Title:
The Effect of Gastrointestinal Digestion on the Allergenicity of Foods (ALLERGEST)

Objectives:
The main objective of this project is to expand current allergenicity assessment strategies regarding GMOs to encompass the whole organism and NOT just the target transgene, and to enlarge the strategies to encompass novel food processing methodologies, novel ingredients and food formulations, especially regarding the development of nutritionally-enhanced foods.

This main objective will be met by working towards a number of other, subsidiary, but complementary objectives, as follows:

- In view of the apparent link between poor digestibility of food proteins and their ability to act as allergens, protein digestion is one of the criteria currently used in assessing the risks which may be posed to consumers by novel food and GMO’s. At the present time the concept has been accepted as relevant for the assessment of the allergenicity even though exceptions to this rule have been observed. The third subsidiary objective of this project is to demonstrate the basis for linking the lack of digestibility of food allergens with their allergenic reactivity.

- Assessment of the degree to which, following passage of food down the gastrointestinal tract, allergenic proteins retain sufficient three-dimensional structure to sensitize susceptible individuals. Whilst considerable efforts have been made to characterize the allergenic determinants in native intact food proteins, the degree to which sensitisation actually occurs towards intact allergens remains unknown. It maybe that denatured forms of allergens found in processed foods, or the proteolytic fragments generated during digestion also play a role in sensitisation. This objective will fill this gap in our knowledge by providing information on the allergenic activity (ability to sensitize) of degraded, compared with intact, allergens.

- Development of an in vitro test system which incorporates the effects of the human digestive system which mimics the conditions found in the gastro-intestinal system of humans and can be manipulated to reflect differences between children and adults. This will provide a superior in vitro alternative to in vivo experimentation using suitable animal models which would allow allergenicity assessments to be made on small amounts of material. It is envisaged that this could be used by regulators, the agro-food industry and researchers, to assess the allergenicity of food proteins.

- The structure of foods is known to influence the release of other nutrients during digestion. The fourth subsidiary objective of this project is to investigate how the release and digestion of allergens is determined by the processing and protein-lipid interactions in foods. This is crucial if knowledge-based strategies are to be developed by the food industry to enable manufacture of high quality low allergen foods suitable for consumption by food-allergic individuals. Such understanding is also important if new minimal processing strategies, such as high pressure, are to be used appropriately to ensure removal of allergenic determinants.

- There is a need to refine the experimental approaches used in the safety assessments made with regards the allergenicity of foods. The fifth subsidiary objective will be to develop a decision tree for determining allergic risks posed by proteins, as part of the toxicological assessment of novel foods. Data generated in the assessment of allergenic activity of the
digestion products of foods will be used to critically assess method performance and to recommend suitable methods and the sequence in which they should be used in safety assessments.

The expected achievements of this project will include the:

- development of an in vitro test system which incorporates the effects of the human digestive system suitable for assessing the allergenic activity of food proteins which has been validated as far as possible using in vivo systems,
- provision of reliable information on how food composition, physical properties and component interactions (particularly with lipids) can affect allergen digestion and presentation to the immune system. Such information would be exploited directly through the industrial partners in the project,
- characterisation of allergenic activity of food protein fragments from digesta and their relationship to size, sequence, and protein conformation,
- correlation of this information in order to define whether allergic individuals are sensitised only to residual intact allergens, or whether large fragments and/or aggregated fragments of allergens can also elicit an allergic reaction. It is anticipated that this new information will help to solve some of the anomalies observed with current in vitro test systems (e.g. the apparently rapid degradation in vitro of caseins, a major milk allergen and a major component of milk, widely held to be one of the most digestible protein sources).

It is envisaged that should the methodology and recommendations be successfully developed, further exploitation would be undertaken within subsequent Demonstration Projects.

Scientific approach:

WP- workpackage
In order to work towards the project objectives described above workpackages have been grouped into three areas with a final work package (WP6) to integrate the results. A workpackage (WP7) has also been included to cover the dissemination and exploitation of new information arising form the project. Table 1 gives a list of Workpackages, Figure 2.1 shows the timescales of workpackages and constituent tasks: whilst Table 2 summarises the current status of Workpackage tasks and milestones.

1) Effects of processing on foods (WP1): The effects of food processing on the digestion and allergenic activity of selected foods will be assessed. Foods will be prepared which represent major allergenic foodstuffs, namely cow’s milk and peanuts, and cover different types of processing employed by the food industry such as (1) The effects of the presentation of the food as particulate matter (i.e. intact nuts) compared with homogenised (i.e. peanut butter) or extruded peanut ingredients; and (2) Liquids versus gel systems e.g. Cow’s milk (freeze dried, condensed and milk-based yoghurts). All foods have a good shelf-life and transport presents no problems for the yoghurt, which will be couriered between partners.

2) Development and validation of in vitro tests of digestibility (WP 2, 3 and 4): The in vitro model will be developed using two-stages to mimic the passage of food into the stomach (stage 1) and then into the duodenum (stage 2). Validation of the in vitro model will be performed in man with food forms based on cow’s milk and peanuts, using gastric and duodenal aspirates. The data will be used to assess the efficacy of the in vitro model at mimicking the in vivo digestion of food and refine the model system to ensure its in vivo relevance. Digesta will be frozen and couriered between partners,
with protease inhibitors to prevent alterations in the peptide profile/allergenic properties in transit, problems eased by the geographical closeness of partners producing and analysing digesta.

(3) Biochemical characterisation and assessment of allergenic activity of digestion products (WP4 and 5): An important element of this research will be assessing the allergenic activity of the digesta obtained in vitro and in vivo. Allergenic activity is defined as the ability of protein and peptide fragments in digesta to sensitisie the immune system to produce an IgE-response. A combination of in vitro assays using human allergic sera and animal experiments will be used, in particular IgE-binding studies will be used to characterise the destruction, (and possible development of ‘new’) allergenic epitopes during digestion. The ability of digesta to sensitise (i.e. to induce a specific IgE response in a naïve individual, or trigger a response in a sensitised individual) will be determined using a Brown Norway rat model using newborn and adult animals. This will address the need to assess the effects of ageing on allergic responses in view of the preponderance of peanut and cow’s milk allergic subjects under the age of 7 years. Allergenicity data will be complemented with biochemical data on the size distribution of intact proteins, and peptides in digesta generated in vitro and in vivo. Wherever possible the sequences of allergen fragments will be obtained, particularly with regards to identifying the fate of known allergenic epitopes.

Results:
Over the last 2 years the project has held four consortium meetings (Year 1: Norwich (partner 2) and Paris (partner 4); Year 2: Copenhagen (partner 8) and Unilever Colworth (partner 6). Additional meetings have been held at Dg Research (July 2003) and Norwich (September 2003). In addition to posting a project website (http://allergest.technion.ac.il/), two issues of a newsletter about the project has been printed.

Progress towards the specific objectives has been as follows:

sObj 1: Basis for linking allergenicity and digestibility
& sObj 2: Assessing the retention of allergen structure and activity following digestion

- Development of protocols for the large scale purification of the major peanut allergen, Ara h 1 able to prepare ~80mg of protein per run with a purity of around 99% (as judged by SDS-PAGE).

- Knowledge of the composition of the digesta is critical to understanding the effect of digestion on allergenic potential. Analysis by RP-HPLC showed the digestion profiles of β-LG to be reproducible and confirmed the inhibitory effect of phosphatidylcholine (PC) on Phase 1+2 digestion of this protein. The breakdown of β-casein, a more labile protein, was little affected by PC. Overall it was shown that this protein is readily broken down, but forms into aggregates, yielding a very complex peptide profile.

- Residual intact allergen, especially in the Phase 1 digesta of β-lactoglobulin (β-LG), has precluded detailed serological characterisation because of residual intact protein. Consequently, a pure peptide fraction of phase 1 β-LG digesta has been prepared. Residual intact β-casein was found in phase 1 digesta, albeit at lower levels than for β-LG. This has again precluded detailed serological characterisation and consequently a pure peptide fraction is being prepared for serological characterisation. As for β-LG, β-casein peptides were also present in aggregated forms in digesta.

- Assembly of the allergic sera collection required to investigate the IgE-binding characteristics of components of digesta is well underway. Sera have been obtained from
well-characterised milk- and peanut-allergic individuals and specific IgE levels determined. Whilst recruitment of allergic patients to the project has experienced some difficulties and has been slower than originally envisaged, these have highlighted some interesting new pieces of information regarding (1) the fact that IgE responses to β-LG tend to be transient in nature in infants, with the major allergen of cow’s milk appearing to be β-casein; (2) patterns of peanut allergen reactivity differ between central Europe (Austria) and the Mediterranean (Greece). Peanut allergic sera from Austria recognise the major allergen Ara h1 and yet those from Greece do not, despite a clear clinical reactivity to peanuts.

- Phase 1+2 β-LG digesta activated basophils, an important effector cell in the allergic response, as effectively as intact β-LG, whilst β-casein digesta were slightly less effective than intact β-casein. This may reflect the different levels of intact allergen present in digesta. Studies using peptide fractions of digesta will confirm if this is so.

- The ability of β-LG and β-casein digesta to induce proliferation of lymphocytes from milk allergic individuals appeared to correlate with the extent to which the protein remained intact. Thus, phase 1 β-LG digesta stimulated proliferation as effectively as the intact protein, but the more degraded phase 1+2 β-LG digesta as less effective. Similarly, β-casein phase 1 and phase 1+2 digesta, which were both more extensively degraded than the β-LG digesta, had a reduced ability to stimulate proliferation of lymphocyte from milk-allergic individuals.

- Ara h1 was found to be highly susceptible to pepsinolysis and was present only as residual peptides following phase 1 digestion; however, further work is needed to demonstrate whether they are aggregated.

sObj 3: Development of an in vitro digestion test system
- The finding that including phosphatidyl choline (PC) in the gastric compartment (phase 1), retards the breakdown of the cow’s milk allergen β-lactoglobulin (βLG) in the duodenal compartment (phase 2) has been shown to be reproducible. However, the breakdown of β-casein was not affected by the inclusion of PC in phase 1 digestion.

- An investigation into in vivo human gastric (phase 1) digestion of a cow’s milk powder and peanut meal suspension has begun with a time course study using six human volunteers. Analysis of these digesta and comparison with in vitro digesta of the same foods forms an important comparison and will allow the in vitro model to evolve in an informed way. The second part of the human study involves sampling of the duodenal contents and is much more invasive in nature. Expertise in these procedures is lacking at Partner 2 and the local hospital and hence a new clinical subcontractor is being sought at the University of Nottingham, in order to complete this work.

sObj 4: Effect of food structure and processing on allergenic properties of food proteins
- The finding that processing of the peanuts (i.e. used raw, boiled or roasted) modifies the IgE-binding capacity of the whole protein extract has been shown to be highly reproducible. Boiling reduced the IgE binding capacity of Ara h1, Ara h2 and whole peanuts extracts, with much of the immunoreactive peanut material finding its way into the cooking water. Hence the apparent drop in boiled nut immunoreactivity may relate to loss of allergens from the nuts rather than their destruction. In contrast, roasting increased the IgE binding capacity of Ara h1, but not of whole peanut preparations. Shock wave treatment of peanuts apparently reduced the immunoreactivity of peanut proteins whilst leaving their composition unchanged.
• Milk and peanut food products have been prepared and distributed to partners. These included three milk powders and three types of yoghurt together with four peanut products. In addition, fat-free raw peanut meal was prepared for Partner 8.

• In addition to the scheduled milk products, Partner 7 also prepared a drinking yoghurt (kefir) and sent to Partner 5 to evaluate its reactivity in cow’s milk allergic individuals. Skin prick testing indicated that the most highly modified kefir had reduced allergenic activity. Such data are promising and indicate that a further investigation into the clinical reactivity of such fermented products in allergic individuals is warranted.

sObj 5: Development of a decision tree for assessing allergenic risks

• An integrative approach to assessing the allergenic activity of digesta is being assembled, which combines investigating the sensitising potential using animal models, with measures of allergenic activity in humans which includes elements of T-cell activation, IgE binding and the ability of bound IgE to trigger de-granulation in sensitised individuals.

• Sensitisation of animals to food proteins requires that they have not been exposed to such proteins for at least 3 generations. Consequently a rat breeding program diets free in milk and leguminous protein has now begun, in order to provide animals for the sensitisation studies. ELISAs for detecting allergen-specific rat IgE are being developed for assessing sensitisation. The RBL-2H3 rat basophilic leukaemia cell line cultures have been transferred from Partner 6 to Partner 8. This cell line will provide an additional way of measuring the specific IgE response of the rats, giving in addition an idea of the functional significance of the specific IgE.