Insert into the coding region of an allergen
  - Insert in gene regulatory region
- Possible, suspected if the inserted gene is:
  - A transcription factor
  - Some specific lectins or immunomodulatory proteins.
- Consequence of increased expression – probably unimportant = eat only half, or twice as much allergen before reacting?
- If tested, it should only be the major allergenic crops and tests would be specific serum IgE binding
- NOT a reasonable test for Cry 1 or aAI cowpea!

1D-PAGE and immunoblots (study 2) BASF - SOYBEAN



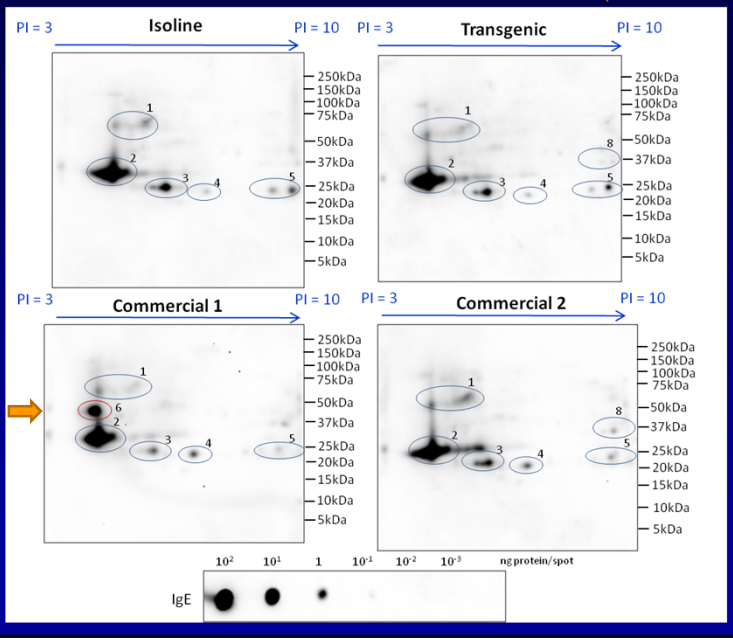
Sera # 20770-MH (CCD binding)

1- Isoline (10µg)
2- Transgenic (10µg)
3- Commercial variety 1 (10µg)
4- Commercial variety 2 (10µg)
5- Commercial variety 3 (10µg)
6- Empty
7- Molecular weight marker
8- Empty
9- Navy bean (10 µg)
10- Empty
11- Peanut (2 µg)
12- Empty
13- Corn (10 µg)

Sera #19392-CS (non-CCD binding)

2D-PAGE and immunoblots (study 2)
BASF SOYBEAN

Sera # 20770-MH
(CCD binding)



Risks of allergy for soybeans

- Someone with soybean allergy MUST avoid all varieties of soybeans to remain symptom free
- There is no selection process for Non-GM commercial soybeans based on potential allergenicity
- Also consider…how we consume soybeans….(next slide)

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GM soybeans are consumed in the US now…~ 10 to 15% food use – Exposure? Labeling?...”soybean”

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Issues - Tests of Unproven Value for allergenicity

- Animal Model Tests – no validated models
- “Heat Stability” – unclear what should be measured
- Targeted Serum IgE tests – most likely to provide false positive results or inconclusive
- Active Post-market surveillance testing – not likely to demonstrate anything
- T cell activation – many T cell types, no tests have been devised to predict allergenicity

Summary

- History of safe, if it is clear, should be the over-riding consideration
- BIOINFORMATICS IS ONE OF THE MOST IMPORTANT STEPS IN THE ASSESSMENT.
  - Is the source of the gene allergenic (or toxic)
  - Is the sequence of the protein highly identical to a known allergen
  - If either is true, appropriate serum IgE tests may be required.
- Example of GM Cowpeas demonstrates that the very conservative limit of 35% identity or matches to "uncertain" allergens….can lead to either product rejection, or unnecessary and complex serum tests.
  - But serum tests and sometimes basophil assays or skin prick tests help differentiate real risks.
  - If you need to do serum test….you certainly will need some expert help. And the serum tests will NOT be simple research methods.

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