1 Introduction

1.1 Background to the study

The prevalence of IgE mediated allergic disease, including rhinitis, asthma, eczema and food allergy, has significantly increased in industrialised countries over the past few decades. Between 1% and 2% of the adult population has IgE mediated food allergy (Sampson, 1997a; Young, 1994), and peanut allergy alone is reported to occur in 0.5% of the UK population (Emmett, 1999). With the increasing prevalence of allergies, the issues of allergenicity of foods have become a major consideration for the food industry and its regulators. The properties of proteins that make them allergenic remain to be determined, as do some of the factors that determine whether an individual will develop an allergy.

The concept for this study arose in 2000, in the Paediatric Allergy Clinic at Southampton General Hospital. An increasing number of young children were referred with allergy to kiwi fruit, often with a history of severe and even life-threatening reactions. A review of the literature at the time suggested that kiwi fruit allergy was a problem predominantly in adults, mainly secondary to a cross reactivity with latex or birch pollen, and that symptoms were generally mild and localized to the oral mucosa. This was at odds with our clinical impression.

From the experience of one clinic, it was not possible to say whether allergy to kiwi fruit was genuinely increasing, or whether local referral patterns had changed. There was also no explanation of why the patients were reacting more severely than those reported in the medical literature. All of the clinic patients were children, and the majority were local to Southampton and its surrounding area. It was possible that the Southampton population was different from those in published case reports and series because of different environmental pollens, age distributions, eating habits or different genetic make-up.

To investigate the clinical suspicion that allergy to kiwi fruit in the UK was increasing, particularly in the paediatric population, and that the allergy was presenting with severe symptoms in young children, a survey of people in the

UK who believe they have an allergy to kiwi fruit was proposed. The objective was to determine the timescale over which people in the UK have developed the allergy, whether they were allergic to pollens or latex, and to characterize their clinical symptoms.

1.2 General review-Food Allergy

1.2.1 Epidemiology

Allergic disease is one of the major causes of chronic illness in developed countries. In the UK, 39% of children and 30% of adults have been diagnosed with one or more atopic conditions (Gupta, 2004). The UK ranks highest in the world for asthma and near the top for allergic rhinoconjunctivitis and atopic eczema (ISAAC,1998). The prevalence of these atopic disorders has been steadily rising for several decades (Gupta, 2003; Upton, 2000), although in the case of asthma, the trend recently appears to be reversing (ISAAC,1998; Fleming, 2000; Linehan, 2005).

High and increasing trends are also apparent in food allergy, although data remains sparse. Studies in Europe and the US estimate the overall prevalence of food allergies to be between 1.4 and 6% (Bock, 1987; Jansen, 1994; Kanny, 2001; Rance, 2005; Young, 1994). Twenty years ago peanut allergy was considered rare, with only a few case reports, but allergy to peanuts has risen substantially, so that by the age of 4 years, 0.5% of children born on the Isle of Wight in 1989 had suffered an allergic reaction to peanut (Tariq, 1996), but of those born in 1994-6, 1.6% had been diagnosed as peanut allergic at 4 years (Grundy, 2002).

The aetiology of allergy is multifactorial. Atopy, the genetic tendency to generate IgE, is the strongest single risk factor for the development of allergic disease and several gene polymorphisms have been associated with susceptibility to asthma and allergy (reviewed in (Arruda, 2005)). Infants born to atopic parents have more than twice the risk of developing atopic disease than infants with no familial risk. However, most atopic disease occurs in children with no familial risk for atopy and in twin studies although the concordance rates for sensitization to aeroallergens by identical twins was high, they were

discordant in their expression of allergy, suggesting that environmental factors have a modifying role (Edfors-Lubs, 1971; Strachan, 2001). Further still, the rapid increase in atopic diseases over recent decades is likely to be a consequence of environmental rather than genetic factors. Early life sensitization to aeroallergens (Kuehr, 1994; Lau, 1989), low sibship size (Jarvis, 1997; Strachan, 1989), lower rates of viral infections (Matricardi, 2000), non-attendance at day nursery (Ball, 2000; Kramer, 1999) and lower endotoxins exposure (Gereda, 2000; von Mutius, 2000) are among the environmental factors associated with an increased risk for development of atopy. Although numerous environmental factors have been implicated as risk factors for atopy, no conclusive explanation for the rising trends has been found.

The hygiene hypothesis was proposed in 1989 as a speculative explanation for the apparent rise in allergic diseases (Strachan, 1989). Strachan suggested that allergic diseases were prevented by infection in early life and that declining family size, improved household amenities and higher standards of cleanliness have reduced opportunities for cross-infection in families, resulting in more widespread clinical expression of atopic diseases. Cytokines produced by T_h2 cells such as IL-4, IL-5, and IL-13 are clearly critical in the initiation, maintenance and amplification of allergic inflammation. It has been suggested that reduced exposure to infections as a consequence of a Westernised lifestyle deviates the immune system from a predominantly T_h1 to a T_h2 profile. Support for this hypothesis was strengthened by the finding that exposure of a pregnant woman or her offspring in the first year of life to farm animals (and their microbes) exerts a protective effect on the child against allergy (von Mutius, 2000). It has been proposed that this protective effect is mediated by the inate immune system as expression of CD14 and toll-like receptor-2 (TLR2) are markedly higher in farmers' children than in non-farmers' children (Lauener, 2002). Binding of microbial components to TLRs activates antigen presenting cells resulting in production of cytokines such as IL-12, IFN-α and INF-γ which promote the development of T_h1 cells and antagonise the development of T_h2 responses. This might reduce the rate of allergic sensitisation, and also modify the allergic response once sensitised. However, the coexistence of atopic

disease with disease processes that are predominantly T_h1, and the simultaneous increased prevalence of these diseases (eg insulin dependent diabetes) has brought the exclusive T_h1/ T_h2 paradigm into question, and alternative explanations for the epidemiological findings are being sought. The role of T-regulatory cells (Treg) in the development of atopy has become a recent focus of attention and it has been suggested that a lower microbial burden promotes allergy by decreasing the activity of T-regulatory cells (Treg) because of reduced stimulation of the immune system. Experimental support for the role of Treg cells damping allergen-specific responses remains sparse (Romagnani, 2004) but is a current area of scientific activity.

The hygiene hypothesis does not explain all of our epidemiological observations. In North American inner cities, asthma is increasing among children who live in very poor housing, and who might be expected to have a high microbial load. In order to explain the increase in allergies, we need to take a broader view and also consider other factors of western lifestyle. For example, it has been suggested that lifestyle changes related to obesity (eg diet, lack of exercise) are associated with asthma (Chinn, 2003).

### 1.2.2 Mechanisms of IgE mediated Food Allergy

Food allergy is a consequence of a malfunction of the normal immune response. The healthy immune system is capable of recognising, but ignoring harmless dietary antigens and commensal bacteria by the process of oral tolerance (Strobel, 1998; Strober, 1998). The pathogenesis of oral tolerance is only partially understood but includes T-cell anergy and the generation of suppressor T cells (reviewed in (Strobel, 1998)). A proposed mechanism for tolerance is that under normal conditions, luminal antigen is processed by the gastrointestinal (GI) epithelial M cells and is presented directly to CD8+ lamina propria T-suppressor cells via the T cell receptor (TCR)/CD3 complex. A secondary co-stimulatory signal via the CD28/CD8 or the CD40/CD40 receptor ligand complexes is not provided and oral tolerance results. However, oral tolerance will not occur if the antigen bypasses processing by the GI epithelial cell and is presented to CD4+ lamina propria T-helper (T_h) cell by conventional antigen presenting cells (eg. macrophages and dendritic cells) which possess

class II major histocompatibility complex (MHC) molecules. The T_h cell is activated to produce an immune response rather than tolerance. The activated T_h cell may predominantly release T_h1 type cytokines (eg IL-2, INFγ) or T_h2 type cytokines (eg. IL4, IL5 and IL10). T_h1 cytokines typically induce cell-mediated hypersensitivity and inflammation whilst T_h2 cytokines promote IgE synthesis and eosinophil infiltration resulting in IgE mediated inflammation. If oral tolerance does not occur, sensitisation may develop to the antigen, which may proceed to food allergy.

IgE mediated hypersensitivity is characterised by mast cell and basophil degranulation with release of inflammatory mediators. The sensitised individual generates plasma cells which secrete allergen specific IgE. The IgE attaches to high affinity $Fc_\varepsilon RI$ receptors on the surface of tissue mast cells or circulating blood basophils. If the individual is re-exposed to the allergen, cross linking of two or more receptor bound IgE causes their associated receptors to be brought into juxtaposition, initiating a complex intracellular signalling cascade. This results in sudden release of preformed and newly generated mediators into the extracellular environment. The mediators stored in granules include histamine, proteases and proteoglycans. The effects of histamine after ingestion of an allergen are 'immediate' and can be severe, including vasodilation, increased vasopermeability, contraction of bronchial and GI smooth muscle and increased mucus production. However, the effects are generally short lived because of the short half life of histamine (≈1 minute). The dominant protease in mast cells is tryptase, which is stored in its fully active state in the granule, as two distinct forms, α-tryptase and β-tryptase. β-tryptase is predominant in allergic reactions, whilst both forms are found in mastocytosis. Chymase is a protease found in the same granules as tryptase in mast cells, but it is not present in basophils. The major proteoglycan is heparin. Once released, heparin stabilises other cell mediators (eg. the active tetramer of tryptase), as well as producing anticoagulant effects, anti-complement effects and growth factor enhancing properties. In addition to release of pre-formed mediators, activation of mast cells induces liberation of cell membrane arachidonic acid, which is oxidised to prostaglandinD$_2$ (PGD$_2$) and leukotriene C$_4$ (LTC$_4$). PGD$_2$ causes bronchoconstriction, is chemokinetic for neutrophils and inhibits platelet

aggregation. LTC_4, which is also made by eosinophils and a variety of other cells, is metabolised to the active LTD_4 in the extracellular environment. Leukotrienes are potent bronchoconstrictors, cause vascular permeability, increased mucus secretion and eosinophil chemoattraction. LTD_4 is converted to a much less potent cysteinyl LT, LTE_4, which is the excretory metabolite and can be measured in the urine.

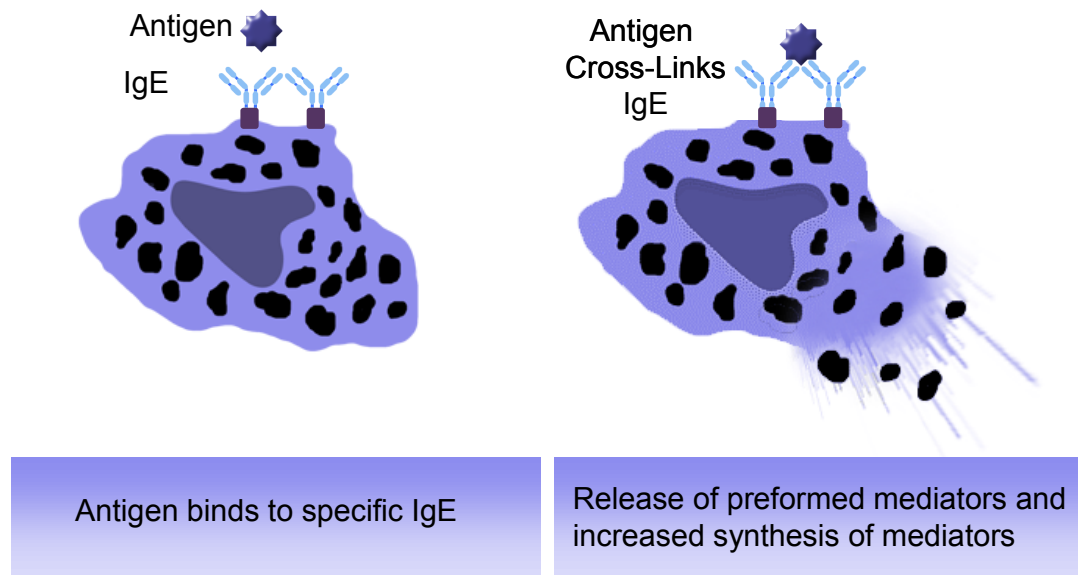*Figure 1.1 Electron micrograph of a human mast cell containing many electron dense secretory granules.*
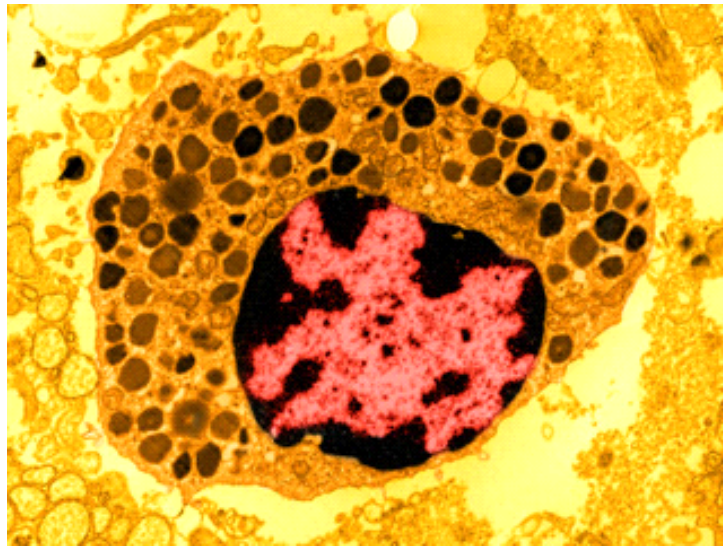
In addition to the mediators, mast cells generate cytokines directed at maintaining allergic inflammation following $Fc_\varepsilon RI$ activation. Mast cells release IL-4 and IL-13, which are involved in switching the B lymphocyte to IgE production, and, IL-5 and GM-CSF which promote eosinophil activation and recruitment. In allergy, the release of mast cell mediators is the initiating response of the early-phase reaction involved in acute anaphylactic reactions. The pathological effects continue in the late phase response approximately 4-24 hours later, characterised by tissue inflammation with neutrophils, eosinophils and mononuclear cells.

Figure 1.2 Schematic representation of mast cell activation.

An allergic response is initiated when allergen cross-links IgE-FcεRI complexes on mast cells. The granules swell, move to the cell membrane, and following membrane fusion, the mediators, including histamine, proteases and proteoglycans are expelled into the local extracellular environment.

Antigen

IgE

Antigen Cross-Links IgE

Antigen binds to specific IgE

Release of preformed mediators and increased synthesis of mediators

1.2.3 Clinical Spectrum of Food Allergy

Food allergy is a term commonly used to describe any unexpected reaction to foods, but its definition is precise, and it should only be used when immunological mechanisms are demonstrated (Johansson, 2001). The EAACI taskforce has proposed that adverse reactions where immunological mechanisms cannot be demonstrated should be called *non-allergic hypersensitivity* (Johansson, 2001).

Food allergy is not caused by a single immunological process. Cows' milk allergy presents an example of the diversity of food allergy pathophysiology and symptoms. The disease process may be mediated by IgE, or may be T-cell dependent. An example of T-cell mediated cows' milk allergy in infants is food protein-induced enterocolitis, which presents with vomiting, diarrhoea, and potentially dehydration, acidaemia and shock (Sicherer, 2005). Atopic dermatitis can be triggered by cows' milk, but the underlying disease processes are less

clearly understood. Some individuals appear to have a predominantly T cell associated process, whilst others additionally have IgE involvement (Eigenmann, 1998). The individual with IgE cows' milk allergy may present with one or more of several manifestations including urticaria, angioedema, abdominal pain, emesis, rhinoconjunctivitis and anaphylactic shock.

It is interesting that the same underlying immune mechanism (IgE-mediated) can result in a diverse spectrum of disease within or between individuals. This can in part be explained by whether the reaction is triggered by direct exposure of the involved organ to the food, or by systemic absorption and distribution of the allergens. Direct exposure may result in local urticaria around the mouth, rhinitis from food aerosols or isolated gastrointestinal reactions. Oral allergy syndrome (OAS) is a localized phenomenon which has been described in approximately 50% of adults with seasonal allergic rhinitis (Ortolani, 1988), resulting in pruritis, erythema or oedema restricted to the oral cavity on eating raw fruits and vegetables. It is believed that initial respiratory sensitisation results in IgE antibodies to pollen proteins that are homologous to those found in some fruits or vegetables. For example, antigens in birch pollen and apples share allergenic epitopes leading to cross reactivity that may cause clinical symptoms of OAS when a birch pollen allergic subject eats an apple (Ebner, 1991). These homologous fruit allergens are heat labile, and cooking denatures the epitopes sufficiently to avoid symptoms.

Systemic absorption of allergens leads to a generalised release of mediators in susceptible individuals, but the consequences range from mild urticaria to life-threatening anaphylaxis with cardiovascular collapse, intractable bronchospasm and severe angioedema. The reason for this discrepancy in reactions remains unclear. Dose of allergen plays a part, but some severe reactions results from trace amounts of food. The UK Fatal Anaphylaxis Register demonstrates that those dying from food allergy had usually had previous reactions but these were typically not severe (Pumphrey, 2004). Features associated with fatal anaphylaxis include a personal history of asthma (Roberts, 2003) and eating away from home (Pumphrey, 2000). Alcohol ingestion and exercise are

important associations with food induced anaphylaxis, perhaps because of increased food absorption.

Various *in vitro* and *in vivo* studies have attempted to identify features that might predict the severity of an individual's immune response, but results have generally been disappointing. Skin prick wheal size does not correlate well with symptoms experienced during a food challenge (Hourihane, 1997). The double blind placebo controlled food challenge (DBPCFC) is considered the gold standard for the diagnosis of food allergy (Atkins, 1985) (Bock, 1988), however, the symptoms experienced are not always the same as those originally described by the patient under 'normal' conditions (Grimshaw, 2003; Warner, 1999). Measurement of specific IgE to peanut has resulted in conflicting results with respect to severity of reactions to peanuts. Hourihane *et al* found no correlation between levels of allergen-specific IgE and the clinical manifestation in a non-challenge situation (Hourihane, 1997). However, using a scoring system combining dosage and symptom grades during DBPCFC, the same research group studying different subjects, found that levels of peanut specific IgE and challenge score correlated significantly (Lewis, 2005). A study from USA demonstrated a median value of serum specific IgE 15-fold higher in patients with a history of severe reactions in comparison with people with moderate and mild symptoms (Bernard, 2003). Quantification may therefore have a predictive value for at least some patients with severe symptoms. There are accumulating data suggesting that differences in epitope recognition might be a useful prognostic marker of persistence of allergy. In the cases of certain egg (Cooke, 1997), milk (Jarvinen, 2002) and peanut (Beyer, 2003) allergens, individuals with persistent allergy have been shown to recognise a larger number of sequential epitopes compared with patients who outgrow there food allergy. However, a similar link has not been shown with severity of symptoms. A study of 65 patients with hazelnut allergy, 7 of whom had a history of anaphylaxis, found that all sera from patients with anaphylaxis, but none with milder symptoms, reacted to a 9 kDa protein in hazelnut extract (Pastorello, 2002). Studies of other food have failed to identify IgE binding patterns predictive of severity. Current areas of research to predict reaction severity

include examining diversity of epitopes and looking at avidity and affinity of IgE binding to epitopes.

1.2.4 Why are some proteins allergenic?

Foods contain a wide variety of proteins yet only a few are allergens. Storage proteins are an example of plant allergens that are abundant. However, proteins that occur in minor amounts can also be major food allergens. The immunogenicity of the protein therefore appears more important than the amount of protein. There are no physico-chemical characteristics that define an allergen, but some features are more likely to be present. There is no definite range of molecular weights, but in practice most have a molecular weight of 10-70 kDa. Allergens are typically stable to changes in heat and pH, and to digestion. Allergens require at least two epitope sites to allow cross-bridging by IgE. They generally have an acid PI and are soluble for absorption across the GI tract. However, many non-allergenic proteins also show these properties.

With the increasing prevalence of allergies the issues of allergenicity of foods has become a major consideration for the food industry and its regulators. New foods including genetically modified (GM) foods need to be scrutinized prior to entry to the market to assess their allergenic potential. A joint World Health Organisation and Food and Agriculture Organization of the United Nations recommended the use of a decision tree for pre-marketing assessment (Joint FAO/ WHO Consultation, 2001). The decision pathway focuses on the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunochemical binding of the newly introduced protein with IgE from the serum of individuals with known allergies to the transferred genetic material, and the physico-chemical properties of the newly introduced protein. It was recommended that the properties assessed in the newly expressed protein include susceptibility to enzymatic degradation and heat stability. The guidelines for assessment of allergenicity in GM foods remain under the review of the Codex FAO/ WHO Task Force. Since 2001 they have agreed that an integrated, stepwise approach should be used, but there has been a divergence of opinion as to whether this should be presented as a decision tree (Codex Alimentarius Commission, 2002). It is commonly felt that the decision tree does not provide

enough insight into the judgements needed at each stage, and a preferable approach includes the assessment steps outlined in the tree, but with a holistic approach for interpreting the information and data derived.

Table 1-1 The WHO/FAO Decision Tree which has been advocated for assessing the potential allergenicity of novel proteins in foods.
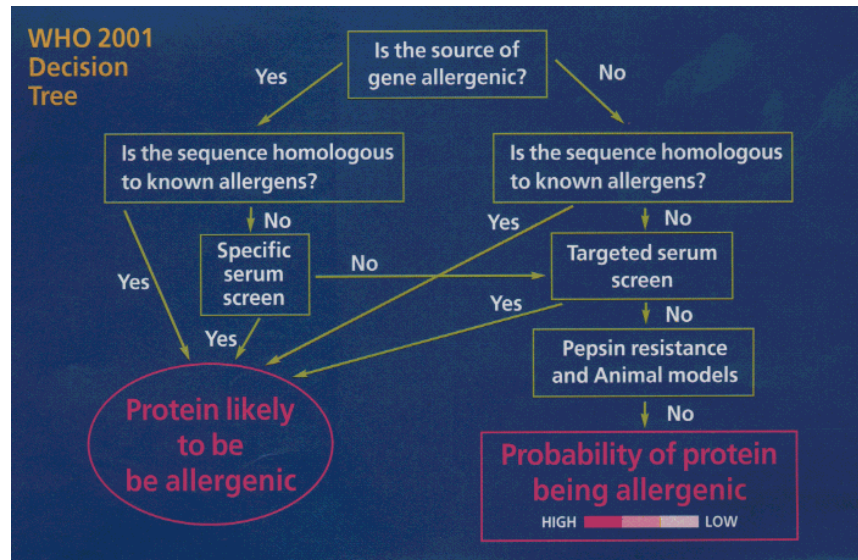
However, whether a decision tree or a weight-of-evidence approach is used, our current poor understanding of the characteristics of a food allergen limits the sensitivity and specificity any process to identify potential allergens.

1.2.5 Why do individuals develop food allergy?

In prevalence studies approximately 25-35% of parents report that their children have adverse reactions to at least one food (Bock, 1987; Eggesbo, 1999), but true food allergy can only be confirmed in less than 10% of children with a peak incidence at ≈1 year of age (Bock, 1987; Eggesbo, 2001; Schafer, 2001; Young, 1994). Likewise, approximately 15-20% of adults perceive an allergy to foods, but the true incidence is less than 2% (Young, 1994). This emphasises that perceived food allergy is extremely common, and that the true incidence is much less, but is still common, affecting approximately 8% of young children and 1.8% of adults. Even a highly allergenic food such as peanut only affects 0.5% of adults and 1.6% of children (Emmett, 1999; Grundy, 2002). Therefore even if a protein has all the requirements of an allergen, most people will not react adversely to it. There is no way of determining who will react to a given

food allergen. There are certainly individuals who have a genetic predisposition to atopic sensitization. In a twin study, it was confirmed that genetic factors increase susceptibility to aeroallergen sensitization. (Strachan, 2001), and heredity has also been shown for food allergies (Hourihane, 1996). However, identical twins were often discordant in their expression of atopy, suggesting a substantial modifying role for environmental factors.

A number of factors can influence the prevalence of a food allergy in a given population. Generally allergy occurs where foods are more commonly eaten. For example, in Europe rice is considered a hypoallergenic food and may be used as the basis of a restriction diet. However, rice allergy presents a significant health problem in Japan (Ikezawa, 1992). This may be a simple consequence of a dose effect on sensitisation. However, the majority of peanut and egg allergic reactions in children occur on the first known exposure (Ford, 1982; Hourihane, 1997), suggesting that low dose exposure resulted in sensitisation, or that sensitisation occurred *in utero* or during breast feeding. Perhaps the timing of exposure is critical in deciding whether an individual develops tolerance or sensitisation. A food that is abundantly available is more likely to be used as a weaning food, perhaps at a crucial time when the immature gut and immune system is vulnerable to sensitisation. For example allergy to lentils occurs in early childhood in Mediterranean countries where lentils are used as a weaning food (Pascual, 1999).

The route of exposure is another variable that may be crucial in determining whether allergy or tolerance prevails. Cutaneous sensitisation to food allergens has been shown to occur in experimental animal models (Strid, 2005) and has also been identified as a significant factor in a retrospective cohort human study (Lack, 2003), and it is possible that other routes, such as inhalation of food aerosol, are important.

1.3 General review- Kiwi Fruit Allergy

Kiwi fruit (Actinidia) is a plant native to the Yangtze Valley of China. Seed was taken to New Zealand in 1904, and almost all kiwi cultivars outside China are descended from the two female and one male plant grown from this single introduction of seed (Vietmeyer N.D., 2003). Commercial plantings began in New Zealand in the late 1930s, and exports to the USA started in 1962. Californian kiwi fruit found their way onto the US market in 1970, and for the past three decades kiwi fruit has been increasingly available worldwide, with producers now in New Zealand, USA, Japan, Italy, Greece, Spain, Australia and Chile.

Acute allergy to kiwi fruit was first described in 1981 (Fine, 1981) and there have since been reports of the allergy presenting with a wide range of symptoms from localised oral allergy syndrome (OAS) to life-threatening anaphylaxis (Falliers, 1983) (Garcia, 1989; Mancuso, 2001; Novembre, 1995; Shimizu, 1995; Veraldi, 1990). The association of kiwi fruit allergy with allergies to pollen and latex has been widely reported in recent years and cross reactivity has been confirmed by inhibition studies with birch pollen (Gall, 1994; Moller, 1997a; Pastorello, 1996; Voitenko, 1997), timothy pollen (Pastorello, 1996), avocado (Diaz-Perales, 1999; Moller, 1998), banana (Moller, 1998), latex (Diaz-Perales, 1999; Moller, 1998) rye (Vocks, 1993) and hazelnuts (Vocks, 1993). Three of the possible major allergens responsible for kiwi allergy have recently been isolated and characterised (Gavrovic-Jankulovic, 2002b; Moller, 1997a; Pastorello, 1998), but much remains unknown about this increasingly common allergy.

1.3.1 Clinical Characteristics

Clinical information about kiwi fruit allergy is mostly based on a handful of case reports and small case series, in addition to the extraction of data from scientific papers primarily written to explore cross-reactivity. The first reported case of kiwi fruit allergy in 1981 was of a 53 year old atopic woman who developed urticaria, wheeze and laryngeal oedema on handling the fruit (Fine, 1981).

Since then there have been a number of reports of kiwi allergy in adults, mostly presenting with oral symptoms (Falliers, 1983; Garcia, 1989; Mancuso, 2001; Novembre, 1995; Shimizu, 1995; Veraldi, 1990). In addition some of these individuals have had more generalised reactions including urticaria (Garcia, 1989), vomiting (Garcia, 1989; Shimizu, 1995) respiratory compromise (Novembre, 1995) and cardiovascular collapse (Novembre, 1995). A recent case report described a 29 year old women who had several episodes of severe anaphylaxis after eating kiwi (Mempel, 2003). The most concerning episode had been elicited by a minute amount of kiwi left on a knife that was subsequently used to prepare a strawberry dessert served to the patient in a restaurant. To date there have been no reports of death from kiwi allergy.

All case reports of kiwi allergy except one (Garcia, 1989) have involved atopic subjects. Case reports of children remain very limited. A 12 year old atopic boy in Japan developed localised oral symptoms, urticaria and dizziness having eaten the fruit (Shimizu, 1995), and a hypotensive response to kiwi has been described in a 3 year old boy (Rance, 1992).

There have been a number of studies investigating cross-reactivity of kiwi fruit with pollens, and these publications may have over-emphasised the mild reactions to kiwi fruit, many subjects with pollinosis having symptoms localised to the oral mucosa. A German study of 25 subjects with birch pollen and kiwi allergies reported that 23 had isolated oral symptoms (OAS) and 2 had urticaria (Moller, 1997b). In the same year, a Danish study of eight subjects with allergies to birch pollen and kiwi fruit had oral allergy syndrome as an entry requirement (Voitenko, 1997). Diez-Gomez *et al* recruited 29 subjects with allergy to plant derived foods, including 12 individuals with symptoms to kiwi, and investigated the subjects for pollen allergy (Diez-Gomez, 1999). The subjects with associated pollen allergy were likely to have oral allergy syndrome, whilst four of five subjects with no pollen allergy had anaphylaxis or wheeze. A similar association has been reported with allergy to Rosaceae fruit, with subjects more likely to have systemic symptoms and anaphylactic shock in the absence of pollinosis (Fernandez-Rivas, 1997).

Since this PhD study started, a Spanish group has reported data from 43 kiwi allergic subjects, including one child, from a birch-free area (Aleman, 2004). Half of their subjects had a history of symptoms restricted to the oral cavity, and 9% of their subjects had anaphylaxis. 21% of their subjects had no pollinosis, and all of these subjects experienced systemic reactions. No studies have addressed whether children respond differently to adults with kiwi allergy, and there are no data about the natural history of the allergy.

1.3.2 Different species of kiwi fruit

The genus *Actinidia* contains about 60 species, but until recently only one has been eaten regularly in the Western world. Green kiwi fruit, *Actinidia deliciosa,* originated in China, but has been cultivated in New Zealand since 1904. It has become a common food throughout the world over the last 30 years, and is now grown in many countries including Italy, Japan, USA and Chile. *Actinidia chinensis* is a species very similar to *Actinidia deliciosa* and until 20 years ago they were classified in the one species. However, there are distinct differences between the two. *Actinidia chinensis* fruit is almost hairless and the flesh ranges from a lime green colour to bright yellow. The fruit are generally much sweeter than *Actinidia deliciosa. Actinidia chinensis* seeds were collected in China in 1977 and taken to New Zealand. Fruit from a mother plant with yellow flesh was identified as particularly good, and is now grown commercially, and marketed under the name Zespri [TM] Gold. Exports were first made to the UK in 2000, and this novel food is increasingly available in North America and Europe. In 2000-1 Zespri[TM] Gold accounted for approximately 7% of New Zealand's kiwi production, and in only two years this had increased to 13% (www.zespri.com). Other species of kiwi fruit are starting to appear in the market eg. baby kiwi (*Actinidia argunta).*

During the progress of this PhD, we reported the immunological cross reactivity of green and gold kiwi fruit allergy in abstract form (Lewis SA, 2003), and the immunological findings were subsequently confirmed by a European collaboration (Bublin, 2004). No assessment of gold kiwi's clinical reactivity had been made prior to this study.

Figure 1.3 Varieties of Actinidia in cross section

The genus Actinidia has approximately sixty species, but only deliciosa and chinensis are readily available on the European market. (Permission given to publish picture by Dr Elspeth McRae, HortResearch, NZ).

1.3.3 Clinical investigations

1.3.3.1 Skin tests

Skin testing with fresh kiwi is the most common clinical investigation reported (Aleman, 2004; Dore, 1990; Falliers, 1983; Gall, 1994; Novembre, 1995; Veraldi, 1990; Voitenko, 1997), the main limitation being that skin testing with fresh fruit lacks standardisation. Some authors have produced an extract of kiwi pulp (Fine, 1981; Shimizu, 1995) or of fruit skin for skin testing (Fine, 1981), and others have used commercially available skin test solutions (Aleman, 2004; Gastaminza, 1998). Prick-to-prick with fresh kiwi or skin testing with homemade kiwi extract was positive in reports of all subjects in whom kiwi allergy was suspected. Commercial skin test extracts are significantly less sensitive. In a study of 33 subjects with kiwi allergy, all of whom had positive skin tests with fresh fruit, only 40% of subjects had positive skin reactions to one commercial extract, and 28% to another make of skin test solution (Aleman, 2004) .

Although highly sensitive, the specificity of fresh kiwi fruit for skin testing appears poor in subjects allergic to cross-reacting pollens or latex. Gall's study

(Gall, 1994) included seven controls allergic to birch pollen but not kiwi fruit, all of whom had positive skin test responses to fresh kiwi fruit. Similarly, two latex allergic individuals with no symptoms on eating kiwi fruit have been reported to have positive skin reactions to fresh kiwi (Monreal, 1996). Beezhold described 47 latex allergic individuals, eight of whom had positive skin tests with fresh kiwi, but only one had symptoms to kiwi fruit (Beezhold, 1996). The converse also occurs. Asymptomatic sensitisation to latex may be as high as 86% in fruit allergic patients, but only 11% suffer clinically relevant latex allergy (Garcia, 1989). Screening subjects with fruit allergy by skin test or measuring food-specific IgE levels to other fruits that might share cross-reactive antigens results in an unacceptable number of false positive reactions (Crespo, 2002). This emphasises the need for a detailed symptom history of oral allergy syndrome and the foods involved before selecting a panel for skin testing. The use of purified fruit allergens in diagnostic tests could improve their specificity, but will reduced sensitivity.

However, some groups have found kiwi skin tests to be highly specific, with negative skin tests in all pollen allergic (Fine, 1981; Garcia, 1989), mite allergic (Garcia, 1989) and 'atopic' (Shimizu, 1995) subjects. There are therefore discrepancies in the literature concerning the specificity of kiwi fruit skin tests in atopic groups. The negative predictive value in non-atopic controls approaches 100% (Fine, 1981; Gall, 1994; Shimizu, 1995) (Garcia, 1989).

Prick-to-prick testing with fresh kiwi is simple and sensitive, but as with all skin test procedures there is a small risk of reaction. A 57 year old man who had suffered two anaphylactic reactions when eating kiwi, had a severe systemic reaction on skin testing (Novembre, 1995) performed at home by his daughter.

1.3.3.2 Specific IgE

The role of measuring specific IgE to confirm kiwi fruit allergy is even less clear. Although positive in some case reports of patients with kiwi allergy (Dore, 1990; Mancuso, 2001; Novembre, 1995; Shimizu, 1995), other authors have found it unhelpful (Garcia, 1989; Gastaminza, 1998). In his study of 22 subjects with

kiwi fruit allergy (Gall, 1994), Gall found that although he was able to detect specific IgE in all subjects with severe symptoms, the results were negative in subjects with mild local symptoms. It is possible that his subjects with oral allergy syndrome had IgE confined to the oral mucosa with no detectable circulating specific IgE, or were allergic to a labile allergen not present in the specific IgE assay. However, other studies have found that at least some subjects with oral allergy syndrome have detectable circulating IgE to kiwi fruit (Moller, 1997b; Moller, 1998; Pastorello, 1996; Voitenko, 1997). Reports of sensitivity of measuring IgE vary between 13% (Brehler, 1997) to over 70% (Moller, 1997b). Variation in sensitivity may reflect the different kiwi allergic populations being studied, and the different techniques used to measure specific IgE, with some groups using the commercially available Pharmacia CAP^{TM} system (Aleman, 2004; Gavrovic-Jankulovic, 2002b), but others using home made allergen discs (Gall, 1994) (Moller, 1997b).

The specificity of *in vitro* tests is also unclear. Using the CAP^{TM} method to detect specific IgE to kiwi in 136 latex allergic patients, Brehler (Brehler, 1997) found relatively high specificity (83%). Likewise, Gall (Gall, 1994) found no specific IgE to kiwi fruit in non-kiwi allergic controls with birch pollen allergy, despite having positive skin tests with fresh kiwi fruit. This is in contrast to reports of 4/4 subjects with no symptoms to kiwi, but symptoms of birch pollen allergy, who had detectable IgE to kiwi fruit (Moller, 1997b) and a case report of two subjects with latex allergy who had positive RAST to kiwi despite being asymptomatic (Monreal, 1996). It has been suggested that each individual has a threshold for anti-birch pollen titres to cause oral allergy intolerance with apple (Ebner, 1991) and indeed several studies have demonstrated higher specific birch pollen specific IgE or larger skin test reactions in subjects with oral allergy syndrome (Eriksson, 1982; Eriksson, 1983; Fogle-Hansson, 1993).

1.3.3.3 Food Challenges

Double blind placebo controlled food challenges (DBPCFC) are the 'gold standard' for confirming food allergy (Atkins, 1985) (Bock, 1988), but blinded food challenges have rarely been used in the context of OAS, and further work

is required to evaluate their role. Only one group has published DBPCFC data concerning kiwi fruit (Aleman, 2004). In their study of 33 subjects, DBPCFC confirmed allergy to kiwi in only 66%, despite all subjects reporting symptoms and all having positive skin tests. In addition, 5 of 8 subjects with negative challenges had positive Western Blots. This may reflect the non-specific nature of SPTs and Western Blots, but alternatively may indicate that their DBPCFC was not 100% sensitive. Most of the subjects had oral allergy syndrome, and the results may reflect the difficulty of performing challenges in a group who by definition have predominantly subjective symptoms. There is also evidence that such subjects may only have reactions to the cross-reacting fruit during the pollen season (Asero, 1996).

1.3.4 Management of kiwi fruit allergy

As with other food allergies, avoidance of kiwi fruit is the mainstay of treatment, with rescue therapy determined by the severity of reactions (McLean-Tooke, 2003). Evidence based data on the management of fruit allergy are lacking, so advice varies from just avoiding the fruit, to restrictive diets of an entire food group. In subjects with a diagnosis of IgE mediated fruit allergy, SPT and measurement of specific IgE to known cross reacting fruits will result in excessive positive test results in the absence of clinical symptoms (Crespo, 2002). Unnecessary restriction of food choice may occur if these clinical investigations are used as a basis for prescribing elimination diets. This stance needs to be balanced against the lack of information about the long-term outcome of subjects with asymptomatic sensitisation in the absence of clinical allergy.

Successful treatment of OAS to fennel, cucumber, melon and apple by pollen specific injection immunotherapy has been described (Asero, 1998; Asero, 2000a). A recent case report of a woman with several episodes of severe anaphylaxis to kiwi fruit described sublingual swallow allergen immunotherapy (SLIT) with kiwi fruit extract (Mempel, 2003). The SLIT caused an increase in IgG4 antibodies to the kiwi 30 kDa allergen, and the patient had a reduced SPT reaction to kiwi fruit. It is possible that this may prove to be a management

option for people with severe reactions to fruit, although it has only been tried in one subject, and she has not been challenged.

1.3.5 Allergens

With regards identifying major allergens, work has resulted in conflicting and confusing results (*Table 1-2*). Different studies have reported different dominant allergens. This could be due to different experimental procedures and/or differences in the study population used (e.g. genetics, differing eating habits). Pastorello (Pastorello, 1996) studied 27 Italian subjects with kiwi fruit allergy, diagnosed by clinical history of OAS, a positive skin test, and a positive open challenge. 87% of subjects also had IgE antibodies to timothy pollen and 73% to birch pollen. They identified 12 IgE binding proteins, one of which, a 30kDa protein, was recognised by all of the subjects, later identified as actinidin.

A Danish study (Voitenko, 1997) using the sera of 8 subjects with pollinosis associated kiwi allergy, and one subject with isolated kiwi allergy had conflicting findings. Their one subject with kiwi fruit allergy but no pollinosis had a band at 30kDa and one at 18kDa. However, none of the other subjects' sera recognised the 30 kDa protein. Instead, they showed proteins in the 10-12 kDa and 22-24 kDa regions to be the most common allergens in kiwi extract. A study from birch free area in Spain found a 30 kDa IgE binding band was recognised by 54% of their kiwi allergic subjects, a 24 kDa band by 29% and 8% recognised a 12 kDa band.

Möller's studies (Moller, 1997b; Moller, 1998) found that proteins of molecular weight 43 and 67 kDa were recognised by over 50% of 22 German subjects with birch pollen allergy, five of whom also had latex, avocado or banana allergy. In addition they detected minor allergens of 13, 22 and 30 kDa. The major allergens, 43 kDa and 67 kDa, were only slightly detected by total protein staining of the blot with Indian ink or by silver staining of the gel, indicating the low amount of these allergens in kiwi extract. On the other hand, proteins found in larger amounts in their extract, with molecular weights of 20, 23 and 26 kDa, did not represent potent allergens. Fahlbusch[42], using sera from 9 kiwi allergic

subjects, 5 with birch pollen allergy, reported that eight subjects recognised a
30 kDa protein. IgE binding proteins were also seen at 23, 43 and 80 kDa.

Table 1-2 A summary of the molecular weights of IgE binding proteins that have been identified in clinical studies.

Reference	Subjects	Country Where The Study Was Based	Molecular Weights of IgE binding proteins (kDa)	Dominant Allergens
Pastorello *et al* 1996 (Pastorello, 1996)	30 subjects with OAS to kiwi- (27 localised symptoms, 3 systemic)	Italy	12, 14, 17, 20, 22, 24, 28, 30, 32, 38, 41, 64.	30 (100%)
Voitenko *et al* 1997 (Voitenko, 1997)	9 kiwi allergic subjects (8 kiwi allergic and birch pollen allergy, 1 kiwi allergic only)	Denmark	10-12, 15-18, 20-25, 30, 38-40. (30 kDa recognised by only 1 subject with mono-allergy.	10-12 (56%) 20-25 (56%)
Möller *et al* 1997 (Moller, 1997b)	22 subjects with birch pollen allergy and sp IgE to kiwi>0.7. - (1 urticaria, 20 OAS, 1 asymptomatic.)	Germany	13, 22, 30, 43, 67	43 (68%) 67(55%)
Möller *et al* 1998 (Moller, 1998)	7 subjects with kiwi allergy, 5 also allergic to banana, avocado and/or latex. - 6 OAS, 1 asymptomatic.	Germany	See Möller *et al* 1997 (above)	43 (71%) 67 (57%)
Fahlbusch *et al* 1998 (Fahlbusch, 1998)	9 subjects with kiwi allergy, 5 also allergic to birch pollen.	Germany	23, 30, 43, 80, >80.	30 (89%) 23 (56%)
Gavrovic-Jankulović *et al* 2002 (Gavrovic-Jankulovic, 2002b)	7 subjects with kiwi fruit allergy	Yugoslavia	24, 25, 27, 30, 43, 67, >67	24 (100%), 25 (100%), 27(100%) 30 (100%)
Aleman 2004 (Aleman, 2004)	43 subjects with history of kiwi fruit allergy	Spain	12, 24, 30, 66 (of 23 subjects with positive DBPCFC, 9 had negative blots)	30 (54%) 24 (29%)
Bublin 2004 (Bublin, 2004)	75 patients with reported kiwi fruit allergy, but blotting with sera from only 20.	Italy Austria Netherlands	Italy (n=11) 27, 22. Austria (N=9) 27	On ELISA, Actinidin Italy 47% Austria 87%

A recent study compared the sensitisation patterns of kiwi allergic patients from different European countries (Bublin, 2004). The majority of patients had OAS. Using ELISA and immunoblotting techniques, distinct differences were found in IgE recognition patterns. Patients from Austria and the Netherlands commonly (90%) recognised actinidin, previously identified as a major allergen in kiwifruit (Pastorello, 1998), but less than 50% of the Italian sera recognized actinidin. The majority of Italian patients' sera reacted to a 22 kDa thaumatin-like protein.

Different extraction methods may account for some of the discrepancy between major allergens identified by different groups. Voitenko (Voitenko, 1997) used two different extraction procedures for kiwi fruit (extraction in borate-buffered saline or phosphate-buffered saline), producing similar protein profiles but slightly different IgE binding profiles. He therefore used a mixture of both extracts in an attempt to provide a more complete allergen extract. The same group also found protein patterns varied according to whether reducing or non-reducing running conditions were applied during electrophoresis. The groups working on kiwi fruit allergens have each used different gels, blockers, dilutions of sera and probing methods and, the precise methodology is unclear in some reports. This emphasises the need for very clear reporting of laboratory techniques by authors working in this field, and where appropriate a need for standardisation of techniques between groups working in the same field. However, a study comparing IgE recognition in different countries (Bublin, 2004), and using the same methodology for all nationalities, indicates that differences in IgE reactivity to green kiwi are real, and may originate from different genetic or environmental factors. Differences may originate from regionally distinct pollen sensitisation patterns (birch, grass and mugwort pollens in Central Europe; grass, cypress, olive and plane pollen in Mediterranean countries), or may reflect regional eating habits.

So far 3 major kiwi allergens have been reported – Act c 1 (30 kDa) (Pastorello, 1998), Act c 2, a thaumatin-like allergen (24kDa) (Gavrovic-Jankulovic, 2002b) and a 43 kDa allergen (Moller, 1997a). Allergens are named according to the guidelines defined by the World Health Organisation/ International Union of

Immunological Societies Allergen Nomenclature Subcommittee. However, traditional, green kiwi fruit was reclassified some twenty years ago from *A chinesis to A deliciosa.* It has therefore been suggested that Act c 1 be renamed Act d 1, and Act c 2 renamed Act d 2 (Bublin, 2004).

Act c 1 is an unglycosylated thiol protease with a mean isoelectric point of 3.5 (Pastorello, 1998). The protein has been partially sequenced and comparison with the Swiss Protein bank showed that this was Actinidin. Actinidin is secreted in an inactive form as actininidin, which has a molecular weight of 39 kDa. Bromelain (from pineapple) and papain (from papaya) are also thiol proteases, with similar modes of action. Although amino acid composition, isoelectric points and molecular weights differ, there are many structural similarities, as demonstrated for actinidin and papain in which polypeptide backbones are extremely similar (Kamphuis, 1985). However, only a very weak cross-reactivity has been found between these thiol proteinases [46]. There is also extensive similarity between the N-terminal amino acid sequence of Der p1 allergen and actinidin (Simpson, 1989), although cross reactivity has not been established.

Act c 2 is a thaumatin-like protein with a molecular weight of 24 kDa and isoelectric point of approximately 9.4 is a major allergen (Gavrovic-Jankulovic, 2002b).

In a study of 22 German patients with birch pollen allergy and kiwi allergy, Moller *et al* described a major allergen with a molecular mass of 43 kDa and an isoelectric point of 6.9 (Moller, 1997a).

1.3.5.1 Kiwi allergens associated with pollen allergy

The clinical association of pollinosis with allergy to fresh fruit including kiwi is well-recognised (Ortolani, 1988). The allergenic components of kiwi fruit that cross react with allergens from timothy and birch pollen have been characterised. Kiwi allergens of 41, 38 and 22 kDa were completely inhibited by timothy grass extract grass pollen, and kiwi allergens with mw 41, 38, 24, 22 and 14 kDa were completely inhibited by birch pollen, suggesting complete identity between the relevant kiwi and pollen allergens (Pastorello, 1996). Other kiwi allergens, for example the 30 kDa allergen (actinidin, Act c 1), were only partially inhibited, suggesting much weaker cross reactivity. In another study (Voitenko, 1997), cross reactivity between birch pollen and kiwi allergens was partly explained by a protein of 10-12 kDa, however, the inhibition was poorly expressed in only half the subjects, perhaps suggesting that only a minor allergen was involved in the cross reactivity.

It remains unproven whether pollinosis always precedes food allergy in OAS. Most of the cross reacting allergens are more abundant in the pollen than in fruits, giving support to the hypothesis that OAS is caused by a primary sensitisation to pollen allergens[54]. In a study of adults with OAS (Kazemi-Shirazi, 2000), a combination of recombinant and natural pollen extracts almost completely inhibited IgE binding to plant extracts, whereas IgE reactivity to pollen allergens was poorly inhibited by recombinant plant food allergens. Based on an assumption that the primary sensitising molecule will carry most if not all the relevant IgE epitopes, whereas the secondary cross-reactive allergen will have less reactive epitopes, the authors concluded that the pollen allergens are responsible for the elicitation and maintenance of OAS.

1.3.5.2 Latex associated allergens

Most of the natural rubber in the world is manufactured using the sap of *Hevea Brasiliensis*. Latex allergy is particularly prevalent in health care workers (Yassin, 1994), rubber industry workers (Tarlo, 1990) and children with spina

bifida (Moneret-Vautrin, 1993), but has a low prevalence in a general paediatric population (Bernardini, 1998). Of the more than 150 polypeptides in natural latex, 35 or more can act as allergens and are recognised by IgE antibodies in the sera of latex allergic subjects (Nel, 1998). Different groups of patients appear to be sensitised to different groups of latex proteins, children with spina bifida showing different IgE binding to health care workers[61].

Approximately 30-50% of individuals who are allergic to natural rubber latex show hypersensitivity to some plant derived food, especially fruits such as avocado, banana and kiwi fruit. The association between latex and these food allergens has been named 'latex-fruit syndrome' (Blanco, 1994). M'Raihi *et al* (M'Raihi, 1991) were amongst the first to describe how fruit allergens, in this case banana, could bind latex specific IgE *in vitro*. More recently it has been shown that an avocado protein (a class I chitinase) could bind specific IgE in the serum of latex allergic patients (Posch, 1999). This protein had significant structural homology to a latex antigen, supporting the hypothesis that foods (e.g. chestnut, avocado, banana and kiwi) are able to cause clinically relevant reactions by immunological cross-reactions, in individuals allergic to latex. A study of fruit and vegetable allergic subjects from Spain found that subjects with pollinosis showed a high frequency of IgE reactivity to Bet v 2 (Diez-Gomez, 1999) and all subjects with positive IgE to Bet v 2 also had reactivity to latex. It is possible that Bet v 2 is a pan-allergen causing cross-reactions between latex, fruits and pollens

Kiwi fruit is recognised as part of the 'latex-fruit syndrome' (Beezhold, 1996; Blanco, 1994), and cross reactivity has been confirmed by inhibition techniques (Brehler, 1997; Moller, 1998). Using immunoblot inhibition, Möller showed that allergens from kiwi fruit share common epitopes with allergens from latex, avocado and banana (Moller, 1998).

In a study of fruit allergic subjects (Garcia Ortiz, 1998) 86% had latex sensitisation (SPT and / or CAP^{TM}) although only 12% had a clear history of latex allergy. Clinical sensitisation to latex was associated with clinical

anaphylaxis to kiwi fruits (Garcia Ortiz, 1998). It has been suggested that sensitisation to latex occurs by inhalation of allergen adsorbing powder and direct contact with latex products[48] followed by allergic symptoms to digestible kiwi proteins. Although the parallel hypothesis in pollinosis associated kiwi allergy appears plausible, evidence that fruit allergy frequently precedes latex allergy and that latex-fruit allergy is often severe (Garcia Ortiz, 1998) would suggest that stable kiwi allergens, different to the allergens in fruit-pollinosis allergy, may-be responsible.

1.3.5.3   *Glycoprotein* associated *cross reactivity*

In eukaryotic cells, most proteins are subject to post-translational modification, of which glycosylation is the most common form. It is estimated that approximately half of all proteins are glycosylated.

Binding of IgE to carbohydrate determinants in protein extracts was first described in 1981 (Aalberse, 1981). It was not clear at this stage whether the IgE-carbohydrate interaction was due to lectins in the extract binding IgE via its carbohydrate side chains, or a carbohydrate-specific IgE interaction, and many allergists were sceptical about the idea of a specific reaction between IgE and carbohydrates. It has since been confirmed that IgE antibodies do react with N-glycans on glycoproteins from plants, invertebrates and Hymenoptera venom. Because of the widespread occurrence of cross reactive carbohydrate determinants (CCDs) in allergens, IgE to carbohydrates often leads to false positive results when using investigations dependent on IgE binding to extracts or allergens, for example CAP, RAST and immunoblots.

Glycoproteins carry two main types of glycans- N-glycans and O-glycans. N-linked glycans are linked to the protein backbone via an amide bond to an asparagine (Asn) residue in an Asn-X-Ser/Thr (serine/ threonine) motif where X can be any amino acid except proline. The glycosylation occurs in the endoplasmic reticulum and Golgi body. O-glycans are linked to hydroxyl groups on serine or threonine. Studies of IgE-carbohydrate moieties have focused almost exclusively on N-linked glycans. Some plant and invertebrate animals

have N-glycans that can contain α(1,2)-fucose or β(1,2)-xylose These monosaccharides do not occur in mammals and are therefore immunogenic. Some glycoproteins carry both sugars, others just one of them. The widespread presence of α(1,2)-fucose and β(1,2)-xylose on N-glycans of plants and invertebrates is the basis for the high degree of cross-reactivity that has been reported for carbohydrate-specific IgE antibodies. Patients with pollen and hymenoptera venom allergy, in addition to having specific IgE to pollen and venom proteins, may develop anti-CCD IgE, leading to cross-reactive recognition of glycans in a range of plant foods and invertebrate foods such as shrimps.

It is generally considered that anti-CCD IgE is of little clinical relevance to the patient. However, anti-CCD may lead to diagnostic errors by causing positive *in vitro* results (RAST, CAP, immunoblots) without apparent clinical significance (Mari, 1999; van der Veen, 1997; van Ree, 2002a). A group of 32 patients with grass pollen allergy and significant levels of anti-peanut IgE in the absence of a history of peanut allergy were tested for the presence of anti-CCD IgE (van der Veen, 1997). 29 of them had detectable levels, and their peanut RASTs were strongly inhibited by CCDs, supporting the view that anti-CCD-IgE does not cause food allergy. The absence of clinical food allergy was explained by poor biological activity of the anti-CCD IgE, as manifest by no SPT reactivity and low reactivity on basophil histamine release tests.

However, some more recent studies suggest that specific IgE binding to carbohydrate moieties of glycosylated allergens may have a role for the elicitation of allergic symptoms in some individuals (Anliker, 2001; Foetisch, 2003; Wicklein, 2004). Vieths' group have reported that CCD-specific IgE was biologically active in a sub-group of patients with tomato allergy, causing histamine release when stimulated with different glycoproteins (Foetisch, 2003). They have also reported allergy to persimmon fruit, which they believe was a consequence of sensitisation to CCDs. In a study designed to determine whether carbohydrate structures on glycoproteins can by themselves elucidate allergic reactions (Wicklein, 2004), the carbohydrate moiety of Phl p 13 (major

allergen of timothy grass pollen) was shown to cross-link IgE receptors via carbohydrate chains and elicit IL-4 release from basophils. The importance of carbohydrate moieties therefore remains a matter of debate.

The glycan structures in kiwi fruit have been studied by incubating kiwi extract after SDS-PAGE blotting, with different lectins (Fahlbusch, 1998). Lectins are proteins or glycoproteins which are isolated from a wide variety of natural sources and bind highly specifically to carbohydrates. A range of lectins were used but only a lectin from *Aleuria aurantia* which binds specifically to terminal α-fucose, and a lectin from *Canavalina ensiformis* (specific for α-mannose, α-glucose, α-N-acetylglucosamine) bound to kiwi proteins. The *Aleuria aurantia* intensely bound to a range of proteins in the mass range 35-92 kDa, and the *Canavalina ensiformis* reacted weakly to a protein of 67 kDa. In the same study, the researchers investigated IgE binding to carbohydrate structures using sera of patients with OAS to kiwi fruit. Using an ELISA system, they treated kiwi fruit with periodate to destroy carbohydrate moieties. The IgE binding to kiwi extract by individuals was reduced to different extents, from >50% to no reduction at all. However, the periodate treatment may have caused conformational changes to the proteins, or oxidation to amino acids, so that the reduction in binding may not have been due to loss of carbohydrate epitopes. In an attempt to confirm the presence of anti-CCD IgE and its cross reactive nature, they preincubated the sera with proteinase-K digested kiwi as a source of CCDs and then performed an ELISA on kiwi coated plates. This supported the findings when using periodate treated kiwi fruit. Protein digestion of an extract to obtain CCDs is a standard techniques and has been used in similar inhibition studies of other allergens (van Ree, 1999). However, the digest is likely to contain structures other than CCD that may interfere with the assay, for example lipids.

1.4 Aims and hypotheses of this study.

Very little information about kiwi fruit allergy existed at the start of this study, and it was therefore necessary to start with a descriptive analysis of the problem in the UK. The original aims were:

1. **To describe the clinical characteristics of kiwi fruit allergy in the UK.** This included identifying the ages of subjects with the allergy, when they had their first reaction, their symptoms, treatment and details of other allergies.
2. **To investigate the use of clinical investigations in the diagnosis of kiwi fruit.**

Achieving these aims will result in a refinement of the clinical approach to kiwi allergy both in terms of understanding history, having tests of good sensitivity and specificity.

The response to the first part of the study indicated that kiwi fruit allergy was a significant problem in the UK, and it confirmed my clinical observation that the allergy can cause severe symptoms, particularly in young children. Hypotheses were derived from the findings of the descriptive study, and these formed the basis of the remainder of this work. The hypotheses for the study are:

1. **Allergy to kiwi as a result of cross reactivity produces only mild symptoms such as oral allergy syndrome, while *de novo* sensitisation leads to a full range of reactions including anaphylaxis.**
2. **The characteristics of the responsible allergens including lability to pH and digestion, determine the severity of symptoms.**
3. **Gold kiwi fruit is not allergenic in subjects with kiwi fruit allergy.**

2 Materials and Methods

2.1 Study population and questionnaire

2.1.1 Ethics

All studies and sample collection were approved by the Southampton and South West Hampshire Joint Research Ethics Committee. Details of the LREC submission are summarised in *Table 2-1*.

Table 2-1 Summary of ethical approvals granted.

Title	LREC no.
Clinical investigations in the management of kiwi fruit allergy	045/00
Clinical investigations in the management of kiwi fruit allergy	055/01
The differences between adults and children with kiwi fruit allergy	028/03/t
Food allergen induced mediator release	319/03/t

2.1.2 Study population

Patients with self reported kiwi fruit allergy were recruited from three sources: the paediatric and adult allergy clinics at Southampton General Hospital, respondents to an advertisement in The Anaphylaxis Campaign Magazine and people who contacted the study following a press release on national radio and in newspapers. Four hundred and twenty patients were sent a self-administered postal questionnaire (Appendix A), with 291 (69%) completed questionnaires being returned between April 2001 and September 2002. There is no agreed standard for an acceptable minimum response rate, but survey response rates of 75% or more are considered good, and less than 50% may be difficult to publish (Barling A, 2005). The slightly low response rate in this study may have introduced selection bias. The study had been funded to investigate approximately 60 patients by questionnaire, but the response rate to the press release was considerably greater than anticipated. There were therefore considerably more questionnaires completed than expected so non-respondents were not followed up.

Patients who completed a questionnaire and reported symptoms suggestive of IgE mediated allergy to kiwi fruit were invited to come to the centre for clinical investigation of their reported symptoms to kiwi fruit. The study was funded sufficiently to perform clinical investigations on 40-50 patients, and 46 patients were recruited. There is no agreed number of patients to participate in clinical studies to characterise food allergens, but most studies have reported between 20 and 50 challenges. A review of all double blind, placebo controlled food challenge (DBPCFC) studies designed to characterise a food allergy between 1995 and 2005 showed that most had challenged between 20 and 50 patients. For example mustard N=24 (Morisset, 2003), cherries N=24 (Ballmer-Weber, 2002), hazelnuts N=86 (Ortolani, 2000), melon=53 (Rodriguez, 2000), celery N=32 (Ballmer-Weber, 2000), wheat=39 (Majamaa, 1999), codfish N=10 (Hansen, 1992).

The patients for clinical investigations were primarily selected by their availability and motivation to attend the research centre. This pragmatic approach was taken because of limited funds for patient expenses, but scientifically, a randomised approach would have been preferable. Patients were chosen to be representative of all ages (over 6 years) and to include people with isolated oral symptoms as well as those with a history of systemic reactions. Patients with a history of severe anaphylaxis were excluded from challenge. In addition skin tests and specific IgE to kiwi fruit were measured in 5 atopic and 5 non-atopic controls who eat kiwi fruit with no adverse symptoms.

2.1.3 Questionnaires

2.1.3.1 Development of the questionnaire

The questionnaire was developed from one previously used in Child Health to characterise peanut allergy (Hourihane, 1997). It was adapted to include questions to identify allergy to allergens known to cross react with kiwi fruit. A pilot copy of the questionnaire was sent to 20 patients with symptoms of kiwi fruit allergy. Following responses from all 20 pilot questionnaires, minor changes were made to clarify questions, and to generate precise responses from patients.

2.1.3.2 The questionnaire

The postal questionnaire included questions on age of onset of allergy, frequency of kiwi ingestion prior to a reaction, symptoms, treatment received and coexisting allergies and atopic diseases (see Appendix A). Reported symptoms were considered mild if they involved a tingling or sore mouth, or a rash; moderately severe if they included abdominal pain, a tight throat, facial swelling or breathing difficulties other than wheeze. Severe symptoms were those involving wheeze, cyanosis or collapse (Hourihane, 1997). Patients were considered to have OAS if they had only ever had reactions to kiwi fruit localized to the oral or pharyngeal mucosa. Patients were considered to have systemic symptoms if they had ever developed a rash, had angioedema other than in the oral pharynx, had breathing difficulties, collapsed or became cyanotic.

2.1.3.3 Validation of the questionnaire

Two hundred and ninety one questionnaires were returned. 18 were excluded because on review, the symptoms were not suggestive of IgE mediated kiwi fruit allergy. Two hundred and seventy three valid questionnaires were therefore analysed. Double blind placebo controlled food challenges (DBPCFC) are considered the gold standard for confirming food allergy (Atkins, 1985) (Bock, 1988). However, this study found DBPCFC not to be 100% sensitive (chapter 4). Skin prick test (SPT) may be more sensitive but is less specific (chapter 4). Specific IgE measurement in sera is poorly sensitive (chapter 4). The responses to the questionnaire were therefore validated against specific IgE, DBPCFC and SPT (Table 2-2), recognising that none of these are perfect gold standards in the confirmation of kiwi fruit allergy.

Table 2-2 *Results of clinical investigations of people who completed the questionnaire and reported symptoms suggestive of IgE mediated kiwi fruit allergy.*

Patients were investigated by prick-to prick SPT with fresh kiwi, specific IgE to kiwi and DBPCFC using protocols explained in 2.2. Fifteen percent of DBPCFCs were inconclusive.

Clinical investigation	Number of people tested	Percentage of total study population (N=273)	Clinical investigation positive
SPT	46	17%	87%
Specific IgE	117	43%	37%
DBPCFC	45	16%	61%

Since none of the clinical investigations are gold standards, the questionnaires were not excluded from analysis on the basis of investigation results. However, it is possible that approximately 10 to 30% of questionnaires were completed by people who are not currently allergic to kiwi fruit (N=27 to 81).

When validated by SPT to birch pollen in 44 of the study population, the question 'do you think or know that you have allergy to tree pollens?' had an excellent negative predictive value (NPV-92%) but poorer positive predictive value(PPV- 61%). The SPT was specifically birch pollen and the poor PPV may have been higher if a mix of tree pollens had been used. Alternatively some of the patients may have misidentified the aeroallergen responsible for their symptoms.

2.1.4 Sera

One hundred and seventeen patients supplied up to 20ml of blood each. Patients were invited to attend the Southampton Wellcome Trust Clinical Research Facility (WTCRF) for venesection, or if they preferred it was arranged for their General Practitioner to take the blood and send it to the research office

in appropriate packaging. Blood samples were centrifuged at 3000rpm for 20 minutes. The supernatant was removed and stored in 1ml aliquots at -80°C.

A pool of sera from kiwi allergic individuals was produced from 9 patients with a history of kiwi allergy and specific IgE> $0.5kU_A/L$. The purpose of the pool was to provide a positive control for Western blotting and ELISA studies. It was not intended to be representative of the study population. The characteristics of patients used for the pool are shown in table (Table 2-3). The patients were chosen to fulfil the following criteria:

- The individual should have sufficient serum in storage to be used throughout the study for the pool i.e. >3 ml.
- Sera were selected from individuals to provide a pool containing IgE reactive to a range of predicted major and most minor allergens.

An atopic sera pool was created from 5 atopic individuals who eat kiwi fruit without allergic symptoms. A non-atopic sera pool was created from 5 non-atopic individuals who eat kiwi fruit without symptoms.

For each pool, equal volumes of patient sera were used to create a 4500μl pool. The sera were stored as 220μl aliquots at -80°.

Table 2-3 Characteristics of patients in the kiwi allergic pool

Age	Kiwi allergy symptoms	Other Allergies	SIgE kiwi KU$_A$/L
12 year	Throat swelling, difficulty breathing, tingling mouth	Peanut, tree nut, tree pollen, grass pollen, House dust mite	0.6
12 year	Wheeze, tight throat, swelling of mouth, angioedema, rash	Nil	0.7
20 year	Anaphylaxis	Banana, latex, grass pollen, dog	4.6
35 years	Wheeze, rash, angioedema, tight throat	Peanut, tree nuts, egg, shell-fish, tree pollen, grass pollen	3.9
58 year	Tingling mouth, throat tightening, vomiting	Nil	0.6
63 years	Tingling sore throat, difficulty swallowing	House dust mite, latex	2.4
68 year	Tingling mouth, throat tightening	Nil	2.4
69 years	Tight throat, throat burning, vomiting	Nil	2.1
71 years	Tingling mouth	Nil	2.6

2.2 Diagnostic investigations

All clinical investigations were performed in the WTCRF, which is fully equipped for physiological monitoring and resuscitation in children and adults.

2.2.1 Skin Tests

Prick-to-prick skin testing was performed using fresh pulp of kiwi on the volar aspect of the distal forearm. The fruit was cut, and the lancet was pricked into the pulp and then immediately afterwards into the skin at 90 degrees. Prick-to-prick testing was similarly performed using fresh pulp of apple, banana and avocado.

Patients were also skin tested with a panel of common allergens (birch pollen, latex 1:10 w: v, house dust mite (*Der p*), egg, milk- all ALK-Abelló), and with any allergen of clinical relevance to the individual patient. A negative control of

saline 0.9% and a positive control of histamine 10mg/ml were used. The reaction was regarded as positive if the mean wheal diameter was at least 3mm in the presence of appropriate reactions to the positive and negative controls. 19 patients also had skin testing using a commercially available kiwi extract (Alyostal, France).

2.2.2 IgE measurement

Serum was tested for specific IgE to kiwi fruit (f84) using the FEIA-CAP^{TM} (fluorescent enzyme immunoassay) system (Pharmacia) in the hospital immunology laboratory. A specific IgE ≥0.35 kU$_A$/L was considered positive. Specific IgE to kiwi fruit was also measured in 5 atopic and 5 non-atopic controls who eat kiwi fruit with no adverse symptoms.

2.2.3 Double blind placebo controlled food challenge

The recipes for DBPCFCs for this study were devised by Kate Grimshaw (WTCRF Research Dietician), because there were no previously published protocols for DBPCFC to kiwi fruit. It was decided to use fresh kiwi fruit masked in another food, because using freeze-dried or concentrated foods masked in capsules or other foods can alter allergenicity. Capsules have the further disadvantage that oropharangeal symptoms do not occur.

The recipes were designed to challenge all patients with reported allergy to kiwi fruit, both patients with oral allergy syndrome, and those with more generalised reactions. The challenges were started at a low dose (1mg fruit; ≈0.1mg protein), reflecting the amount of kiwi some patients had indicated as the minimum dose that causes them to react. Prior to performing the challenges on patients, a panel of non-allergic patients confirmed that the active and placebo mixtures were indistinguishable in all ways, including texture, taste, smell and appearance.

Patients were asked to avoid antihistamines in the week preceding the challenge. In addition to being medically assessed, patients had facial

photographs both prior to the challenge, and following the development of visible signs. A contemporary diary of signs and symptoms was maintained by the patient and research nurse throughout the challenge. A dietician (Kate Grimshaw) and clinician (Jane Lucas) reviewed the challenge symptom diaries whilst blinded to the sequence of active and placebo doses. Challenges were considered positive if the patient developed an objective sign e.g. urticaria, angioedema or a fall in peak expiratory flow, or if the had subjective symptoms which conformed to the pattern of active/ placebo doses. Patients who had a negative or inconclusive DBPCFC were offered an open challenge with whole kiwi fruit (60g). The open challenge was only considered positive if the patient developed physical signs indicative of an IgE mediated response.

The first 8 challenges used an ice cream vehicle at room temperature (recipe 1).

Recipe 1: Peeled, pureed pulp of kiwi fruit, including seeds, was masked in a sorbet or ice cream vehicle that contained ground dried tea leaves and liquid food colouring. 4 placebo doses were randomly dispersed with 9 active doses in incremental doses of kiwi fruit (1mg, 10mg, 50mg, 100mg 500mg 1000mg, 2000mg, 4000mg, 8000mg). The doses were given to the patient at intervals appropriate to the reported reaction time. This was generally 15 minutes.

Using Recipe 1, two patients with inconclusive DBPCFC and one patient with a negative challenge subsequently developed objective signs when openly challenged, thereby suggesting that the challenge was not sensitive. Although open challenges are susceptible to placebo effects, the signs were considered convincing by nursing and medical staff.

Problems with delayed symptoms have been reported when challenging patients to peanuts using a high fat vehicle (Grimshaw, 2003), and it was proposed that a similar "matrix effect" was occurring. The recipe was therefore adapted to contain low fat yoghurt (recipe 2).

Recipe 2: Peeled, pureed pulp of kiwi fruit, including seeds, was masked in yoghurt that contained a mix of orange pulp, tealeaves and liquid food colouring. 3 placebo doses were randomly dispersed with 4 active doses in incremental doses of kiwi fruit (2.5g, 10g, 20g, and 60g). The doses were given to the patient at intervals of 15/ 30 minutes.

Using recipe 2, there were again problems with patients complaining of severe oral symptoms that did not correlate with the dosing schedule. Some of these symptoms were undoubtedly due to placebo, but some of the patients with OAS may have had persistence of symptoms from one dose to the next or fluctuating severity of symptoms, that did not necessarily coincide with the doses. The protocol was changed again, this time giving intervals of at least half an hour between doses, and having greater incremental increases in the doses. Some patients with OAS continued to have inconclusive challenge results, and ethical approval was gained to investigate the use of measuring mast cell mediators in saliva to provide objective measurements during challenges (2.2.4).

One patient had an open challenge due to known allergy to the challenge vehicle.

2.2.4 Oral mediator release

Interpreting DBPCFCs in patients with oral allergy syndrome proved difficult. Using the hypothesis that in patients with OAS, mast cell degranulation occurs in the mouth in response to allergen exposure, with the release of mediators including histamine, a protocol (LREC 319/03/t) for a pilot study was designed in an attempt to develop an objective clinical investigation to confirm OAS.

2.2.4.1 Recovery of mast cells from the buccal mucosa

Firstly a feasibility study was conducted using non-allergic volunteers to see if mast cells could be retrieved from the buccal mucosa. If successful, the plan would be to obtain oral mast cells from food allergic patients and incubate them

with the index allergen and anti-IgE (positive control), measuring histamine release using commercial immunoassays.

Buccal mucosa swabs were taken from inside the cheek of the healthy volunteers (N=2) using cytology brushes. The brushes were washed into 500mcl of phosphate buffered saline (PBS). 30mcl of kimura stain was added to 30mcl of the PBS and the total number of cells estimated by hemacytometer. To confirm the differential count two cytocentrifuge preparations (Shandon cytospin) were made using each volunteer's cell suspensions. The preparations were fixed in acetone and stained with Giemsa's stain.

2.2.4.2 Buccal challenge

Since no mast cells were obtained using the cytology brush, a modified food challenge protocol was devised. Patients with OAS, who had already had a positive DBPCFC were invited to undergo a buccal food challenge. Patients were asked to avoid antihistamines for a week, and cigarettes, food and drinks for 3 hours prior to the challenge. The dose of allergen for buccal challenge was determined individually for each patient as the minimum dose that provoked a clinical reaction during the DBPCFC.

Kiwi fruit was placed in the pouch between the lower molar teeth and the cheek and left *in situ* for 15 minutes. Saliva from the cheek pouch adjacent to the site of the challenge was collected at 0 min, 15 min, 30 min, 60 min, 120 minutes, following removal of allergen from the oral cavity. A sample was also taken prior to the challenge. The saliva was collected by placing a cotton swab salivette (Sarstedt Ltd) in the cheek pouch for 2 minutes. To recover the saliva from the salivettte, it was immediately placed in the salivette centrifuge container and centrifuged at 4˚C for 10 minutes at 13000 rpm. The saliva was immediately put on ice and transferred to a -80˚C freezer. For each allergic patient, a non-atopic volunteer also had a buccal kiwi challenge simultaneously, with identical food dose and saliva sampling. The researcher (JL) was blinded to which specimens were from the allergic patients, and which were from the healthy volunteers.

2.2.4.3 Histamine ELISA

Histamine in saliva was measured at each time point using a histamine enzyme-linked immunosorbent assay (ELISA) (IBL Immuno Biological Laboratories, Hamburg). The ELISA is based on the competition principal. An unknown amount of histamine in the saliva sample and a fixed amount of enzyme-labelled histamine compete for binding sites on the wells coated with antibodies. After substrate reaction the intensity of the developed colour is inversely proportional to the amount of antigen in the sample. The immunoassay is designed to measure histamine in plasma, and the manufacturers instructions for use with plasma were followed because there was no available information concerning histamine quantification in saliva. The limits of detection for this assay were 0.55ng/ml to 123 ng/ml of histamine.

In brief, the histamine in samples was alkylated by adding 20mcl of acylation reagent into each 100mcl of plasma standard (IBL), patient saliva and control saliva. The specimens were incubated for 30 minutes with indicator buffer, and then 750mcl of assay buffer was added and vortexed. 50mcl duplicates of each standard, control and patient sample was pipetted into the wells of a microtitre plate which had been coated with goat anti-rabbit antiserum. 50mcl of a solution containing histamine conjugated with peroxidase was added to the wells and then 50mcl of histamine antiserum (rabbit).The plate was sealed and incubated at room temperature on an orbital shaker for 3 hours. After washing the wells, 200mcl of TMB substrate was added to each well and incubated at room temperature for 40 minutes. The reaction was stopped and the optical density (OD) measured at 450nm. The obtained mean OD of the standards was plotted (y-axis, linear) against their concentration (x-axis, logarithmic) using Excel (Microsoft software). The concentration of the samples was read from the standard curve.

2.2.5 Gold kiwi fruit
Five patients, who had positive DBPCFCs with green kiwi fruit, were invited to return for further investigations using Zespri[TM] Gold. Patients had prick-to-prick

skin testing with fresh pulp of green and gold fruits using the above protocol. They underwent a DBPCFC to gold kiwi fruit using recipe 2.

2.3 Laboratory methods

2.3.1 Allergen preparation

2.3.1.1 Development of method to prepare a kiwi fruit protein extract

There have been several published methods for the extraction of kiwi fruit proteins (Diaz-Perales, 1999; Fahlbusch, 1998; Gavrovic-Jankulovic, 2002b; Moller, 1997a; Moller, 1998; Pastorello, 1996; Voitenko, 1997). Three of these extraction methods (Diaz-Perales, 1999; Moller, 1997a; Pastorello, 1996) were compared in our laboratory (Adam Pearce, 4th Year Medical student, University of Southampton 2002). The extraction methods varied little in terms of protein yield or the patterns of proteins isolated from them, but Pastorello's method (Pastorello, 1996) was considered the most comprehensive, containing all of the higher and lower molecular weight proteins separated by sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE). This method was therefore used as the basis for further optimisation.

Pastorello (Pastorello, 1996) homogenized the pulp with potassium phosphate buffer (PPB), but this was found to have no advantage over phosphate buffered saline (PBS). Pastorello (Pastorello, 1996) dialysed against phosphate buffer, but again this was not found to have any advantages over dialyzing against water. Moller (Moller, 1997b) did not dialyze the supernatant, but this method resulted in a paucity of higher molecular weight proteins . For this study the extract was therefore homogenized with PBS and dialyzed against water. The use of water also avoided potential problems associated with high salt content of samples during subsequent electrophoresis.

Because kiwi fruit has a high protease content, extracts were compared when prepared by extraction in PBS with and without protease inhibitor cocktail tablets (Roche, UK). The fruit was peeled, weighed and placed immediately in 100ml of PBS +/- inhibitor (1 tablet to 50 ml) at 4°C. The volume of PBS was made up to 1:2 w:v. Extraction was then completed as outlined below. SDS-

PAGE analysis and Western blots were identical in extracts made with and without the protease inhibitor tablets, and their use was discontinued (*Figure 2.1*).

Figure 2.1 *Western blot to compare IgE binding by pooled kiwi-allergic sera to extract made without protease inhibitor (lane 1) and with protease inhibitor (lane 2).*
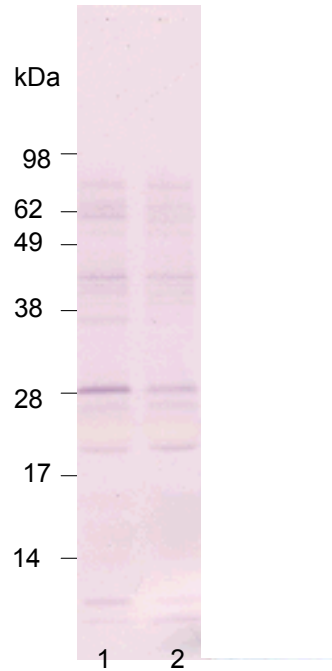
2.3.1.2 Standard Method to make kiwi fruit extract

Many fruit proteins are labile, and additionally kiwi fruit contains proteases making autodigestion possible. Care was therefore taken to keep the kiwi fruit at approximately 4˚C throughout the extraction and dialysis process. Extracts of kiwi fruit protein were made from fresh green and gold kiwi fruit. Fresh fruit was peeled, pureed and some of the seeds were crushed. The pulp was homogenized in 1:2 wt/vol in PBS (pH 7.4), and stored on ice whilst vortexing every 15 minutes for 2 hours. The resulting homogenate was centrifuged (3500rpm, 30 minutes, 4˚C), and the supernatant dialysed over 48 hours at 4˚C against water, changing the water after 4 hours and then approximately 12 hourly. The specimen was frozen at -80˚C and then lyophilised under suction at -60˚C over 5 days. The resulting protein powder was frozen at -80˚C until required.

2.3.1.3 Validation and repeatability of method to extract kiwi fruit proteins

To confirm the quality of the extracts made during the study, comparison was made with kiwi fruit extract provided by Dr Elspeth McRae (HortResearch, Auckland, New Zealand). The protein and allergen profiles obtained by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (*Figure 2.2*) and Western blotting using pooled kiwi allergic sera did not reveal any significant differences between the HortResearch extract and study extracts.

Comparisons were also made between batches of extract made during the study to ensure repeatability. Very minor differences were seen between batches of study extract, possibly reflecting true differences in protein profiles of fruits bought at different times (*Figure 2.2*). The same extract (batch F1) was therefore used for all definitive studies unless stated.

Figure 2.2 *Seven different extracts from kiwi fruit ran on 12% SDS-PAGE gels under reducing conditions and stained with EZblue (Sigma.)*

Lane 1: extract UK- from Southampton grown fruit; lane 2: extract It- extract from Italian fruit; lane 3: K- green kiwi, origin unknown; lane 4: F_2- green kiwi, origin unknown; lane 5: F_1- green kiwi, origin unknown; lane 6: NZ- extract made from NZ Hayward variety by HortResearch, NZ; lane 7: HRA- affinity purified actinidin from HortResearch NZ. The protein band containing actinidin is indicated.
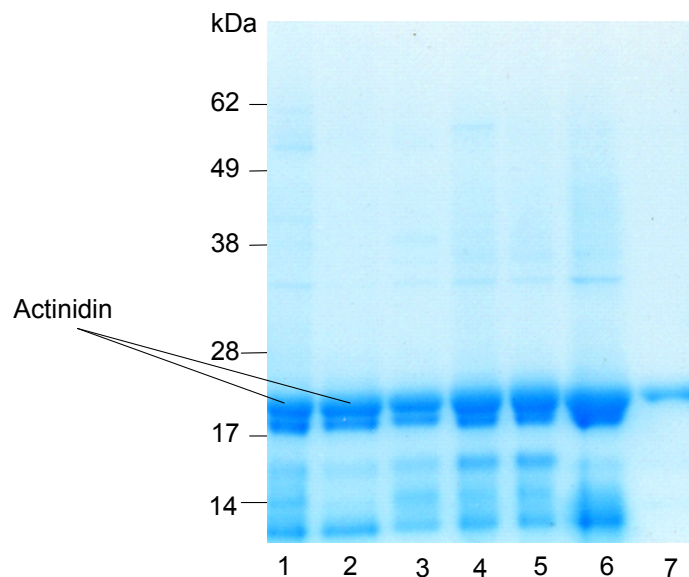
2.3.1.4 Actinidin extract

Extracts of actinidin were supplied by Dr Elspeth McRae (HortResearch, NZ). Extracts supplied were:

1. HRA- Affinity purified actinidin from kiwi fruit.
2. A1- Recombinant actinidin type 1 (acidic isoform) produced in *E coli* and purified under denaturing conditions.
3. A2- Recombinant actinidin type 2 (basic isoform) produced in *E coli* and purified under denaturing conditions.
4. TER- Recombinant TERSYN control protein produced in *E coli* and purified under denaturing conditions.

All three recombinant proteins had a 6x his tag attached at the N terminus adding approximately 5 kDa to the protein size.

2.3.1.5 Kiwi seed protein extract

A kiwi seed protein extract was made by sieving a puree of kiwi, followed by careful drying of the seeds and gentle rubbing to remove any pulp. The seeds were then washed and ground in 1:2 wt/vol in PPB. Vortexing, centrifuge and dialysis were conducted as for the kiwi fruit extract. The extract was then frozen at -80°C and lyophilised under suction at -60°C over 5 days. The resulting protein powder was frozen at -80°C until required.

2.3.1.6 Extracts used to inhibit IgE binding to kiwi fruit proteins

Birch pollen (NIBSC 84/522), twelve grass pollen (NIBSC 77/616) and house dust mite (NIBSC 82/518) standardized lyophilized extracts were obtained from the National Institute for Biological Standards and Controls (Potters Bar, UK). Lyophilized peanut extract was made in-house by Dr Stella Lewis. Defatted peanut protein was prepared from roasted peanut flour (Golden Peanut Company, GA, USA) based on the methods of De Jong (de Jong, 1998b). The peanut flour was extracted 5 times with 100% hexane (1:1 w:v). Each time, the flour and hexane was stirred for 30 minutes and the hexane removed by vacuum filtration. The defatted flour was then mixed with cold 0.1M ammonium bicarbonate solution and stirred for 4 hours at 4°C. This mixture was then centrifuged at 15,000g for 1 hour, 4°C and the resulting supernatant dialysed

against double distilled water for 24 hours at 4°C with regular water changes. A 3kDa cut off dialysis tubing was used. The dialysate was then lyophilised and the resulting powder frozen at -80°C until use.

2.3.2 Protein quantification.

The DC Bio-Rad Protein Assay was used to quantify the protein content of the allergen extracts. This is a colorimetric assay based upon the Lowry assay. Duplicates of five doubling dilutions in water of bovine serum albumin (BSA Protein Standard, Sigma) containing 0.0625-1 mg/ml, plus a blank, were used to prepare a standard curve.

Duplicate samples of extract were made containing 1 mg protein powder/ml, in water. The DC Protein Assay reagents were added to 20µl of standards and samples. After 15 minutes standards and samples were read at 750nm in a spectrophotometer. A standard curve was created by plotting the known protein content of the standard (x axis) against the mean of the optical density readings (y axis). For the standard curve to be accepted, it was required to have a correlation coefficient ≥ 0.99, and the protein content of the samples had to lie within the analytical range of the standard curve. The mean of the duplicate readings of the samples was used to calculate the protein content of the extract from the standard curve (acceptable coefficient of variation ≤10%).

2.3.3 1D SDS-PAGE

2.3.3.1 Optimisation of method for SDS-PAGE of kiwi fruit extract

Size fractionation of the kiwi proteins was performed under reducing conditions by SDS-PAGE to determine the protein profiles of extracts. Initial electrophoresis was performed using 10% NuPAGE[TM] Bis Tris gels with morpholinoethanesulfonic acid (MES) running buffer. Most of the proteins were found to have molecular weights in the range 20-60 kDa and inspection of the manufacturer's manual showed that better resolution of proteins in this range would be obtained by using 12% NuPAGE[TM] Bis Tris gels with morpholinopropanesulfonic acid (MOPS) running buffer.

Protein loading of gels varying from 5-80μg of protein extract were compared. Higher molecular weight protein bands were not visible if the quantity of extract was <20 μg, and at high quantities of extract the resolution of protein bands was poor. Therefore 20μg of protein per lane was considered optimal.

Unless otherwise stated, extracts were run under reducing conditions. However protein patterns under both reducing and non-reducing conditions were compared. For non-reducing conditions the extracts were treated as described below (2.3.3.2), except no reducing agent was added to the extract, and no antioxidant was added to the inner chamber during electrophoresis. Only minor differences in the apparent protein profiles (and IgE binding on Western blotting) were seen under the two conditions.

2.3.3.2 Standard method for SDS-PAGE of kiwi fruit extract

Kiwi extract was dissolved in an appropriate volume of water to make a solution of 1 mg protein per ml. 130μl of the extract solution (≈ 130μg protein) was refrigerated with 50μl of LDS sample buffer for 10 minutes and then heated for 10 minutes at 70˚C with 20μl of reducing agent (dithiothreitol, DTT). Extract containing 20μg of protein was loaded in each well of a NuPAGE^{TM} 12% Bis Tris Gel (Invitrogen). Reference markers with known molecular weights (SeeBlue plus 2 (Invitrogen) for Western blots; Magic Marker or Mark 12(Invitrogen) for staining), were run on the same gel in X Cell SureLock^{TM} Mini-Cell for 45 minutes at 200V constant using 650ml MOPs running buffer in the outer chamber and 200ml MOPS running buffer with 0.5ml of NuPAGE^{TM} antioxidant (Invitrogen) in the inner chamber.

2.3.3.3 Attribution of apparent molecular weight to protein bands

Molecular weight markers may not always be accurate. To confirm that no systematic error would be caused by the choice of marker, the extract was run with three different standards- SeeBlue plus 2 (Invitrogen), Mark 12 (Invitrogen) and Magic Marker (Invitrogen). The apparent molecular weights of the three standards were in agreement (data not shown).

2.3.4 2D SDS-PAGE

The kiwi protein extract was cleaned using 2-D Clean-Up Kit (Amersham Biosciences), and was resuspended in rehydration buffer (8M urea, 2% CHAPS, 0.5% (v/v) ZOOM® Carrier Ampholytes, 0.002% bromophenol blue). 400 µg of protein extract was loaded onto gels to be used for Mass Spectroscopy (MS) and 200 µg for staining. The extracts were incubated with 7cm pH 3-10 (non-linear) gradient strip (ZOOM® Strip, Invitrogen) for 16 hours at room temperature. Subsequent isoelectric focusing was performed using a ZOOM®IPGRunner^{TM} in a Mini Cell using a step voltage protocol (200V for 20 minutes, 450V for 15 minutes, 750V for 15 minutes, and 2000V for 2 ½ hours). The focused proteins were then further resolved by SDS-PAGE electrophoresis. The strips were incubated for 15 minutes with NuPAGE SDS Sample Buffer, followed by 15 minutes incubation with alkylating solution. 2-D electrophoresis was performed at 200V for 40 minutes using a NuPAGE Novex Bis-Tris ZOOM Gel (4-12%), using SeeBlue Plus2 (Invitrogen) as the molecular weight standard. The gel was stained with EZBlue stain (Sigma).

2.3.5 Staining SDS-PAGE separated proteins

2.3.5.1 Coomassie stain

The standard stain for proteins used throughout the study was EZBlue Gel staining reagent (Sigma). EZBlue is a solution containing Coomassie Blue G-250 and is reported to detect 10 ng of protein.

Following electrophoresis the gel was rinsed three times for 5 minutes with an excess of deionised water to remove the SDS in the gel. The proteins were then fixed using 50% methanol/ 10% acetic acid for 15 minutes. The gel was rinsed again for 10 minutes, before adding 30 ml of Coomassie stain (EZBlue, Sigma). The gel with stain was placed on a gentle shaker for one hour, followed by destaining in deionised water for 2 hours.

2.3.5.2 Silver stain

A silver stain (ProteoSilver kit, Sigma) was used for staining SDS-PAGE gels when EZBlue was found not be sufficiently sensitive. The manufacturer's

protocol for staining SDS-PAGE mini-gels was followed. Silver nitrate binds to selective amino acids on the proteins. The protein bound silver ions are then reduced by formaldehyde at alkaline pH to form metallic silver in the gel. This method is highly sensitive for protein detection, staining 0.2ng of protein.

2.3.6 Scanning and drying gels

Scanning of stained gels was performed prior to, as well as after drying, because cracking of the gel can occur during the drying process. However where possible images of the dried gels were used because the images are better.

Following staining, all gels were scanned (Epson perfection 1660 Photo Scanner) and saved as 400dpi TIF image files on a personal computer.

To dry the gels they were equilibrated in Gel-Dry^{TM} drying solution (Invitrogen) for 15 minutes. Two pre-cut cellophane sheets were soaked in the drying solution for 15 seconds. A cellophane-gel-cellophane sandwich was assembled on an open frame, so that the sandwich was exposed to the air allowing passive evaporation over night. The dried gel was then placed under a heavy weight for 24 hours. The gel was scanned again, and the dried gel stored in a file.

2.3.7 Identification of proteins by MS

Care was taken during the running and handling of gels to minimize keratin and other types of contamination. Protein spots were chosen for analysis because preliminary work to optimize 2D Western blots suggested that they might be allergens (work not presented in this thesis). The protein spots were excised from the 2D-gel and transferred to a 96-well plate with ultra-pure water to prevent dehydration. Mass spectrometry (MS) analysis was performed by Paul Skipp, Centre for Proteomic Research, University of Southampton. Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) MS was used to measure the masses of the peptides derived from the enzymatic digestion (trypsin) of the gel-separated proteins. Peptide mass fingerprinting was used in an attempt to identify proteins, but no positive matches were identified.

Electrospray ionisation (ESI) NanoLC tandem MS was then used to obtain fragmentation data from each peptide and *de novo* sequence obtained. A BLAST search was used to look for homology to any protein in the NCBInr database. Pub Med (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed), PIR (http://pir.georgetown.edu/pirwww/) and The Biotechnology Information for Food Safety Allergen Database (http://www.iit.edu/~sgendel/foodallr.h^{TM}) were searched against to determine if the homologous proteins were known allergens.

2.3.8 Western blotting

2.3.8.1 Development of method for Western blots

The optimum conditions for immunoblotting proved difficult to establish. Loadings of extracts at quantities varying from 10-40 µg per lane were compared under different conditions. A loading quantity of 10 µg per lane was found to be optimal.

To achieve maximum sensitivity with minimal background or non-specific binding, many different blocking agents and blotting conditions were tried (BSA 3% in tris buffered saline (TBS) +/- tween 0.1%, I-Block(Applied Biosystems), 5% skimmed milk). Ultimately, BSA 3% withTBS- tween (TTBS) provided the best blocking with minimal background. Different diluents were also investigated (1% BSA/ 10% goat sera, 1% BSA/1% goat sera, 1% BSA, 0.3% BSA, 0.1% milk, 1% milk). 0.3% BSA in TBS provided the best results.

Non-specific binding bands persisted in Western blots using kiwi extract (*Figure 5.2, lane 2*). Seven different secondary antibodies were studied (*Table2.4*) over a range of concentrations (1:500- 1:4000), and incubation times (30 minutes to 1 hour). Altering the concentration of the secondary antibodies did affect the intensity of binding, but it was not possible to find an antibody concentration at which non-specific binding was eliminated but sensitivity kiwi allergens was maintained.

Table 2-4 *Secondary antibodies used in optimisation studies for Western blots.*

Biotin labelled affinity purified goat antibody to human IgE (KPL)
Monoclonal mouse IgG2b isotope, ALP conjugate (Sigma, A3076)
Monoclonal mouse IgG2a, ALP conjugate (Pharmingen 555859)
Rabbit anti-human IgE specific for epsilon chains (Dako A0094)
Monoclonal mouse IgG2a anti human IgE ('home-made' HB121)
Purified mouse IgG2a antihuman IgE (Pharmingen 555894)
Biotin conjugated mouse anti-human IgE monoclonal antibody (Pharmingen 555858)
Biotinylated goat anti-human IgE (Vector BA3040)

Non-specific bands were present when the blots were incubated in diluent with no sera (0.3% BSA, 0.1% Tween in TBS) excluding non-specific IgG binding as a cause. The non-specific binding was present if milk or I-Block were used as a blocker and diluent, refuting the possibility that contaminating immunoglobulins from the sera derived blocking agent (BSA) was responsible. The bands were absent if no secondary antibody was incubated with the extract. Therefore non-specific bands were not caused by direct activation of the developer by peroxidase or phosphatase activity, or by biotinylated protein in the extract reacting with the extravidin. In the optimisation studies, the concentration of ALP- or HRP-extravidin had little influence on the intensity of the non-specific binding.

The most likely explanation is that the non-specific binding was due to a direct binding of the secondary antibodies to a kiwi protein.

Reviewing published immunoblots, one group reported clear kiwi blots using non-atopic sera under extreme blotting conditions, blocking with 15% fetal calf sera (Aleman, 2004), but 39% of their patients with positive DBPCFC had negative blots. Some researchers have not reported the results of control sera (Pastorello, 1996). Other research teams have produced clear control blots but I was not able to replicate their results (Gavrovic-Jankulovic, 2002b; Moller, 1997b).

For the purposes of this study, patient bands were ignored at sites that also produced non-specific binding by non-atopic control sera (ie. For green kiwi fruit ≈20 and 36 kDa).

2.3.8.2 Standard method for Western blots

10µg of kiwi proteins were separated by 1D SDS-PAGE using the above protocol, with prestained molecular markers (SeeBlue Plus2, Invitrogen). The extracts were transferred onto polyvinylidinedifluoride (PVDF) membranes (0.2µm pore, Invitrogen, UK) at 30V for 1 hour using an XCell II blotting apparatus (Invitrogen, UK). Following transfer the lane of markers was air-dried. The remaining blot was placed in 3% BSA in TTBS for 1 hour, to block non-specific protein binding sites. The blot was then cut into strips, each strip equating to one lane of separated kiwi extract. Sera from individual patients were diluted 1 in 10, in a diluent of 0.3% BSA in TBS, and incubated at room temperature over-night with the strips. Strips were then incubated in 1:500 biotin-labelled goat anti-human IgE (Vector BA 3040) followed by 1:120,000 ALP-linked extravidin (Sigma, UK) each for 45 minutes. Following each incubation, membranes were washed twice with TTBS and twice in TBS. IgE binding sites were identified by developing with BCIP/NBT for 15 minutes.

2.3.8.3 Analysis of IgE binding bands on Western blots

The binding patterns were examined by two methods. First they were looked at visually and the apparent molecular weights of bands estimated and recorded in the SPSS data file. On a separate occasion the bands were analysed by BioRad Discovery One software as follows. The Western blots were scanned (Epson perfection 1660 Photo Scanner) and saved as 400dpi TIF files on a personal computer. Bio-Rad Quantity One software (Bio-Rad, UK) was used to analyse the images. In brief the background for each lane was deleted and the molecular weights of the standards (SeeBlue Plus2, Invitrogen) were defined. The bands on the patient and control strips were then detected by the software and confirmed by visual inspection. Bands present on control lanes were subtracted from the patient blots. Peak intensity (as an semi-quantitative

estimate of IgE binding to a band) and estimated molecular weight were calculated for each patient band and the data transferred to SPSS v.11 (Chicago, Illinois, USA).

However, neither method was perfect. Visual inspection does not give intensity data, and on 9% of blots, bands were missed, especially bands of high or low molecular weight. This method is less accurate at estimating apparent molecular weights of the allergens. Using the Discovery One software, some bands that were clearly visible by eye were not detected using the software, particularly if one band was in close proximity to another band. It was therefore decided to use a combined approach, inspecting visually first, followed by analysis by Discovery One software. The results of the first 8 blots (60 patient strips) were validated by a second researcher (Dr Lewis) using visual inspection and Discovery One software.

Using the first 8 blot studies, the Discovery One software was used to estimate the molecular weights of the IgE binding bands, using SeeBlue Plus2 (Invitrogen) as the standard. For each band that was recognised by sera from different patients, the mean molecular weight of that band on different strips was calculated (coefficients of variation 0.75-6.7%). Using the mean molecular weight from the first 80 strips for each IgE band, the bands were 'named' according to their apparent molecular weight, and these names were used for all future blots. No new bands were identified after the first 8 studies.

The IgE binding bands (kDa) were:

6, 15, 25, 28, 30, 34, 38, 40, 42, 58, 62, 72

2.3.8.4 Validation and repeatability of Western blots

For each blot, sera from the non-atopic pool were incubated with a strip as a negative control, and sera from the KAP as a positive control.

One strip from each blot was repeated on a different occasion. If there was any discrepancy between the two strips, all strips from the two blots were re-run. This only happened once out of 22 studies.

The IgE binding patterns reported in this thesis are different to those reported by groups in central and northern Europe. I have attempted to obtain sera from the European study groups to confirm that the differences are genuine, and not because of differences in methodology, but the sera have not been forthcoming.

2.3.9 Immunoblot inhibition

2.3.9.1 Sera

Pools of sera were made. The patients for sera pools all had kiwi fruit allergy, diagnosed by a convincing history of a IgE mediated reaction to kiwi fruit plus a positive DBPCFC or kiwi specific IgE >0.5 kU_A/L. Patients used to assess cross reactivity to birch pollen (N=5) had a history of rhinoconjunctivitis in the tree pollen season with a positive skin test reaction to birch pollen. Patients used to assess grass pollen cross reactivity (N=11) had a history of seasonal rhinoconjunctivitis in the grass pollen season (patients had not been skin tested with grass pollen). Patients used to assess house dust mite (HDM) cross reactivity (N=10) had a history of asthma or eczema and a positive skin test to HDM. Patients whose sera was used for the peanut inhibition pool had a history of severe reactions to peanuts (N=8).

2.3.9.2 Extracts of inhibitors

Defatted peanut protein was prepared from roasted peanut flour (Golden Peanut Company, GA, USA) in our laboratory (Dr Stella Lewis) using a method based on that of De Jong *et al* (de Jong, 1998a) The peanut flour was extracted 5 times with 100% hexane (1:1 w:v). Each time, the flour and hexane was stirred for 30 minutes and the hexane removed by vacuum filtration. The defatted flour was then mixed with cold 0.1M ammonium bicarbonate solution and stirred for 4 hours at 4°C. This mixture was then centrifuged at 15,000g for 1 hour, 4°C and the resulting supernatant dialysed against double distilled water

for 24 hours at 4°C with regular water changes. A 3kDa cut off dialysis tubing was used. The dialysate was then lyophilised and the resulting powder frozen at -80°C until use.

Birch pollen (NIBSC 84/522), twelve grass pollen (NIBSC 77/616) and house dust mite (NIBSC 82/518) standardized lyophilized extracts were obtained from the National Institute for Biological Standards and Controls UK.

2.3.9.3 Inhibition protocol

10μg of kiwi and other proteins (HDM, birch pollen, mixed grass pollen and peanut) were separated by 1D SDS-PAGE. Blotting and blocking was carried out as previously described (2.3.8.2). IgE binding to kiwi fruit proteins and the inhibitor proteins was confirmed by incubating strips of the separated extracts in the appropriate pool of sera. A pool of sera from 5 non-atopic patients was used as a negative control. Immunodetection of IgE binding was performed as previously described (2.3.8.2).

For inhibition of IgE binding, green kiwi proteins were blotted onto a PVDF membrane, and blocked. 0.1mg of each inhibitor extract was incubated in 1ml of the appropriate diluted sera pool (1:10) for 1 hour. 0.1mg/ml BSA and 100μl of kiwi extract (0.1mg) were incubated with kiwi allergic pool sera as negative and positive controls retrospectively. Strips of the PVDF were then incubated with each of the sera mixtures overnight. Immuno-detection of IgE binding was performed as previously described.

2.3.10   ELISA Inhibition

The protein extracts and sera pool used for immunoblot inhibition (2.3.9.1) were also used for ELISA inhibition studies. A Corning half area 96-well plate was coated with green kiwi extract 100μg/ml in carbonate-bicarbonate buffer (50μl/well), and incubated overnight at 4°C. A second plate was coated with BSA (100μg/ml; 50μl/well) as a background control. Plates were washed with PBS and blocked with BSA 3% in PBS (150μl/well) for 2 hours at room temperature. After washing with PBS, 50μl of patient and control sera diluted

with 1% BSA in T-PBS were added to the wells. For the standard curves, sera were serially diluted (1 in 5; 1 in 10; 1 in 20; 1 in 40; 1 in 80; 1 in 160). Inhibition experiments were conducting by pre-incubation of patient sera (1 in 10) with serial dilutions of the inhibitor in 1%BSA and TPBS (inhibitor concentrations: 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125 mg/ml). The inhibition mixtures (including sera with no inhibitor and sera with kiwi extract 0.1mg/ml as controls) and standard curves were dispensed in the wells (50µl/well) and incubated at room temperature for 2 hours. The plates were then incubated with anti-human IgE (Vector BA3040) 1:500; 50µl/well, followed by HRP-labelled Neutravidin (Pierce, IL, USA) 1:5000; 50µl/well. IgE binding was detected by TMB (BD UK Ltd) 50µl/well, and the reaction stopped after 30 minutes with the addition of 1M sulphuric acid 100µl/well. The absorbance (OD) in each well was measured at 450nm. The absorbance in wells of the control plate coated with BSA were subtracted from the absorbance in the kiwi coated wells to account for non-specific binding. Assays were performed in duplicate. Percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = 100 \times \frac{(OD_{\text{of serum with no inhibitor}} - OD_{\text{of serum with inhibitor}})}{(OD_{\text{of serum with no inhibitor}} - OD_{\text{no sera, no inhibitor}})}$$

2.3.11   Glycoproteins in kiwi fruit

2.3.11.1   Staining for glycoproteins

The kiwi extract was run under reducing conditions (2.3.3.2) with 20µg protein per lane. Horseradish peroxidase (Sigma), reported as having a carbohydrate content of 16%, was used as a positive control with 5 µg in one lane. The gel was stained with a Glycoprotein Detection Kit (Sigma) based on a modified Periodic acid-Schiff method. Following SDS-PAGE, the gel was gently agitated in 200 ml of 50% methanol for 60 minutes to fix the glycoproteins. The gel was then washed in water (2x20 minutes; 200 ml), before oxidizing the glycoproteins with periodic acid (100ml) for 60 minutes. Following another wash stage the gel was stained with 100 ml Schiff's reagent for 2 hours until the bands turned magenta with a light pink background. The gel was placed in sodium

metabisulfite, a reducing agent, for 2 hours, and was finally washed, with four changes of water, over 3 hours.

2.3.11.2   Carbohydrate cross-reactive determinants

Carbohydrate cross-reactive determinants (CCD) were made for inhibition studies, to determine IgE binding to glycoproteins in kiwi fruit assays. CCDs were made using a method based on that described by van Ree *et al* (van Ree, 1999) who made CCDs from grass pollen extract by enzymatic digestion of the proteins. Optimisation studies showed that higher concentrations of proteins, and a longer incubation time were needed to achieve complete protein digestion in this study. 5mg of Proteinase K (Sigma) was dissolved in 1000μl of water, vortexed and 100μl aliquots stored at -20°C. 10mg of kiwi fruit protein extract was added to 2ml of TBS and vortexed. 250μl of proteinase K was added to 1ml of the kiwi solution. 250μl of TBS was added to another 1ml aliquot of kiwi solution as a control. The extract was heated to 40°C for 24 hours, and then heated to 100°C to inactivate the enzyme. The proteinase-K treated extract started with 5mg protein in 1250μl (4mg/ml). To confirm complete digestion of the protein, a SDS-PAGE gel was run under reduced conditions. The extract that would have contained 10μg protein prior to digestion was run in one lane. 10μg of extract incubated with TBS, and 10μg of untreated extract were run as controls. The gel was stained with silver stain to confirm that all protein had been digested.

2.3.11.3   Inhibition blots with CCDs

Kiwi allergic sera was used from patients with birch pollen allergy (SPT birch and history of rhinoconjunctivitis). Kiwi allergic sera from patients with mono-allergy were used as controls. Inhibition immunoblots were conducted as detailed in section 2.3.9. Sera were pre-incubated with 25μl CCD for one hour prior to incubation with separated kiwi extract blotted onto PVDF membrane. Kiwi extract was preincubated with sera as a positive control, and TBS as a negative control.

2.3.12   Digestion of kiwi extract

2.3.12.1   Simulated gastric fluid

For gastric digestion studies, simulated gastric fluid (SGF) was made. A ratio of 10 U of pepsin activity/μg of test protein was used, based on an evaluation of the average activity of pepsin recommended in the US Pharmacopoeia (2000) and used in previous studies (Thomas, 2004). Pepsin A (Sigma) was purchased as lyophilised powder with 2235 U activity/mg solid.

3M HCL was added to 30ml of water until the pH reached 1.5. 61mg of NaCl was then added to 30ml of HCL solution (35 mM NaCl). 28mg of pepsin A was added to 3.5ml of solution. The SGF was heated to 37˚ in a water bath.

2.3.12.2   Digestion of Extract

34mg of lyophilised kiwi extract protein was re-suspended in 1ml cold (4˚C) water and 0.08ml (2.72mg protein) was added to 1.56ml of warmed SGF, vortexed and immediately placed in a 37˚ water bath. Samples of 60μl were removed at 0.5, 2, 5, 10, 20, 30 and 60 minutes after initiation of the incubation. Each 60μl was immediately quenched by addition of 70μl sodium bicarbonate solution (pH 10), 50μl LDS sample buffer (Invitrogen) and 20μl reducing agent (Invitrogen). They were then heated to 70˚C for 10 minutes and then stored at -20˚C.

The zero time point protein digestion samples were prepared by quenching the pepsin in the sodium bicarbonate/ sample buffer solution before adding the protein extract.

2.3.12.3   Kiwi digestion under different pH conditions

To determine whether pH conditions influence digestion of kiwi extract and its immunogenicity, solutions were made as for SGF, but with pH of 2, 3, 5, and 7.5.

2.3.12.4   Controls

Control samples for protein stability (kiwi extract in water at 37˚C, no SGF), pH stability (kiwi extract in SGF without pepsin) and pepsin auto-digestion (pepsin without kiwi protein) were treated in exactly the same way.

β-lactoglobulin (BLG) was used as a control allergen which is resistant to digestion. A 34mg/ml solution was made using lyophilised BLG (Sigma), and was treated identically to the kiwi extract solution.

2.3.12.5   SDS-PAGE and Western blots of digested kiwi fruit

For SDS-PAGE, 10µg kiwi extract from each time point was subjected to SDS-PAGE under reducing conditions. For BLG, a single protein, 5µg was used. Gels were stained with EZblue.

For Western blotting, 10µg of extract incubated with SGF was used for each time point. Control kiwi extracts incubated with water at time point zero and 60 minutes were used. One lane was used to run kiwi extract treated as for the standard SDS-PAGE protocol (ie. with no water bath, no SGF, HCl or quenching.) The blots were incubated with pools of sera- a pool of sera from patients with a history of systemic reactions, and a pool of sera from volunteers whose symptoms were always localised to the oral mucosa. The pools were made using equal amounts of sera from every patient in the study with sufficient sera, who had IgE binding to kiwi extract on Western blotting and whose symptoms could be consistently be classified as systemic or localised oral.

2.4 Data entry and Statistics

The data were doubly entered into SPSS Data Entry and were analysed in SPSS for Windows (SPSS version 10.0, Chicago, USA). Data were analyzed using Pearson's X^2 test and Fisher's exact test where appropriate. Correlations between non-parametric independent variables were sought using Spearman's

Rank correlation. To compare parametric data a 2-tailed t-test was used. A p value of less than 0.05 was considered to be significant.

3 Clinical characteristics of kiwi fruit allergy in the UK

3.1 Introduction

The objective of this questionnaire based study was to describe the characteristics of people in the UK with kiwi fruit allergy.

Prior to this study the only literature concerning the clinical features of the allergy consisted of several case reports from Europe (1.3.1). Together with some *in vitro* studies designed to characterize the allergens in kiwi fruit, the available literature suggested that kiwi fruit allergy was a problem almost exclusively of adults, and that the symptoms were almost invariably mild. This was at odds with the clinical impression from the Paediatric Allergy Clinic in Southampton.

This retrospective survey of people in the UK was therefore designed to establish the features of the allergy. The objectives were to determine whether kiwi fruit allergy is a significant problem in children as well as adults, to compare the features of the allergy in children and adults, and also to compare the characteristics of people who have systemic compared with oral allergy syndrome (OAS) response to kiwi.

3.2 Methods

Patients and parents of children, with self-reported kiwi fruit allergy were invited to complete a questionnaire (Appendix A). Data was analyzed in SPSS for Windows (SPSS version 10.0, Chicago, USA). Pearson's X^2 test and Fisher's exact test where appropriate. A p value of less than 0.05 was considered to be significant.

3.3 Results

3.3.1 Study population

Two hundred and seventy three valid questionnaires were analysed, 189 (69%) of which were from female patients. The age range of cases was 5 months to 86 years (mean 38.8 years, median 40.0 years; SD 21.1 years). The age of patients at the time of their first reaction ranged from 4 months-71 years (mean 31.5 years; median 32.0 years; SD 18.9), 13% reacting below the age of 5 years (*Figure 3.1*).

Respondents reported very little allergy to kiwi fruit in the 1970s, particularly in the now adult population who were children at the time. Allergic reactions were increasingly reported in the 1980s, again predominantly in adults. It was not until the 1990s that kiwi fruit allergy was commonly reported in children and young infants (*Figure 3.2*).

Figure 3.1 Age at time of first reaction, and gender of study patients.
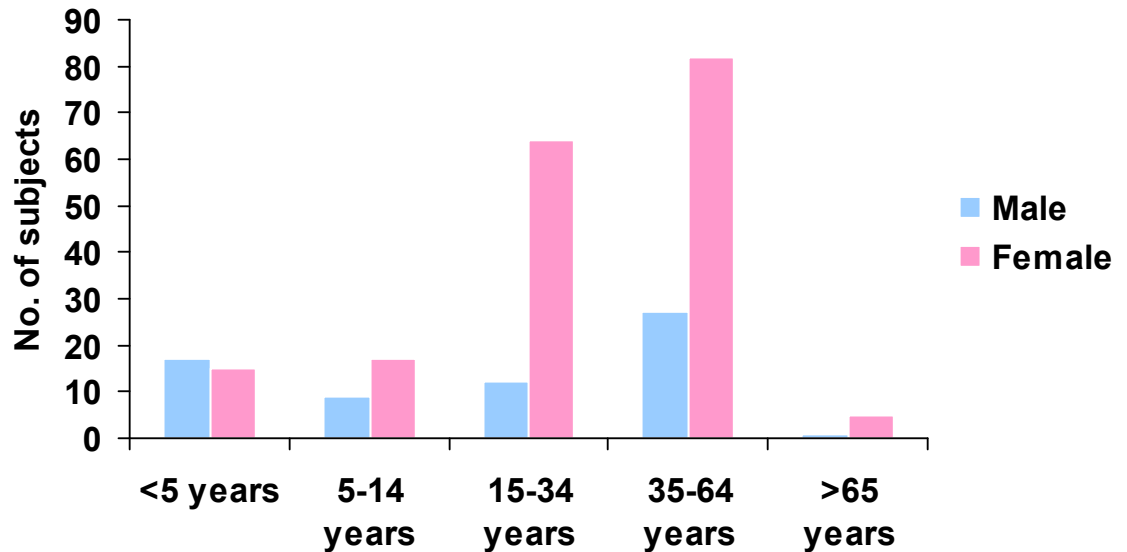
Figure 3.2 The development of allergy to kiwi fruit in adults and children since 1970.

Each point represents one patient plotted in the year of their first reaction, and their age at that time. People that were children at the time of their first reaction are indicated by open circles.
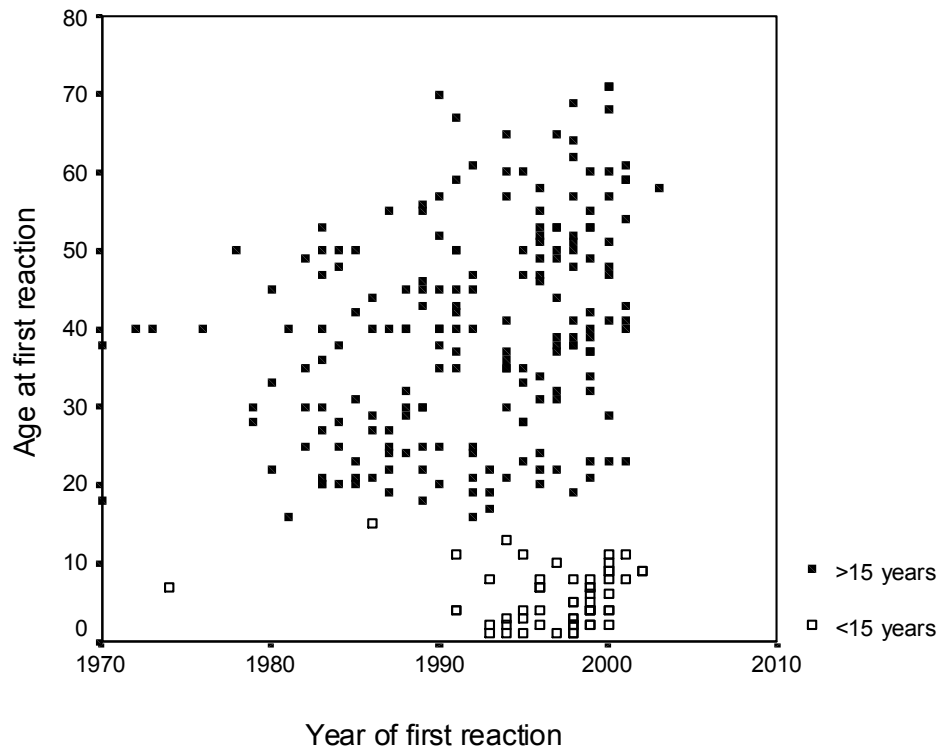
3.3.2 Timing of reactions

73% of children of 5 years or less (22 of 32 children) reacted on their first known exposure to kiwi fruit in comparison to only 21% of patients over the age of 15 years (39 of 189 adults) (Pearson's X^2=33.3, P<0.001).

64% of all patients reported immediate (< 5 minutes) symptoms on contact with the fruit, 90% of all patients had reactions within 30 minutes and only 3% reported a delay of more than an hour. All patients with delayed reactions had mild symptoms.

3.3.3 Associated allergies

Kiwi allergy in this UK population was associated with self-reported latex allergy (9%) and allergies to avocado (5%), banana (6%), apple (6%), grass pollen (29%) and tree pollen (23%). Allergies to allergens not known to cross react with kiwi fruit were also common. Commonly reported co-existing allergies included peanuts (14%), tree nuts (17%), milk (6%) and egg (8%). Children under the age of 5 years were particularly likely to have a strong atopic predisposition. 90% had been treated for asthma, eczema or hay fever. 19 of 32 of these young children (58%) had reported peanut allergy, 15 (45%) tree nut allergy, 5(15%) milk allergy, and 10 (30%) egg allergy.

3.3.4 Allergic symptoms

The symptoms reported on first and most recent reactions are summarised in *Table 3-1*. Oral symptoms such as tingling and localised swelling, were the most commonly described symptoms. The symptoms usually occurred in conjunction with a systemic allergic manifestation, but 81 patients had symptoms that were always confined to the oral cavity and pharynx (OAS). Severe symptoms were reported on the first reaction by 18% of respondents and by 12% of patients on their most recent reaction. The most common severe symptom described was wheeze but patients who had been treated for asthma were not more likely to have a severe reaction (Pearson's X^2=2.00; p=0.40).

Severe symptoms were significantly more likely to occur in young children (<5 years) than adults over 15 years (Pearson's X^2=7.1; p=0.008) (*Figure 3.3*). If patients had severe symptoms on their first reaction, the most recent reaction was also likely to be severe. However, more than 30% of those who initially had a mild reaction subsequently had moderate or severe symptoms (Table 3-2).

3.3.5 Treatment of reactions

15% of respondents had attended hospital on at least one occasion during an acute reaction to kiwi fruit, 3% had been admitted overnight and 3 patients (1%) had been admitted to an intensive care unit. 31% of respondents had used

antihistamines to treat a reaction to kiwi fruit, 7% injected epinephrine, 6% used a bronchodilator and 4% steroids.

Table 3-1 Symptoms reported by patients on their first and most recent reaction.

All 273 reported their symptoms for their first reaction. 206 patients had a subsequent reaction. Actual numbers of patients reporting each symptom are reported; percentages of 276 or 206 are shown respectively in parentheses. Patients could report more than one symptom.

Symptom	First reaction N=273 (%)	Most recent reaction N=206 (%)
Tingling, sore mouth	180 (65)	144 (69)
Throat tightening/ difficulty swallowing	125 (45)	103 (50)
Swelling of lips/ tongue	106 (38)	77 (37)
Face swelling	74 (27)	42 (20)
Rash	60 (22)	31 (15)
Breathing difficulty	49 (18)	33 (16)
Vomiting	49 (18)	35 (17)
Abdominal pain	46 (17)	37 (18)
Wheeze	39 (14)	26 (13)
Collapse	13 (5)	7 (3)
Cyanosis	9 (3)	3 (1)

Figure 3.3 Severity of first reactions reported by patients according to age at the time of first reaction. (Pearson's X^2=7.1; p=0.008).

Reported symptoms were considered mild if they involved a tingling or sore mouth, or a rash; moderately severe if they included abdominal pain, a tight throat, facial swelling or breathing difficulties other than wheeze. Severe symptoms were those involving wheeze, cyanosis or collapse.
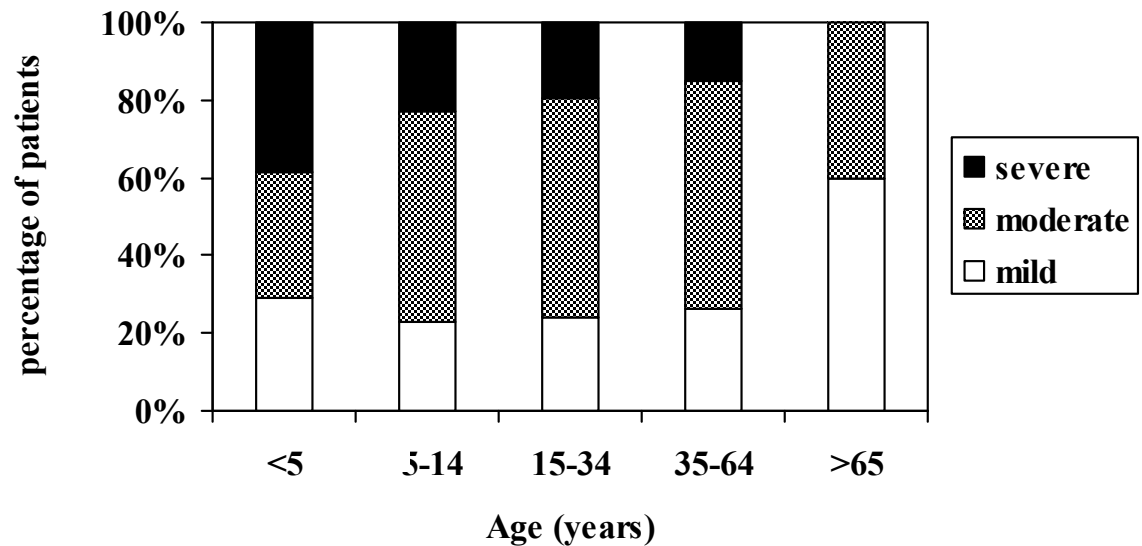
Table 3-2 *Comparison of severity of reported first and most recent reactions.*

Symptoms were considered mild if they involved a tingling or sore mouth, or a rash; moderately severe if they included abdominal pain, a tight throat, facial swelling or breathing difficulties other than wheeze. Severe symptoms were those involving wheeze, cyanosis or collapse.

		First reaction		
		Mild	Moderate	Severe
	Mild	46	7	1
Most recent reaction	Moderate	16	86	5
	Severe	4	5	23

Complete data missing for 13 patients

3.3.6 Comparison of patients with OAS and systemic symptoms

Eighty one patients reported symptoms that were always confined to the oral mucosa, and 165 reported systemic symptoms on at least one occasion. The symptoms reported by the remaining patients (N=27) were difficult to confidently allocate to one of these categories and they were therefore excluded from this part of the analysis.

All 32 of the children who had their first reaction under the age of 5 years reported systemic reactions, whilst 74 of 189 patients over the age of 15 years had OAS (X^2=13.66; p<0.0001).

OAS is said to occur as a cross-reaction to pollen allergens, but only 36% of people with OAS reported symptoms of seasonal rhinitis. 26% of people with OAS thought that they were allergic to tree pollens and 25% to grass pollens. Similarly, 37% of patients with systemic reactions to kiwi reported seasonal rhinitis. 21% thought they were allergic to tree pollens and 30% to grass pollens.

3.4 Discussion

The retrospective questionnaire survey of 273 patients shows that the first people to develop allergy to kiwi fruit were adults, and despite an increasing consumption of the fruit throughout the 1970s and 80s, it was not until the mid 1990s that a large increase in the number of children developing kiwi allergy started to occur. The large number of self selecting respondents to this study suggests that kiwi fruit allergy in the UK may be more common than previously recognized by the medical profession. A recent study from France suggests that kiwi fruit allergy is increasing there, and is perhaps more common amongst French school children than peanut allergy (Rance, 2005). There has been a demonstrable increase in the incidence of food allergy in general (Gupta, 2003), and this has been coincident with an increase in the consumption of kiwi fruit. The combination of these factors probably accounts for the increase in reports of reactions to kiwi fruit. World production figures certainly support a massive

increase in kiwi fruit consumption over the past 20 years. Estimates of world kiwi production of 162,398 tonnes during the three years 1983-6 had increased seven fold to 1,142,316 by 1998-2001 (World Kiwi Fruit Report, Belrose Inc.) Import data suggests that UK consumption is similarly increasing, with imports of 22,193 tonnes in 1996 rising to 31,563 tonnes in 2002 (UKtradeinfo.co.uk, HM Customs and Excise). Increased consumption of food, particularly by young children, has been identified as a possible cause of new allergies in other studies. A recently published study of paediatric patients with sesame seed allergy reported 30% presented with anaphylaxis, and all of them under the age of 1 year (Dalal, 2003). The authors suggested that their findings could be due to both early exposure in life and the heavy consumption of sesame in their population.

Increasing consumption, early life exposure and a general increase in atopic prevalence cannot solely account for the increase in kiwi fruit allergy. Banana is a known allergen (Makinen-Kiljunen, 1994) with a consumer history not dissimilar to kiwi fruit in the UK. It is not a native fruit in the UK and has been available for several more decades than kiwi. It is commonly used as a weaning food. However, anecdotally, an increase in banana allergy has not been noticeable in paediatric clinics.

Some of the studies investigating cross reactivity and kiwi fruit have specifically recruited patients with OAS (Voitenko, 1997). This may have led to misconceptions that kiwi fruit allergy is almost exclusively a 'mild' allergy. Data from this study clearly demonstrates that kiwi fruit allergy can result in severe, life-threatening reactions, particularly in young children. Only 33% of patients had OAS in the absence of any systemic symptoms.

The reasons why children did not start developing kiwi fruit allergy until some 30 years after its introduction into the diet will require further investigation.   It is possible that kiwi fruit has only been consumed by children in the UK in recent years, but there are no data about kiwi fruit consumption in the general population.

Children in this study were reported to react differently to kiwi than the adults in the study. They were more likely to react on their first known exposure to kiwi fruit, whilst adults commonly reported multiple exposures before developing symptoms. Our current understanding of allergic reactions is that prior exposure must occur to allow sensitisation to occur. The issue of routes of sensitisation is important, and hidden routes include topical application (Lack, 2003; Strid, 2005), breast milk (Vadas, 2001) and *in utero* exposure (Jones, 2002). A 4 year old study patient with no history of prior exposure, had a systemic reaction to kiwi fruit when his father who had eaten the fruit kissed him on the top of the head (*Figure 3.4*). The route of sensitisation remains unknown, but the route of exposure that caused the reaction was presumably through intact skin or inhalation of food aerosol.

A Presidential address to the Society for Study of Asthma and Allied conditions in 1942 provided fascinating data concerning absorption of allergens via non-enteral routes (Walzer M, 1942). Passive local cutaneous sensitisation to allergens including cotton seed and peanut was accomplished in non-atopic human and primate subjects using sera from allergic individuals. Rapid absorption of the allergen was confirmed, by reaction of the sensitised site, with allergen from a variety of non-enteral sites including the skin, urinary bladder, nasal mucosa, spinal fluid and conjunctiva. More recently, unpublished data from Poulsen suggests that the buccal mucosa may be a site of absorption (Poulsen, 2005). These data may help explain sensitisation to labile foods in people without pollen sensitivity and also the rapid reactions reported on ingestion of foods by some patients.

Figure 3.4 Systemic reaction following non-enteral exposure to kiwi fruit in 4 year old.

Patient was kissed on the top of his head by father who had been eating kiwi fruit. The 4 year old rapidly became quiet, complained of feeling unwell, developed conjunctivitis and generalised facial oedema. (Photograph provided by mother with permission to use it for medical educational purposes).

Children who reacted to kiwi fruit were likely to be strongly atopic, with 90% reporting atopic disease, and 58% reporting allergy to peanuts. Children were also more likely to have severe reactions. The youngest patient in this study had a severe anaphylactic reaction at 4 months of age, requiring resuscitation with epinephrine and oxygen, having eaten kiwi fruit prepared using a recipe provided by a health professional. The severity of reactions experienced by young children may simply reflect that they represent a population with a strong allergic predisposition, and as such are more likely to have severe reactions. They do not appear to recognize different IgE binding proteins (addressed in chapter 5). Kiwi fruit has only been easily available in the UK for the past 20-30 years, and the adult population must therefore be less likely to have had

exposure to the allergen in early life. Perhaps timing of exposure in relation to immune development is important.

The clinical association of pollinosis with OAS to fresh fruit including kiwi is well-recognised (Ortolani, 1988). However, in this study population, patients with seasonal rhinitis, or reported allergy to pollens, reported similar rates of systemic symptoms to those without pollen related allergy. Indeed, the majority of patients with OAS did not complain of symptoms of allergic rhinitis and did not consider themselves to be pollen allergic. The high negative predictive value (92%) of the question "do you think or know that you have allergy to tree pollens?' for this population would indicate that this finding is true (2.1.3.3). Further more, two patients with OAS who had positive DBPCFCs to kiwi fruit had monoallergy to kiwi. These data challenge our current understanding of the pathophysiology of OAS. Further work is required to determine why some patients react with symptoms localised to the oral mucosa, while others have severe systemic reactions. *In vitro* studies using sera pools from the two groups suggest that the systemic reactors respond digestion stable epitopes (chapter 7). Further analysis, comparing the kiwi allergens that bind IgE from people with OAS and systemic reactions recognise is addressed in chapters 5 and 6. There is no published work comparing different immune responses in the oral mucosa and systemically in patients with OAS and systemic reactions.

This study has limitations. It is not an epidemiological study, and therefore can not provide an estimate of the prevalence of the problem. The patients are self selected, which may explain the greater number of adult females, as well as a fairly high percentage of patients with severe symptoms. As with all questionnaire studies, there is likely to be recall bias. However, this report highlights important features of kiwi fruit allergy that further our clinical progress in the field.

In summary, kiwi allergy is a relatively new allergy in the UK, with most severe reactions occurring in young children. A significant number of patients have required resuscitation with epinephrine, and 3 adult patients had been admitted

to intensive care following ingestion of kiwi fruit. Although kiwi allergy is known to occur as a consequence of cross reactions with pollens most of our patients did not have pollen allergy. This survey brings into question the hypothesis that fruit OAS is always a consequence of cross reactivity with pollen allergens.

4 Clinical investigation of patients with kiwi fruit allergy

4.1 Introduction

The diagnostic value of clinical investigations in the management of kiwi fruit allergy is uncertain with conflicting results from different studies (1.3.3). Skin prick testing using a prick-to-prick technique with fresh kiwi pulp has consistently been reported as highly sensitive, but its specificity in atopic patients varies significantly between studies (Beezhold, 1996; Fine, 1981; Gall, 1994; Garcia, 1989; Monreal, 1996; Shimizu, 1995). The role of measuring specific IgE is also unclear (1.3.3). Although positive in some reports of kiwi fruit allergy (Dore, 1990; Mancuso, 2001; Novembre, 1995; Shimizu, 1995), other authors have found it unhelpful (Gall, 1994; Garcia, 1989; Gastaminza, 1998). Lack of a satisfactory 'gold standard' test to compare skin testing and IgE measurement against remains a problem. Only one study has attempted to conduct double blind placebo controlled food challenges (DBPCFCs) (Aleman, 2004).

The aim of this part of the study was to evaluate the use of skin prick testing (SPT), specific IgE measurement and DBPCFC in the diagnosis of kiwi fruit allergy in the UK population.

4.2 Methods

The methods used for clinical investigations are described in detail in chapter two. In brief, 45 patients were investigated by DBPCFC (plus one by open challenge), prick-to-prick skin testing with fresh kiwi pulp, and specific IgE measurement (2.1.3). 19 of these patients were also skin tested using a commercially available solution (Alyostal, France). In addition SPTs and specific IgE were measured in five atopic and five non-atopic controls that eat kiwi fruit with no adverse symptoms.

Interpreting DBPCFCs in patients with oral allergy syndrome proved difficult.

A pilot study was therefore designed to develop an objective clinical investigation to confirm OAS (2.2.4) and three patients and three healthy controls participated in a buccal challenge.

In addition, SDS-PAGE analysis was used to compare the protein profiles of an extract of fresh kiwi fruit and the commercially available kiwi fruit skin prick test solution (2.2.3).

4.3 Results

4.3.1 Food Challenge

Oral challenges were performed in 46 patients with self-reported allergy to kiwi fruit (45 DBPCFC +/- open challenge, 1 open food challenge). Patients ranged in age from 6 to 64 years (mean 33 years; SD 18 years), and included 12 children under the age of 15 years. 26 (57%) patients had a history of symptoms localized to the oral mucosa, the remaining 20 patients reported systemic symptoms.

30 patients received recipe one, 15 recipe two and 1 child had an open challenge. In total, 24 DBPCFCs were positive, the open challenge was positive, (Table 4-1), 12 were negative and 9 challenges were inconclusive. The inconclusive challenges were in patients with subjective symptoms. They had symptoms that did not strictly correlate with the placebo/ active dose regime, but who had features to suggest their symptoms may have been positive. The child who only had an open challenge developed swelling of the lips and facial erythema after 2.5g of kiwi (≈25mg protein). In addition three patients developed objective clinical signs suggestive of allergy during an open challenge following a negative or inconclusive DBPCFC (Table 4-1). Therefore 24 patients (53%) had kiwi fruit allergy confirmed by DBPCFC, with a further 4 (total- 60%) developing signs suggestive of IgE mediated food allergy during an open challenge. It had been hoped that recipe 2 would eliminate the inconclusive results in patients with OAS, but this was not the case with 2 of 15 patients having inconclusive symptoms with this revised recipe.

Table 4-1 *Clinical manifestations during challenges*

Clinical signs and symptoms	Ages at test (years)
DBPCFC	
Isolated oral symptoms	29, 36 42, 44, 49 53, 53, 55, 57
Facial swelling and oral symptoms	9, 22, 51, 55.
Urticaria +/- angioedema	6, 9, 9, 10
Drop in peak flow, urticaria and facial oedema	9.
Wheeze	42
Urticaria and abdominal pain	9, 10, 26.
Erythema and oral symptoms	15.
Erythema and abdominal pain	46.
Open challenge	
Swelling of the lips and facial erythema	54.
Swelling of tongue (observable)	54.
Urticaria + angioedema	22
Stridor and dysphonia	36

4.3.2 Oral histamine release

4.3.2.1 Oral mast cells

Oral mucosa brushings were taken from non-allergic volunteers to see if mast cells could be retrieved (2.2.4). If successful, the plan had been to incubate oral mast cells from food allergic patients with the index allergen and then measure histamine release.

The cell count from volunteer 1 contained 220,000 cells/ml. The cell count from volunteer 2 contained 315, 000 cells/ml. No cells were mast cells.

Cytocentrifuge preparations stained with Giemsa also revealed no mast cells or any other bone marrow derived cells in the specimens from two volunteer. Therefore, despite good cell counts, no mast cells were obtained. (All work in section 4.3.2.1 was supervised by Dr Mark Buckley).

4.3.2.2 Oral histamine release during buccal challenge

Three patients with OAS and 3 non-allergic control volunteers had a buccal challenge with kiwi fruit. The patients had all had DBPCFC and SPTs on previous occasions that confirmed that their symptoms correlated appropriately with active and placebo doses. The concentration of histamine in saliva prior to the challenge ranged from undetectable to 8.5ng/ml (Figure 4.1). Only one patient had a significant increase in histamine concentration following the challenge, and this patient also developed erythema and urticaria of the oral mucosa. Her peak histamine concentration was 42.3 ng/ml, and the histamine remained elevated throughout the first hour after which she was given antihistamine to alleviate her symptoms . The two allergic patients who did not have an increase in histamine concentration complained of intense oral pruritis, but did not develop objective signs. None of the control volunteers had an increase in histamine concentration.

Figure 4.1 Histamine concentrations in saliva from 3 kiwi allergic patients with OAS (red lines) and 3 non-allergic volunteers.

All had had a buccal challenge during which kiwi fruit was placed in the mouth for 15 minutes. Saliva was collected at 6 time-points (1) prior to kiwi (2) immediately after removal of kiwi (3) 15 minutes (4) 30 minutes (5) 60 minutes (6) 120 minutes after removal of fruit. (There was insufficient saliva from 03a to measure histamine at time point 4.)
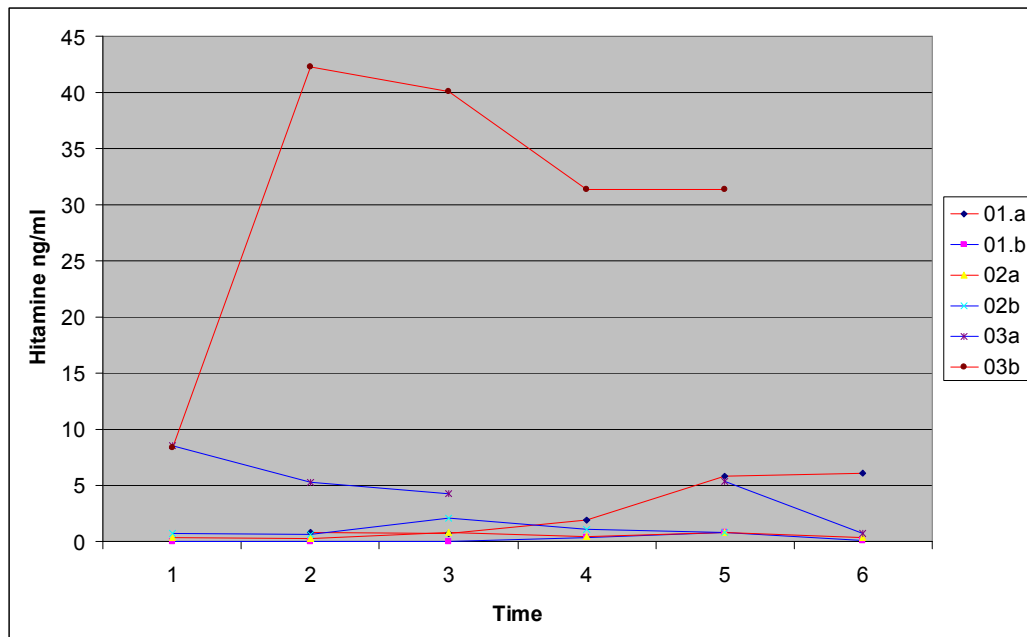
4.3.3 Skin tests

4.3.3.1 Clinical results

Of the 24 patients who reacted during the blinded challenge, only 1 had a negative skin test on prick-to-prick testing with fresh fruit (ie. <3mm). However, there was a high rate of false positive skin test results (Table 4.2). Although people with positive DBPCFCs had a tendency to have larger SPT wheals, there was considerable overlap of wheal size with people who had negative DBPCFCs (Figure 4.2). Using DBPCFC as the gold standard, in this population prick-to-prick skin tests had a sensitivity of 95% and a specificity of 31%. Skin tests were negative in the 5 atopic and 5 non-atopic controls.

Table 4-2 *Skin test results with fresh kiwi fruit pulp in 45 patients and with a commercial extract in 19 patients comparing the results against the 'gold standard' DBPCFC.*

The child who only had an open challenge is not included in the table. She had a positive open challenge and a positive SPT.

DBPCFC result

Fresh kiwi SPT wheal

	Positive	Negative	Inconclusive
<3mm	1	4	1
>=3mm	23	8	8

Commercial kiwi SPT wheal

	Positive	Negative	Inconclusive
<3mm	3	2	2
>=3mm	8	1	3

Figure 4.2 *SPT wheal sizes (mm) using fresh kiwi fruit in 45 patients.*

Patients are divided into those who had positive, negative or inconclusive DBPCFC.
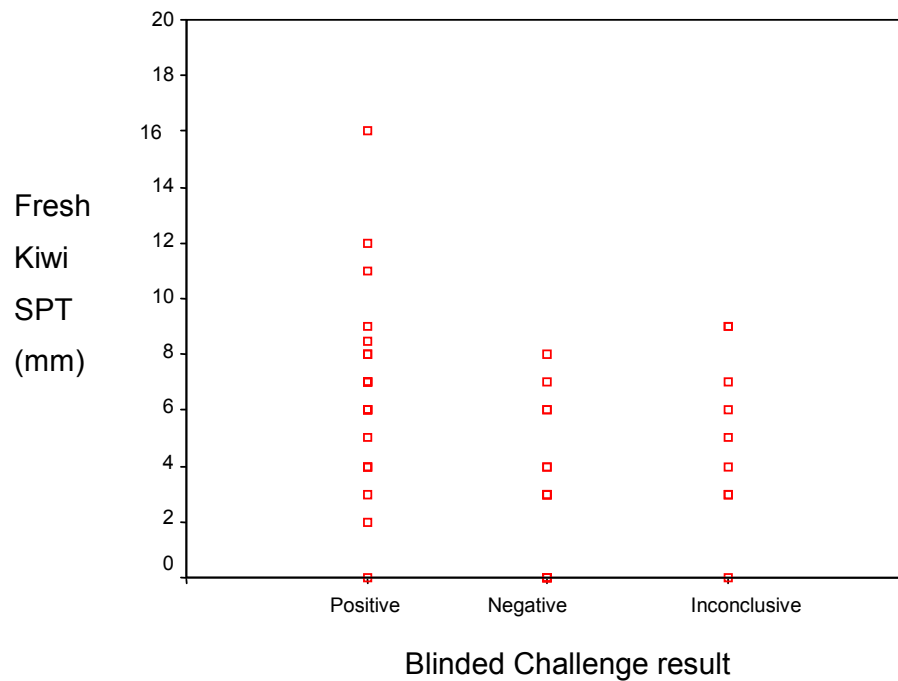
19 patients were also tested with a commercial kiwi skin test extract (*table 5.2*). The commercial solution was significantly less sensitive, but the number of false positive skin test reactions was reduced.

The most common allergens causing positive skin tests in the forty-six patients who under went clinical investigations were birch pollen (14), house dust mite (24), apple (6), banana (4) and avocado (5). In the 24 patients who had a positive DBPCFC the most common sensitisations were birch pollen (7), house dust mite (13), apple (4), banana (4) and avocado (3).

4.3.3.2 SDS-gel of commercial kiwi SPT solution

Protein quantification of the commercial kiwi SPT solution showed that it contained 50mg protein per ml. The protein profiles of the solution, compared with those in an extract of kiwi fruit are shown in

Figure 4.3. The fresh fruit extract had protein bands at ≈14, 15, 17, 22, 25, 36 and 40 kDa. The skin test solution only had one band at ≈23 kDa, even when high protein concentrations were loaded onto the gel, and at a concentration of 20 µg/lane no protein bands were seen.

4.3.4 Serum IgE measurements

The results of Specific IgE in 45 patients who had undergone a DBPCFC challenge are shown in Table 4.3. In this population, the sensitivity of the Sp IgE, using DBPCFC as a gold standard, was 60% and the specificity was 83%. The level of specific IgE did not correlate with reported severity of symptoms or age. One atopic control had measurable kiwi specific IgE. A challenge was not performed because this patient reported regular consumption of kiwi fruit with no problems. None of the other 9 control sera had detectable kiwi sIgE.

Table 4-3 *Specific kiwi IgE results in 45 patients comparing the results against DBPCFC*.

		DBPCFC result		
		Positive	**Negative**	**Inconclusive**
	<0.35	9	10	7
Specific IgE (kU$_A$/l)	**0.4-0.7**	7	1	0
to Kiwi Fruit	**0.7-3.5**	6	1	1
	3.5-17.5	2	0	1

Figure 4.3 *1D-SDS PAGE separation of proteins to compare the protein profiles of an extract of fresh kiwi fruit and Alyostal kiwi fruit skin test solution.*

115mcg, 40 mcg, 20 mcg and 10 mcg of commercial skin test solution protein were loaded per lane, and 40 mcg, 20 mcg, 10 mcg and 5 mcg of kiwi fruit extract protein. Samples were run in 12% NuPAGE gel (Invitrogen) with MES running buffer. Mark 12 unstained standard molecular markers (MW) were run in the same gel. The gel was fixed and stained with Brilliant Blue G Colloidal concentrate.
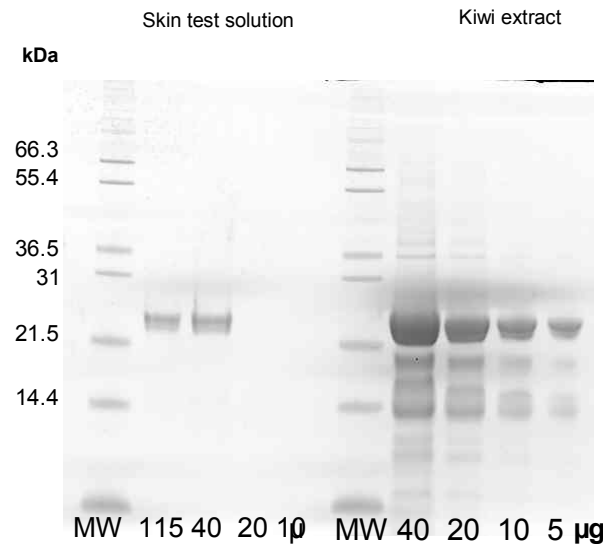
4.4 Discussion

Allergy to kiwi fruit is increasingly reported, but the role of clinical investigations has received little critical evaluation. Skin testing with fresh kiwi fruit is the most common clinical investigation reported in case reports and series (Dore, 1990; Falliers, 1983; Gall, 1994; Novembre, 1995; Veraldi, 1990; Voitenko, 1997) . Previous reports suggest that prick-to-prick with fresh kiwi or skin testing with home made kiwi extract are extremely sensitive in patients in whom kiwi allergy is suspected. This study has also found prick-to-prick testing with fresh kiwi fruit to be very sensitive with only one of 23 patients having a negative skin test but positive challenge (sensitivity 95%). Although highly sensitive, some studies have described poor specificity, with skin test reactivity in patients without symptoms of allergy to kiwi fruit, but clinical reactivity to pollens (Gall, 1994) or latex (Beezhold, 1996; Monreal, 1996). In this study 69% of patients who had a

negative DBPCFC had a positive skin test (specificity 31%). However, given the problems of confirming whether oral challenge was positive or negative in some of our patients (discussed below), the specificity in this study is a worse case scenario. Skin tests were negative in all atopic and non-atopic controls. There has been recent interest to correlate SPT wheal size with the outcome of food challenges in order to generate diagnostic decision points based on the wheal size predicting a positive challenge. Hill *et al* showed that skin wheals of at least 8mm for cow's milk, 7mm for egg and 8mm for peanut were highly predictive ('100%') for a positive food challenge in 467 children (Hill, 2004). Further studies with different foods in a variety of populations are required to confirm these results. Furthermore, for the predictive values to be useful, standardization of extracts and techniques between centres is essential. In this study the problems with interpreting food challenge results made in inappropriate to attempt calculating predictive values.

Commercial skin test extracts were significantly less sensitive. Comparing SDS-PAGE gels of commercial extract and an extract of fresh kiwi fruit, it is evident that most protein bands are absent from the commercial extract, presumably reflecting the lability of kiwi fruit proteins (Figure 4.3). Other groups have reported the superiority of native allergens over commercial extracts when diagnosing food allergy (Rance, 1997).

Reports to date are contradictory about the role of measuring specific IgE to confirm kiwi fruit allergy. Although positive in some case reports of patients with kiwi allergy (Dore, 1990; Mancuso, 2001; Novembre, 1995; Shimizu, 1995), other authors have found sIgE measurement unhelpful (Garcia, 1989; Gastaminza, 1998). In this PhD study the test had poor sensitivity (Sensitivity 60%). It might be that the FEIA-CAP[TM] (fluorescent enzyme immunoassay) system has a similar problem with the lability of allergens as were identified in the skin test solution. The allergen repertoire on the FEIA-CAP is not indicated by the manufacturer.

To increase the specificity of all specific IgE assays, there is currently considerable interest to identify so-called major allergens. The genes of the allergen are then cloned allowing unlimited quantities of recombinant allergen to be manufactured for use in these assays or skin test solutions. However, it will be years until all major allergens are identified, even for the major food allergens. More importantly, as highlighted by this thesis, identification of major allergens may not always be correct (5.3.3 and 9.1.6.3) and validation of results in different populations and study groups is required before resources and time are spent developing clinical investigations which might be useless.

Food allergen-specific IgE concentrations have been correlated with food challenge results to predict the likelihood of a positive reaction on the basis of the specific IgE result. Diagnostic decision points have been described for peanut, egg, milk and fish (Celik-Bilgili, 2005; Perry, 2004; Sampson, 1997b). However the diagnostic decision points vary markedly between study populations. As with SPTs, for this study calculation of diagnostic decision points for specific IgE was not attempted because of the lack of reliability of the challenge data.

Double blind food challenges (DBPCFC) are the 'gold standard' for confirming food allergy (Atkins, 1985), but they are time consuming, expensive and include the risk of severe reactions. Also, they are conducted under artificial conditions and the results do not necessarily reflect the outcome of an exposure in the community. For example, patients are required to be well on the day of the food challenge, they do not drink alcohol and do not exercise.

Blinded food challenges have rarely been used in the context of oral allergy syndrome. The recipes for this study were designed to identically challenge all patients with reported allergy to kiwi fruit, both patients with oral allergy syndrome, and those with more generalised reactions. Some patients with OAS complained of severe oral symptoms but these did not correlate with the dosing schedule. These patients may not be allergic to kiwi fruit, or alternatively, the DBPCFC protocol may not have been efficient at detecting all cases of OAS.

For example some patients had symptoms that appeared to be delayed so that they did not coincide with the doses. The patients were insistent that they experienced symptoms similar to their "usual" symptoms both during the closed and open challenge. These patients were labelled negative or inconclusive in the absence of objective signs. Despite changing the protocol the results continued to be inconclusive, particularly in patients with OAS. This highlights a need to tailor challenges to the individual. Many of the people with systemic reactions, particularly young children, reacted at low doses. However, in order to maximise the safety of the test for the systemic reactors, patients with OAS also started at low doses with relatively small increments. Retrospectively, this group would have been characterised better using even larger increments with longer (e.g. an hour) time intervals between doses.

As highlighted, particularly in OAS, the outcome of DBPCFC can be extremely difficult to interpret, since patients complain of subjective symptoms. More importantly, DBPCFC can provoke life-threatening reactions and needs to be conducted in a specialist centre. The development of a test to assist the interpretation of DBPCFC results, or even to eliminate the need for food challenges would greatly improve the care of food allergic patients.

The immunological mechanisms of OAS have not been studied in great depth, but are presumed to be due to allergic antibody–mediated release of histamine and other mediators of allergic inflammation by mast cells, after exposure to allergenic food proteins. Several studies have found increased levels of tryptase and histamine in tears and nasal fluid after exposure to inhalant allergens in sensitised patients (Ahluwalia, 2001; Bacon, 2000), supporting the role of mast cells in the immediate phase of the allergic response. Only one study to date has measured a mediator of allergic inflammation in saliva after food challenge in patients suffering from OAS, but the increase of tryptase occurred in only one of their nine patients (Vila, 2001). Therefore, a protocol was devised to investigate the release of histamine in the oral cavity in response to food allergens. It was hoped that mast cells could easily be recovered from the oral mucosa using a cytology brush. The cells would then have been incubated with

the food allergen and mediator release from the cells measured. However simply using a cytology brush did not obtain mast cells. Punch biopsies from the oral mucosa would be likely to obtain mast cells, but this method would probably not be acceptable to patients. It was therefore decided to proceed by measuring histamine concentrations in saliva of patients following a food challenge. Three patients and three controls have been challenged to date. The only one to have a rise in histamine concentration relative to their baseline level was a patient who also developed urticaria and oedema of her oral mucosa. The other patients had intense pruritis but no visible manifestations of allergy. The number of patients tested is small, but the data suggest that this method may not be helpful for interpreting subjective symptoms during a challenge. The data challenges our current understanding of OAS, as mast cell degranulation may not be the cause of pruritis. Alternatively the data may reflect the lack of sensitivity of the histamine assay or the lability of histamine. The study need to be continued in more patients, looking at different mediators or other endpoints.

In summary, the investigations used in the diagnosis of food allergy remain inadequate. Since strict avoidance of the responsible allergen is the mainstay of management, accurate identification of the responsible food is essential for patient care. This study highlights the need for better standardization of skin test extracts, improved sensitivity of specific IgE measurement and improved protocols for food challenges.

5 Identification of the major and minor kiwi fruit allergens in the UK population

5.1 Introduction

Several studies have reported the major and minor allergens to kiwi fruit identified in their European populations. The first major allergen to be reported was actinidin (Act c1) (1.3.5). This is the first study to investigate the allergens in the UK population.

5.2 Methods

Kiwi proteins were separated by 1D- and 2D-SDS PAGE analysis and stained with Coomassie (2.3.3 & 2.3.4). Five kiwi fruit proteins were *de novo* sequenced (2.3.7).

To identify the allergens in kiwi extract, immunoblots were made with sera from 76 patients with a history of kiwi fruit allergy (2.3.8), 41 of whom had under gone DBPCFC.

igE binding to purified native actinidin (2.3.1.4) was studied by Western blots in 30 patients, 16 of whom had reacted positively to a DBPCFC. These patients were chosen because they had sufficient sera and had binding to bands in the kiwi fruit extract. Five patients' sera were also used for Western blotting to acid and basic isoforms of recombinant actinidin. Inhibition of IgE binding to kiwi fruit by purified actinidin was investigated by inhibition immunoblots and inhibition ELISAs (2.3.9 & 2.3.10) using a pool of kiwi allergic sera.

The blot images were analysed using Bio-Rad Quantity One software (Bio-Rad, UK) (2.3.8.3) to estimate molecular weight and peak intensity of each band. Peak intensity was used as an estimate of semi-quantitative IgE binding. Associations between the presence or absence of a band, with the following independent variables were sought using Chi Squared tests in SPSS v 11 (Chicago, Illinois, USA): severity of symptoms, age of patients, outcome of DBPCFC and associated allergies. Correlations between these independent

variables and the maximum band intensity recorded for a patient blot, or the sum of band intensities for an individual blot, were sought using Spearman's Rank.

5.3 Results

5.3.1 Kiwi Fruit proteins

1D SDS-PAGE separation of the kiwi extract resulted in detection of protein bands of ≈14, 15, 17, 22, 25 and 36 kDa (*Figure 5.1, B*). 2D SDS-PAGE resulted in the detection of 15 protein spots (*Figure 5.1, A*), five of which were *de novo* sequenced.

Figure 5.1 *SDS-PAGE of kiwi fruit proteins (Coomassie stain) A) 1D and B) 2D*

A)

kDa

38

28

17

14

6

Spot 1:

A BLAST search against thirteen tryptic peptides confirmed that this protein was actinidin (Act c1), a cysteine protease which is the major protein in kiwi fruit. The pI and molecular weight of the protein would also support its identification as actinidin. The tryptic peptide sequences covered 12%, of the actinidin molecule.

The tryptic peptide sequences showed homology with other cysteine proteases that have been reported as allergens. More than 8 continuous amino acids from the tryptic peptides identified with:

- Papaya proteins (P05994) (Baur, 1982; Freye, 1988; Mansfield, 1983; Mansfield, 1985; Niinimaki, 1993; Novey, 1979; Soto-Mera, 2000; Tarlo, 1978; Vandenplas, 1996)
- Papain precursor (P00784) (Baur, 1982; Freye, 1988; Mansfield, 1983; Mansfield, 1985; Niinimaki, 1993; Novey, 1979; Soto-Mera, 2000; Tarlo, 1978; Vandenplas, 1996)
- Bromelain (P14518) (Baur, 1979; Gailhofer, 1988; Nettis, 2001)

Protein Spot 2

- Has sequence homology of 9 consecutive amino acids with thaumatin-like protein (P27357- e-value 0.046), and with other pathogenesis related proteins (P32938, e-value 0.04; P32937, e-value 0.04; P50698, e-value 0.04). These are not listed as allergens on PIR or NBIC, but thaumatin-like proteins (Gavrovic-Jankulovic, 2002b; Krebitz, 2003) and other pathogenesis related proteins (Ebner, 2001; Hoffmann-Sommergruber, 2002) are common food allergens.

Protein Spot 3

- Has sequence homology of 9 consecutive amino acids with an osmotin – like protein and 9 with soya bean P21 protein but these are not recorded as known allergens. Soya bean P21 protein belongs to the thaumatin family some of which are known to be allergenic.

Protein Spot 4

- Has homology with no known allergens.

Protein Spot 5

- Has sequence homology of 7 consecutive amino acids with an allergen from Aspergillus fumigatus (A114-ASPFU allergen Asp F 4).

5.3.2   IgE binding patterns to kiwi protein extract

Despite numerous optimisation studies using a variety of secondary antibodies and blocking conditions it was not possible to eliminate non-specific bands at ≈ 20 and 36kDa. These bands were present when the protein strips were incubated with sera from non-atopic volunteers or without sera (2.3.8.1). Corresponding bands in kiwi allergic patients were therefore not considered significant.

Sera from 2 of 10 atopic volunteers had IgE binding to a 42 kDa protein. Eight atopic controls had binding similar to the non-atopic volunteers (data not shown).

Patient sera identified 12 different binding bands between with molecular weights of 6, 15, 25, 28, 30, 34, 38, 40, 42, 58, 62, 72 kDa. Representative blots are shown in *Figure 5.2*. Because of its high abundance in the extract, actinidin can be seen on the blot as a pale 'negative-band' at 25kDa. There was no IgE binding to actinidin by any patient sera. A 25 kDa IgE binding protein ran just below actinidin (*Figure 5.2*, lane 4) but was not itself actinidin as binding was not inhibited by pre-incubating sera with actinidin.

The 38 kDa band was bound by 69% of patients and it is therefore identified as a major allergen in this population. All other allergens were recognised by <50% of the patients and are therefore not major allergens in this population. A summary of the percentage of 76 patients with IgE binding to each band is shown *Figure 5.3*.

Of the 41 patients who had a DBPCFC with kiwi fruit, 16 of 21 positive reactors had one or more IgE binding bands on their Western blot. Three of the eleven patients who had a negative DBPCFC had binding bands, and 4 of nine patients who had an equivocal challenge. Of 37 patients who were not challenged, 26 had at least one band on their Western blot. There were no differences in binding patterns between people with positive challenges and those who had not had a challenge.

Figure 5.2 Western blots with pooled and individual sera showing examples of IgE binding to green kiwi fruit protein extracts.

Lane 1= pool of kiwi allergic sera; lane 2= pool of non-allergic sera with non-specific binding at approximately 20 and 36 kDa; 3= sera from a patient with binding to 38 and 30 kDa allergens; lane 4=sera from a patient with binding to 25, 30, 38, 40, 42, 62, 72 kDa; lane 5=sera with IgE binding to 38 kDa.
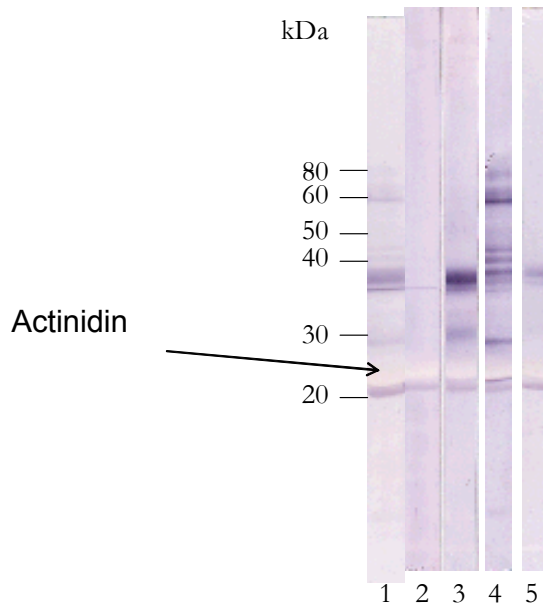
Figure 5.3 IgE binding bands to kiwi fruit by Western blotting using sera from 76 kiwi allergic patients.

The percentage of patients whose IgE binds to allergens of each molecular weight is shown.
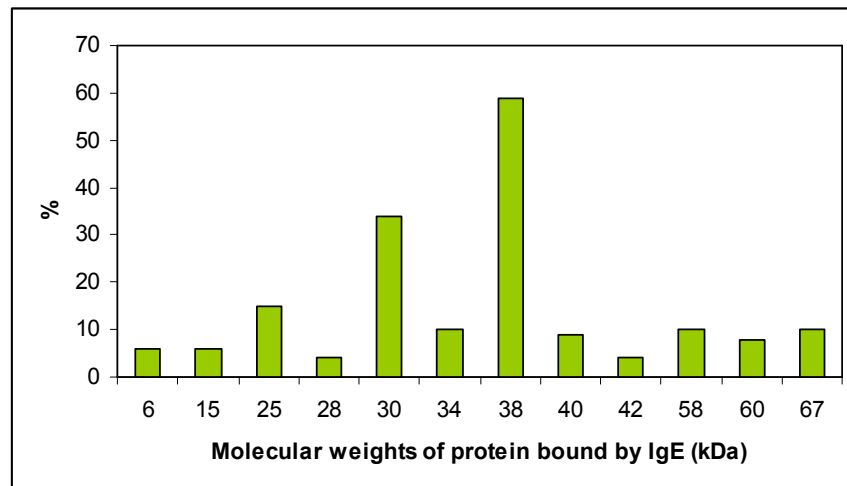
5.3.2.1 Associations

The total number of protein bands that an individual's sera bound to was not related to their age at time of first reaction (p=0.1) (*Figure 5.4)*, result of DBPCFC (p=0.57) (*Figure 5.4)*, severity of their reported symptoms (p=0.15) (*Figure 5.5*), or whether they had OAS or systemic reactions (p=0.63). There was no association between the presence or absence of any particular IgE-binding band and the aforementioned independent variables. The maximum band intensity recorded for a patient blot and the total intensities for all bands in an individual's blot also failed to correlate with these factors.

Figure 5.4 Scatter plot of age of 41 food- challenged patients at time of their first reaction to kiwi fruit and their number of IgE binding proteins on Western blot.

Cases are labelled by DBPCFC result.
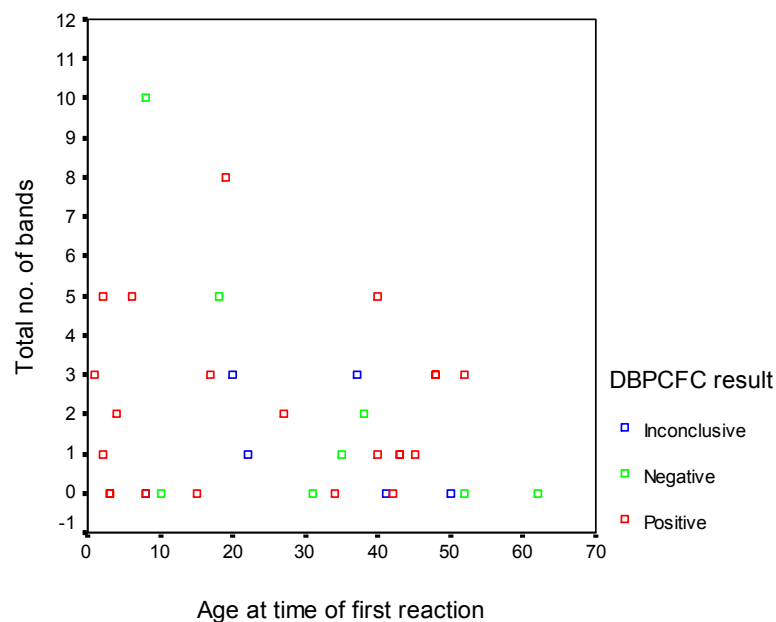
Figure 5.5 Scatter plot of age of 72 food- challenged patients at time of their first reaction to kiwi fruit and their number of IgE binding proteins on Western blot.

Cases are labelled by severity (incomplete data concerning severity was available for 4 patients who were excluded from the plot).
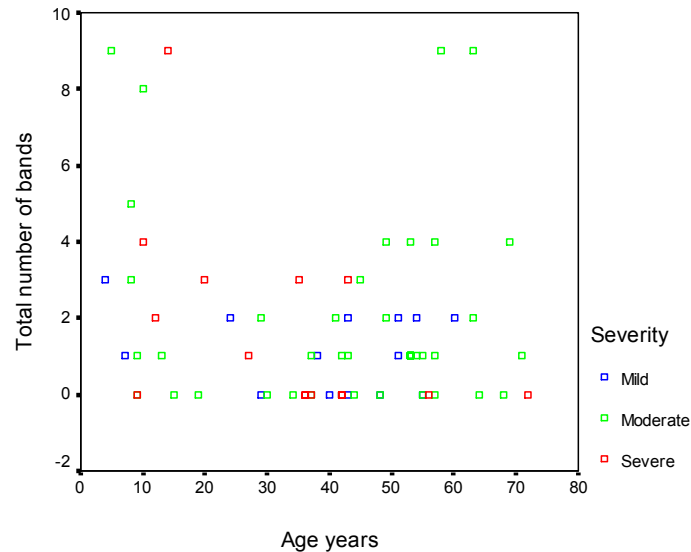
5.3.3 Actinidin

Because of its high abundance in the extract, actinidin is seen on Western blots of kiwi protein extract as a pale 'negative-band' at 25 kDa. No patient IgE (n=76) bound actinidin in kiwi fruit extract Western blots (*Figure 5.2*). A pool of kiwi allergic sera, and individual sera from 30 patients, showed no IgE binding to purified native actinidin on Western blots (*Figure 5.6*). There was no IgE binding by pooled allergic sera (N=9) or individual sera from 6 patients to recombinant actinidin (acidic and basic forms). There was no inhibition of IgE binding to any bands in the kiwi protein extract immunoblots when the pooled kiwi sera were pre-incubated with purified native actinidin (*Figure 5.7*), implying that none of the binding proteins were homologous with actinidin. Furthermore, using pooled allergic sera in an ELISA, there was no IgE binding to actinidin, but sera-dose dependant binding to kiwi extract (*Figure 5.8*). IgE binding to kiwi fruit extract was strongly inhibited by pre-incubating the sera with kiwi fruit (ie. positive control). There was no inhibition relative to negative control by pre-incubating the sera with actinidin (*Figure 5.9*), confirming no IgE binding to actinidin or homologous proteins.

Figure 5.6 IgE Western blots of native purified actinidin using sera from 9 patients with kiwi fruit allergy. Blots from a further 21 patients showed no binding to actinidin.

Lanes 1-9: Sera from individuals with a history of kiwi fruit allergy and binding on Western blots to kiwi protein extract. N&K: kiwi protein extract with non-atopic and kiwi allergic pools of sera.
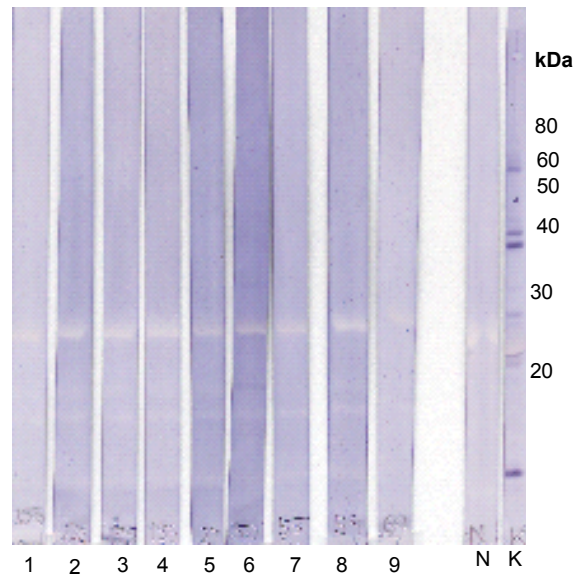
Figure 5.7 Inhibition immunoblot of IgE binding to purified actinidin and kiwi protein extract by pooled allergic sera, inhibited by purified actinidin.

Lanes 1: Binding to kiwi protein extract, no inhibition; lane 2: Binding to kiwi protein extract inhibited by preincubation of the pool with actinidin; lane 3: binding to actinidin; lane 4: binding to actinidin inhibited by preincubation with actinidin.
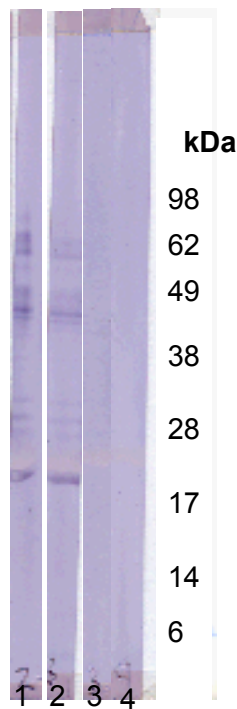
Figure 5.8 ELISA of kiwi-allergic sera IgE binding to purified actinidin and kiwi fruit.

The ELISA plate wells were coated with 50mcl of 100mcg/ml actinidin protein or kiwi extract protein. Six dilutions of pooled allergic sera were incubated in each well overnight, the strongest dilution being 1:5 (sera:diluent).
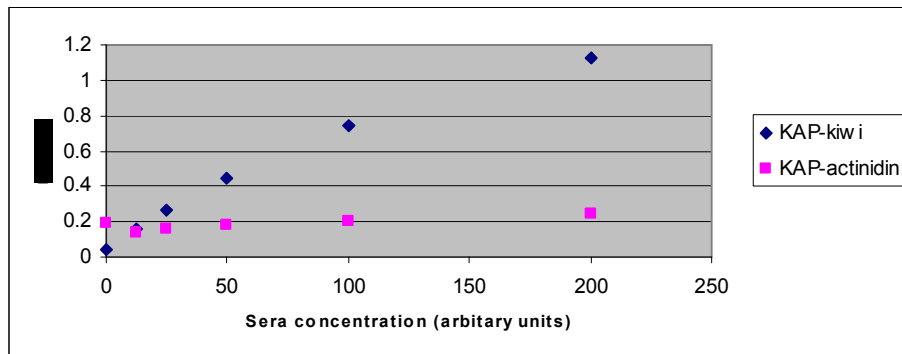
Figure 5.9 ELISA of IgE binding to kiwi fruit extract inhibited by pre-incubating the pooled kiwi allergic sera (1:10) with kiwi extract (100mcg/ml) or actinidin (50mcg/ml).
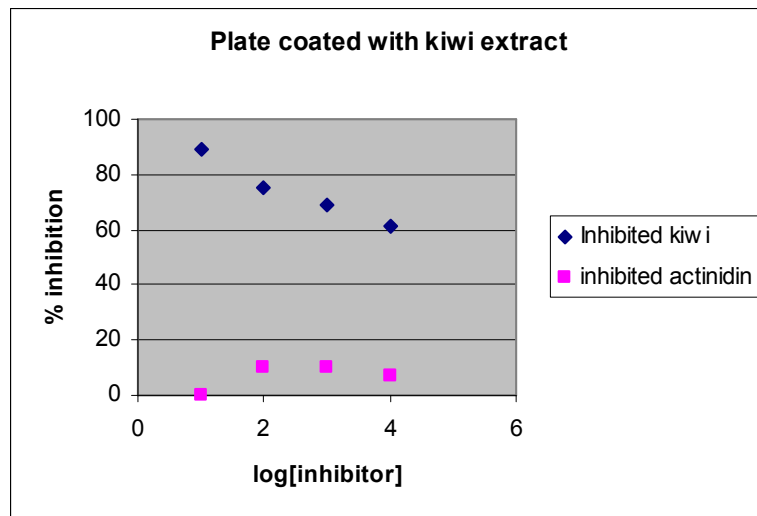
5.4 Discussion

The 1D-SDS gel of kiwi proteins is in keeping with published data (Bublin, 2004). The molecular weight of mature Act c 1 without signal peptide, N and C terminal extensions should be about 24 kDa on SDS PAGE. In some instances, for example in studies by Pastorello, the weight on SDS PAGE has been reported as being 30 kDa, (Pastorello, 1996; Pastorello, 1998). The reasons for this are not clear but are presumably due to slight differences in methodology eg. the degree of reducing conditions. The digestion of some proteins within the extract by proteases may account for the absence of IgE binding to some Western blots using sera from patients who had reacted during the DBPCFC. This is likely to be a particular problem in kiwi fruit extracts as actinidin is itself an active cysteine protease. Also, some allergens are very labile and are easily denatured by extraction and storage procedures (Rudeschko, 1995). Great care was taken to ensure minimal degradation of proteins by working at 4˚C. Studies were performed to compare extracts made with and without protease inhibitors, but they made no discernable differences to the gels or Western blots and were not used for the definitive extract. All extracts were lyophilized and stored frozen to provide the optimal conditions for allergen preservation (Rudeschko, 1995).

There are no published 2D gels of kiwi fruit proteins, but images of the gels were reviewed by plant geneticists from HortResearch NZ, and confirmed to be of a good standard and compatible with their 2D gels of kiwi fruit. The predominant protein in the protein extract was confirmed to be actinidin by MALDI-TOF *de novo* sequencing. The fact that actinidin is so abundant in kiwi fruit makes using the extract difficult to analyse because at normal loading concentrations for 1D and 2D gels, all other proteins are in sparse amounts. *De novo* sequencing and BLAST searches of three other proteins in the extract confirm that they have sequence alignment with potential allergens, which would label these proteins as potential allergens using the WHO/FAO criteria (Joint FAO/ WHO Consultation, 2001).

The results of Western blotting suggest that a protein with a molecular weight ≈38 kDa is a major allergen in this population. No other studies have reported a

38 kDa protein to be a major allergen (Table 1.1). The 38 kDa protein needs to be identified. This has proved difficult because no 38 kDa protein band was visualised in SDS-PAGE so elution could not be performed. The very low protein content of most allergens relative to the total protein content in kiwi fruit (predominantly actinidin) has hampered allergen identification by other groups (Bublin, 2004; Moller, 1997a). If the protein had shown cross reaction with proteins from other plants, some clues to its identity might have been gained, but binding to the 38 kDa protein was not inhibited by pre-incubating the sera with grass pollen, birch pollen or peanut. Removal of actinidin by immunoprecipitation with anti-actinidin antibodies would provide a workable sample to identify other proteins, and this approach is currently being pursued.

Most (Aleman, 2004; Fahlbusch, 1998; Gavrovic-Jankulovic, 2002b; Pastorello, 1998) but not all previous studies (Moller, 1997b; Voitenko, 1997) have reported actinidin to be the major allergen. In this PhD study, no patient IgE bound to actinidin in the kiwi extract, or to purified native or recombinant forms of actinidin. Using Western blots, an Italian study reported that 100% of their patients had IgE binding to a 30 kDa protein (Pastorello, 1996), 100% in Yugoslavia (Gavrovic-Jankulovic, 2002b), 54% in Spain (Aleman, 2004) and German studies have varied between 89% (Fahlbusch, 1998) and 13% (Moller, 1997b). Most of these studies have not confirmed the identity of the 30 kDa protein, but have assumed it to be actinidin. Many proteins in kiwi fruit, including some potential allergens have molecular weights similar to the abundant actinidin *(Figure 5.1, B)*, which may result in misidentification. In this study there was binding to a 30 kDa protein but it was not actinidin. The abundant actinidin band was clearly visible at a slightly lower molecular weight, and significantly, binding to the 30 kDa band was not inhibited by pre-incubating the sera with purified actinidin.

Pastorello *et al* proceeded to identify their 30 kDa protein as actinidin (Pastorello, 1998) by purifying the protein by high performance liquid chromatography and identifying it by isoelectric focusing and amino acid sequencing. A pool of sera from 30 kiwi allergic patients was used to confirm

IgE binding to actinidin by Western blotting (Pastorello, 1998). Because a pool of allergic sera was used, it is impossible to confirm how many of the patients reacted to actinidin. Their published blots show non-specific binding to actinidin, particularly by atopic controls. Perhaps their blocking conditions were inadequate, as they only used PBS with 0.5% tween. However, the intensity of the band produced by IgE binding of the allergic pool is significantly stronger than those by individual non-kiwi allergic controls, implying that some of the binding may have been specific.

A study comparing kiwi allergic patients from Italy, Austria and the Netherlands used ELISAs to investigate IgE binding to purified actinidin (Bublin, 2004). 28 of 31 (90%) Austrian patients showed IgE reactivity to green kiwi protein extracts and 27 (87%) recognised actinidin. Similar results were reported for the sera from the Netherlands. In contrast 29 (85%) of 34 Italian sera contained kiwi specific IgE, but only 47% reacted to actinidin. Additionally the study had a group of 14 Italians with positive skin tests to kiwi fruit but without symptoms when eating the fruit. 93% of this group had IgE reactivity to kiwi protein extract, and 47% to actinidin. Thus their results in sensitised patients with and without kiwi fruit allergy are the same indicating that that their assays were not specific and interpretation of the results require caution.

Because the results of this UK study were so different from previously published works, particular care was taken to confirm the finding that IgE did not bind to actinidin in kiwi protein extract. To establish that lack of binding was not due to a problem with the extraction process, IgE binding by pooled sera to 4 different batches of kiwi extract were compared, but none showed binding to actinidin. An extract was then obtained from a HortResearch which uses different extraction techniques, but again no IgE binding occurred when using pooled sera.

HortResearch also supplied purified native actinidin, and basic (pI 9.30) and acidic (pI 4.06) isoforms of recombinant actinidin. No sera showed IgE binding on Western blots to the native actinidin, or recombinant isoforms. The absence

of inhibition of binding to any band on the blot, when the kiwi allergic pooled sera was pre-incubated with actinidin, excludes misidentification of the actinidin band.

ELISAs using kiwi protein extract and purified actinidin were studied, providing more native conditions. Again IgE binding did not occur to actinidin, and binding to kiwi protein could not be inhibited by pre-incubating the sera with actinidin.

Differences in recognition of major allergens between populations of kiwi allergic patients from different countries may be due to genetic, dietary or other environmental differences, or to variations in the kiwi fruits that are consumed. Previously reported populations with kiwi fruit allergy have demonstrated significant cross-reactivity between pollen and kiwi proteins, which was not the case with this UK population (chapter 6). Therefore, perhaps the difference in allergen recognition is because the UK patients predominantly had a primary sensitisation to kiwi fruit, whilst the European population became sensitised as a cross reactivity to pollens. Different routes of sensitisation might result in sensitisation to different proteins. The reasons for differences in the way the British have become sensitised to the fruit is more difficult to explain. There is a large overlap of pollen exposure between the UK and Northern and Central Europe. Perhaps dietary habits are responsible. There are large variations in consumption of kiwi fruit, for example Spain consumes twice as much per capita as Germany and France, and four times that of the UK (O'Rourke, 2004).

Discrepancies in allergen recognition by different populations have been recognised in peanut allergy. Ara h 1 was recognised by over 95% of patients in a study from North America (Burks, 1991), but the same allergen was recognised by only 35% (de Jong, 1998b), 65% (Kleber-Janke, 1999), or 70% (Clarke, 1998) of patients in three European studies. A study was conducted to see if these serological differences could be explained by differences in allergen composition in peanuts grown in different parts of the world, but it was found that peanuts of different varieties and from different parts of the world contain

similar quantities of proteins including Ara h1 and Ara h 2 (Koppelman, 2001). Kiwi fruit is grown in many countries. SDS-PAGE gels and Western blots using protein extracts of kiwi fruits grown in Italy, New Zealand and the UK showed no significant differences in proteins (*Figure 2.2*) or IgE binding by pooled allergic sera (data not shown).

Regionally distinct IgE recognition patterns have already been reported in kiwi fruit allergy. A study comparing patients with symptoms of kiwi allergy from Austria, Italy and the Netherlands reported immunoblots using selective sera (Bublin, 2004). The figure in their publication showing immunoblots from 9 Austrian and 11 Italian patients does not agree with the reporting in their text. The bands are spread making it difficult to define them as 22 and 27 kDa as reported by the researchers. The authors report positive binding by patient sera to bands that are no more intense than those of the buffer control. Importantly the buffer control lanes on the Austrian blot and the Italian blot show different results, demonstrating lack of reproducibility, but also invalidating any comparison between the blots. It is therefore difficult to confirm from this study that the differences between the two countries are genuine.

The finding that actinidin is not a major allergen is supported by the fact that only one of our patients reported allergy to pineapple and no patient reported reactions to papaya. Pineapple and papaya contain bromelain and papain respectively, which are also cysteine proteases, with similar modes of action. Although amino acid composition, isoelectric points and molecular weights differ, there are many structural similarities, as demonstrated for actinidin and papain in which polypeptide backbones are extremely similar (Kamphuis, 1985). In the study from 3 European countries only 2 of the 90 patients had pineapple allergy (Bublin, 2004). Furthermore, for the majority of food allergens, the major allergenic proteins constitute a relatively small proportion of the total protein content of the native food. Thus actinidin is not fitting in with the usual pattern of major allergens which again casts doubt on its relevance.

Attempts have been made to exchange sera with the European groups who have reported actinidin as an allergen, but this has not been possible.

In summary, none of the patients in this *in vitro* study have IgE to actinidin which has previously been described as the major allergen. A 38 kDa protein is the major allergen recognised by this study, and on going studies will identify it. Importantly a review of the literature surrounding this study demonstrates lack of standardisation in methodologies used for identifying allergens. Appropriate negative controls must be used and reproducibility of conditions confirmed by negative and positive controls.

6 IgE cross-reactivity between kiwi fruit and other plant proteins

6.1 Introduction

Cross reactivity by proteins in kiwi fruit has been described in relation to a wide range of unrelated plant proteins. Cross reactivity has been reported to grass pollens (Gavrovic-Jankulovic, 2002a; Pastorello, 1996), tree pollens (Pastorello, 1996; Voitenko, 1997) (Moller, 1997b), latex allergens (Beezhold, 1996; Blanco, 1994; Brehler, 1997; Moller, 1998). and other fruits (1.3.5). Cross reactivity of plant food allergens with pollens is in part a consequence of cross-reactive carbohydrate determinants (CCDs) (1.3.5.3). A study of German patients with kiwi fruit allergy and birch pollen allergy found that the cross reactivity between kiwi and birch pollen allergy was mainly due to CCDs in their population (Fahlbusch, 1998).

Pollens, latex and the 'latex-fruit syndrome' foods are the only allergens to have been examined with regard to kiwi fruit cross reactivity. In this study of UK kiwi allergic patients, house dust mite was the most common allergen to which patients were sensitised (24 of 46 SPTs) (4.3.3.1). There is extensive similarity between the N-terminal amino acid sequence of Der p1 allergen and the cysteine proteases actinidin and papain (Simpson, 1989), but immunological cross reactivity has never been assessed. The incidence of peanut allergy was extremely high amongst the children in the study (3.3.3), and 4% of questionnaire responders (N=12) reported that they had seed allergy in addition to kiwi fruit allergy. It was postulated that their symptoms on eating kiwi fruit might be caused by allergy to the multiple seeds in the fruit.

The aim of this study was to establish the relevance of cross reactivity in a UK group of patients with kiwi fruit allergy. It was decided to examine cross reactivity between kiwi fruit and birch pollen and a mix of grass pollens, as examples of pollen cross reactivity, and to assess the role of CCDs. Cross reactivity between kiwi with Der p1 and peanut was also assessed.

6.2 Methods

6.2.1 Laboratory methods

ELISA inhibition (2.3.10) was used to assess inhibition of IgE binding in a semi-quantitative way using pooled sera from appropriate patients (6.2.2). Western blot inhibition was used to examine which kiwi proteins were inhibited by the relevant allergen (2.3.1.6 & 2.3.9). Western blots were used to compare IgE binding to kiwi fruit extract (including seeds) and an extract made exclusively of kiwi seeds (2.3.8). CCDs to determine cross reactivity with glycoproteins were used for inhibition immunoblots (2.3.11).

6.2.2 Patient Sera

All patients had kiwi fruit allergy, diagnosed by a convincing history of an IgE mediated reaction to kiwi fruit plus a positive DBPCFC or kiwi specific IgE. Because of the difficulties encountered interpreting DBPCFC (2.2.3), patients with an equivocal DBPCFC were included if they had a highly suggestive history and a specific IgE to kiwi>0.5KU$_A$/l. Details of the patients are summarized in *Table 6.1*.

Patients whose sera were used to assess for cross reactivity to birch pollen had a history of rhinoconjunctivitis in the tree pollen season with a positive skin test reaction to birch pollen. Patients used to assess for grass pollen cross reactivity had a history of seasonal rhinoconjunctivitis in the grass pollen season (patients had not been skin tested with grass pollen). Patients used to assess for Der p1 (house dust mite (HDM) allergen) reactivity had a history of asthma or eczema and a positive skin test to HDM. Patients whose sera were used to assess reactivity to seed allergens had a history of systemic reactions to seeds. They also complained of symptoms to kiwi fruit, but did not necessarily have specific IgE to kiwi fruit using the FEIA-CAP^{TM} system. An additional 4 patients who had kiwi allergy but not seed allergy, also had sera used for Western blots to kiwi seed extract. These 4 patients were chosen because they had a good history of kiwi allergy and demonstrated IgE binding to kiwi extract, and are not necessarily representative of the study population.

Table 6-1 Characteristics of patients whose sera was used for in vitro studies to assess cross reactivity between kiwi fruit and a) birch pollen 5 patients b) grass pollen11 patients c) Der p 1 10 patients d) peanut 8 patients d)seed 9 patients.

a) BIRCH POLLEN	Age (years)	DBPCFC	History of hay fever	SPT birch	sIgE kiwi	Symptoms to kiwi
	9	Positive	✓	4mm	3.6	Angioedema, rash, sore mouth
	9	Positive	✓	6mm	0	Angioedema, rash, oral symptoms
	48	N/A	✓	4mm	0.8	Anaphylactic shock
	46	Positive	✓	6mm	0.7	Angioedema, oral symptoms, vomiting
	52	Equivocal	✓	3mm	1.8	Tight throat, tingling of oral mucosa

b) GRASS POLLEN	Age (years)	DBPCFC	History of hay fever	sIgE kiwi	Symptoms to kiwi
	9	Positive	✓	3.6	Angioedema, rash, sore mouth
	9	Positive	✓	0	Angioedema, rash, oral symptoms
	48	N/A	✓	0.8	Anaphylactic shock
	10	Equivocal	✓	0.6	Tingling mouth, breathing difficulty
	18	Positive	✓	4.6	Angioedema, wheeze, oral symptoms
	9	N/A	✓	4.1	Rash, wheeze, oral tingling
	35	N/A	✓	3.9	Anaphylactic shock, wheeze
	21	Positive	✓	1.2	Oral swelling and tingling
	37	Equivocal	✓	7.7	Pharyngeal symptoms, abdominal pain
	5	N/A	✓	17.9	Anaphylactic shock
	52	Equivocal	✓	1.8	Tight throat, tingling of oral mucosa

c) Der p1	Age (years)	DBPCFC	SPT HDM	SpIgE kiwi	Symptoms to kiwi
	9	Positive	3mm	3.6	Angioedema, rash, sore mouth
	9	Positive	3mm	0	Angioedema, rash, oral symptoms
	48	N/A	6mm	0.8	Anaphylactic shock
	10	Equivocal	3mm	0.6	Tingling mouth, breathing difficulty
	9	Positive	3mm	1.9	Rash, vomiting
	10	Positive	3mm	2.35	Rash, vomiting, oral symptoms
	26	Positive	6mm	0	Oral tingling
	21	Positive	4mm	1.2	Oral tingling, tongue swelling
	55	Positive	3mm	0	Oral tingling, oral swelling
	49	Positive	5mm	0.5	Oral tingling, oral swelling, tight throat

d) PEANUT	Age (years)	DBPCFC	sIgE kiwi	Symptoms to kiwi
	10	positive	0	Angioedema, swollen tongue, Oral symptoms
	9	positive	1.9	Rash, vomiting
	10	positive	2.35	Rash, vomiting, oral symptoms
	9	N/A	4.1	Rash, wheeze, oral tingling
	9	positive	0	Vomiting, abdominal pain
	35	positive	3.9	Angioedema, breathing difficulties, wheeze
	5	N/A	17.9	Anaphylaxis
	4	N/A	3.62	Anaphylaxis

e) SEED	Age (years)	DBPCFC	sIgE kiwi	Symptoms to kiwi
Sesame	9	positive	1.9	Rash, vomiting
Sesame, sunflower	24	N/A	0	Swelling and tingling of oropharynx
Sesame	42	N/A	0	Swelling and tingling of oropharynx. Wheeze
Sunflower, pumpkin	72	N/A	0	Abdominal pain and diarrhoea
Sunflower	55	N/A	0	Tingling of oropharynx
Not seed allergic	14	N/A	1.7	Anaphylaxis
Not seed allergic	10	Positive	0.6	Swelling and tingling oral cavity
Not seed allergic	58	N/A	0.6	Oral pruritis
Not seed allergic	14	N/A	0.7	Anaphylaxis

6.3 Results

6.3.1 ELISA inhibition- HDM, grass pollen, birch pollen, peanut extract.

The ELISA inhibition was confirmed to work by inhibiting IgE binding to green kiwi fruit by pre-incubating the sera with green kiwi fruit (positive control) and gold kiwi fruit (a closely related fruit). Pre-incubating the sera with BSA (negative control) showed no inhibition of IgE binding. Pre-incubating appropriate pools of sera with extracts of HDM, birch pollen, grass pollen and peanut showed no inhibition of IgE binding to kiwi fruit (*Figure 6.1*)

Figure 6.1 ELISA inhibition of IgE binding.

Serial dilutions of inhibitors (HDM, birch pollen, grass pollen and peanut extracts) and controls (green kiwi and gold kiwi) were incubated with serum from kiwi allergic patients who were also allergic to the inhibitor allergen. Unbound IgE was captured on kiwi coated ELISA plates and immunodetection performed with anti-human IgE. 100% inhibition was defined by no IgE binding (i.e. OD equivalent to the ELISA well which was incubated with diluent with no sera). 0% inhibition was defined as the OD following development of a well in which sera which had not been pre-incubated with inhibitor allergen.
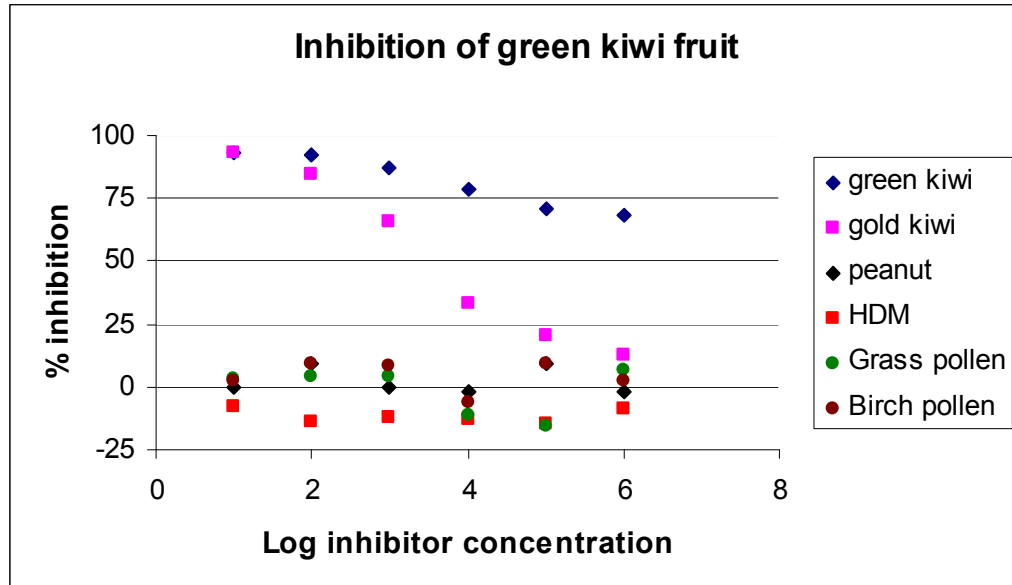
6.3.2 Western Blot and Western blot inhibition

6.3.2.1   House dust mite extract

IgE from the pool reacted to proteins in the HDM extract (*Figure 6.2* , lane 1), and to a kiwi fruit protein of 38 kDa (lane 2). There was no inhibition of IgE binding to the kiwi 38 kDa protein (lane 3) by pre-incubating the sera with Der p1.

Figure 6.2 Inhibition Western blot using pooled sera from patients with HDM and kiwi fruit allergy.

Lane 1 IgE binding to HDM extract; Lane 2 IgE binding to kiwi extract; Lane 3 Sera pool inhibited with HDM extract, IgE binding to kiwi extract.
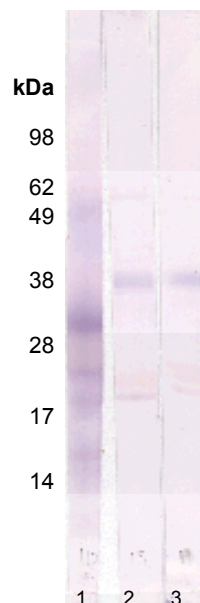
6.3.2.2   Grass pollen

IgE in the serum pool of kiwi and grass pollen allergic patients recognized multiple grass pollen allergens (*Figure 6.3*, lane 1) and kiwi allergens of 38 kDa and 30 kDa (figure 5, lane 2). IgE binding to the 30 kDa kiwi allergens was inhibited by incubation of the sera with grass pollen extract (*Figure 6.3*, lane 3), but the major allergen in this study (38 kDa) was not inhibited.

Figure 6.3 Inhibition Western blot using sera pool from patients with grass pollen and kiwi fruit allergy.

Lane 1 IgE binding to grass pollen extract; Lane 2 IgE binding to kiwi extract; Lane 3 Sera pool inhibited with grass pollen extract, IgE binding to kiwi extract.
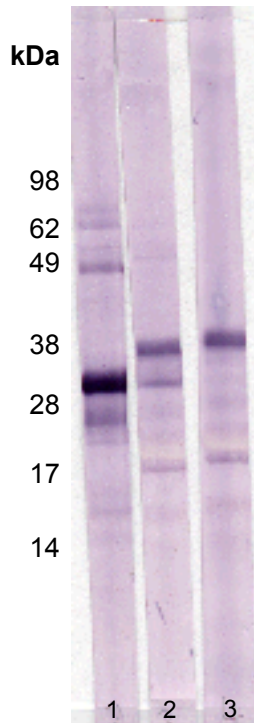
6.3.2.3   Birch Pollen

Despite the pool being from patients with a history of tree pollinosis and a positive SPT to birch pollen, only weak IgE bands were detected following incubation of the pool with the blotted birch extract (*Figure 6.4*, lane 1). Repeat experiments with different ampoules of the same extract confirmed this finding. The pool recognized kiwi proteins at 38, and 30* kDa, but these were not inhibited by pre-incubation of the sera with birch extract (*Figure 6.4*, lanes 2 and 3 respectively).

(*The 30 kDa band was not easily seen on scanned image, but was present on the original blot).

Figure 6.4 Inhibition Western blot using pooled sera from patients with kiwi fruit allergy and birch pollen sensitization.

Lane 1 IgE binding to Birch pollen (BP) extract; Lane 2 IgE binding to kiwi extract; Lane 3 Sera pool inhibited with birch pollen extract, IgE binding to kiwi extract.
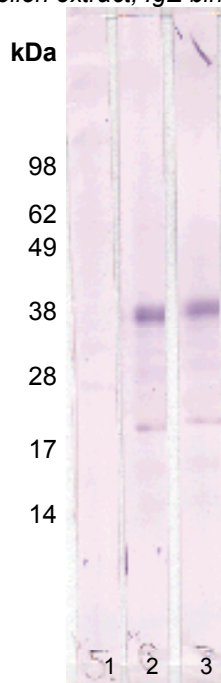
6.3.2.4   Peanut

The pool of sera used for this study contained IgE that reacted to a range of peanut proteins including Ara h 1 (approximately 63 kDa) and Ara h 2 (doublet at approximately 17-20 kDa) (Figure 6.5, lane 1). The pool recognized kiwi proteins with weights of 38 kDa and 15kDa. IgE to the lower weight allergen was from one patient in the pool. IgE binding to 15kDa was inhibited by pre-incubation of the sera with peanut extract (Figure 6.5, lanes 2 and 3 respectively), but binding to the 38 kDa protein showed no inhibition.

Figure 6.5 Inhibition Western blot using pooled sera from patients with allergy to kiwi fruit and peanuts

Lane 1 PN/ kiwi pool IgE binding to peanut (PN) extract.; Lane 2 PN/kiwi pool IgE binding to kiwi extract; Lane 3 PN/ kiwi pool inhibited with PN extract, IgE binding to kiwi extract.
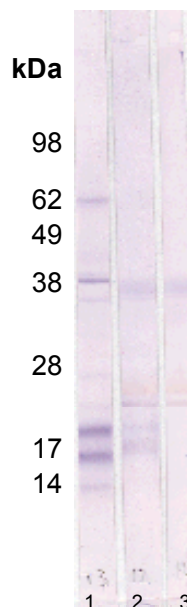
6.3.2.5   Seeds

SDS PAGE of kiwi extract and kiwi seed extract showed distinct protein profiles (*Figure 6.6*). The 25 kDa band that corresponds to actinidin in the green extract is significantly less abundant in the seed extract. However, there are relatively more high molecular weight proteins (>30kDa).

Figure 6.6 1D-SDS PAGE of kiwi extract and kiwi seed extract

(MW= molecular weight markers; Mark 12, Invitrogen).
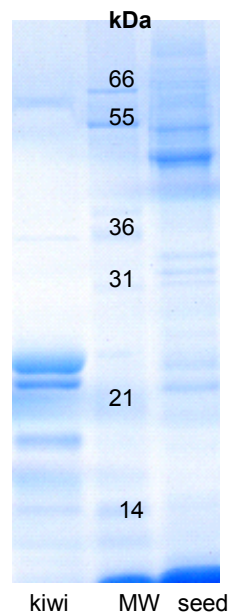
A pool of sera from 9 patients with kiwi fruit allergy, but not seed allergy, showed multiple IgE binding bands to kiwi extract (*Figure 6.7*; Lane 1) and seed extract (Lane 2). The binding patterns were distinctly different for the two extracts. A child with systemic symptoms to kiwi fruit and a history of seed allergy had IgE binding to a 38 kDa band in the kiwi extract, but no reaction to the seed extract (Lanes 5&6). Three patients with convincing histories of reactions to kiwi and seeds, but a negative specific IgE to kiwi, had no IgE binding to kiwi extract, but did have bands to the seed extract at ≈98 kDa, 62 kDa and 45 kDa. Additionally, all 4 patients with a history of kiwi fruit allergy but not seed allergy, had IgE binding to both kiwi fruit extract and the seed extract (data not shown).

Figure 6.7 Western blot with kiwi extract (K) and kiwi seed extract (S).

Lanes 1&2 kiwi allergic pool; 3&4 non-atopic pool; 5-14 individual sera from patients with a history of kiwi fruit allergy and seed allergy.
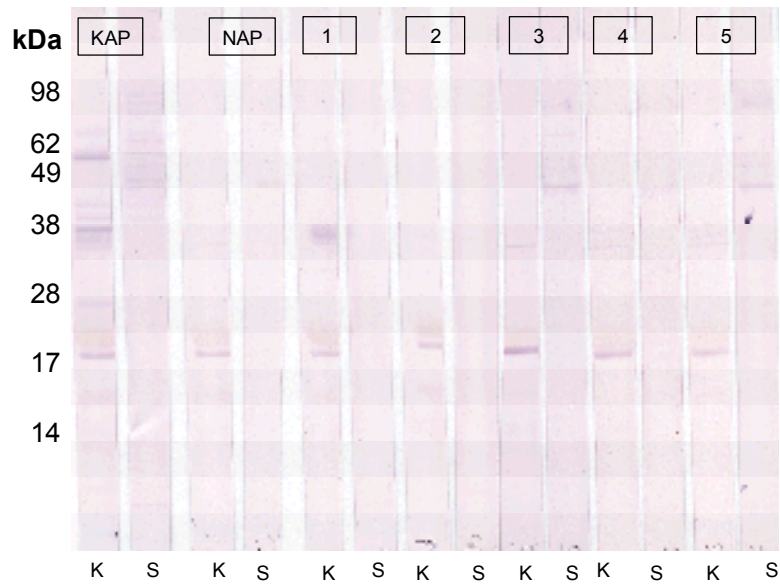
6.3.3 Cross reactivity to glycoproteins

SDS-PAGE of kiwi protein extract stained using the Periodic acid-Schiff (PAS) method to detect glycoproteins showed that the proteins with molecular weights of 17, 22 and 36 kDa are glycosylated (*Figure 6.8*). Actinidin (MW 25 kDa) was not glycosylated.

Figure 6.8 SDS PAGE of kiwi protein extract stained with Coomassie (blue) to detect proteins and PAS (pink) to detect glycoproteins.
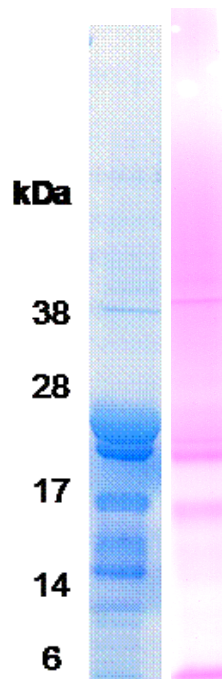
To confirm the presence of anti-CCD IgE and to establish their cross reactive nature, 14 individual sera of patients with both pollen allergy and kiwi allergy were pre-incubated with proteinase K-digested kiwi protein extract (1.3.11.2), prior to Western blotting. Inhibition of at least one band was seen using sera from 5 patients, but all patients had bands that persisted. Binding to proteins of 15, 30, 40, 42, and 62 kDa were inhibited in some but not all patients. Examples of the inhibition blots are shown in *Figure 6.9*. IgE binding to proteins 30. 40. 42 and 62 kDa by patient A was inhibited by pre-incubation with CCDs, but interestingly binding persisted to these proteins by patient C. As expected, pre-incubating with kiwi protein extract (positive control) caused complete inhibition

of all IgE binding to kiwi fruit. These results show that in this population CCDs are responsible for IgE binding to some kiwi proteins, but probably in a minority of patients.

Figure 6.9 Inhibition Western blot, inhibiting IgE binding to kiwi fruit by pre-incubation of sera with CCDs.

Lane 1: non-atopic pool (not inhibited); Lane 2 kiwi allergic pool not inhibited; lane 3 kiwi allergic pool pre-incubated with kiwi protein extract; Lane 4 kiwi allergic pool pre-incubated with CCDs; Lane 5 patient A sera not inhibited; lane 6 patient A sera pre-incubated with CCDs; lane 7 patient B sera not inhibited; lane 8 patient B sera pre-incubated with CCDs; lane 9 patient C sera not inhibited; Lane 10 patient C sera pre-inhibited with CCDs. M=Molecular weight markers.
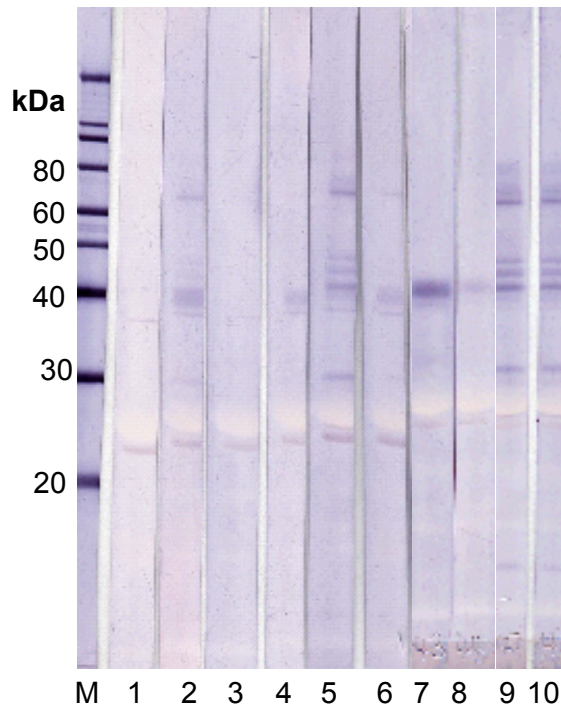
6.4 Discussion

The relevant cross-reactivity in this population was to seeds. People with seed allergy may react to the seeds in kiwi fruit rather than the pulp. These people describe symptoms on eating kiwi fruit, but have a negative specific IgE (CAP-FEIA) to the fruit. Further work is required to determine the incidence of allergy to fruits caused by seed allergy. It is likely that other fruits with an abundance of seeds are also implicated in these phenomena eg. strawberries and raspberries. Seed allergy has not previously been reported as a cause of fruit allergy, and this is an important finding of clinical relevance. Patients with kiwi allergy, but not seed allergy reacted to the seed extract as well as kiwi fruit extract. This is not surprising because kiwi seeds will contain many of the same proteins or homologous proteins as the pulp.

Allergy to fruits and vegetables has frequently been described in patients with pollinosis. The common explanation for these observations is cross-reactive IgE that binds to similar epitopes in the two allergens. Several studies have reported *in vitro* cross reactivity between kiwi fruit and pollens (Gavrovic-Jankulovic, 2002a; Moller, 1997b; Pastorello, 1996). However, cross-reactive IgE showed little relevance in this UK population. *In vitro* cross-reactivity occurred between some epitopes in kiwi fruit and those in grass and peanut. However, the clinical relevance of this is limited because inhibition did not occur to major allergens in the kiwi fruit. *In vitro* cross-reactivity to kiwi proteins was not shown to occur in this population with birch pollen and house dust mite. However, IgE binding to the birch pollen extract was poor and the results may reflect problems with the extract. The experiment was repeated using a different ampoule of extract, but the extracts were from the same source and perhaps lacked the relevant epitopes for this UK population.

It is important when reporting cross reactivity, to discriminate between cross-immunogenicity, (an *in vitro* immunological mechanism) cross-allergy (a clinical response to cross-immunogenicity) and associated allergenicity (clinical allergy to more than one allergen without cross-immunogenicity). If *in vitro* cross-immunogenicity is shown, this does not necessarily confirm *in vivo* cross-

immunogenicity. For example, patients with pollen and hymenoptera venom allergy, in addition to having specific IgE to pollen and venom proteins, may develop anti-cross-reactive carbohydrate determinants IgE (anti-(CCD IgE), leading to cross-reactive recognition of glycans in a range of plant foods (reviewed in 1.3.5). However, it is generally considered that anti-CCD IgE is of little clinical relevance to the patient. If true cross reactivity is to be confirmed, confirmation of clinical allergy to the fruit and the pollen needs to be sought, and *in vitro* cross allergenicity demonstrated.

Most studies to date have fallen far short of confirming co-existing cross-immunogenicity and cross-allergy, and results are therefore difficult to interpret. For example, Pastorello *et al* conducted a study to investigate the allergenic components of kiwi fruit and to evaluate their cross-reactivity with timothy and birch pollens (Pastorello, 1996). Thirty patients were recruited on the basis that they had kiwi fruit allergy, and this was confirmed by open food challenge. The patients' sera was tested for specific IgE to pollen, and 26 had timothy pollen specific IgE and 22 had birch pollen specific IgE. However, it is not reported whether the patients had pollen related allergy. The specific IgE to pollen and the *in vitro* cross reactivity reported to kiwi fruit may have been a consequence of irrelevant anti-CCD IgE. Moller *et a*l (Moller, 1997b) recruited patients with symptoms of birch pollen allergy and kiwi fruit allergy, and demonstrated *in vitro* cross-reactivity. To prove the relevance of this cross-immunogenicity, they would need to show that it did not occur in patients who were only allergic to the pollen or only allergic to the fruit. A German study designed to determine the cross-reactive allergens of kiwi fruit and pollens (Gall, 1994)recruited 7 patients who tolerated kiwi fruit as a control group. These patients had positive skin tests to kiwi fruit in the absence of clinical reactivity, emphasising that *in vivo* cross-sensitization does not confirm clinical relevance. In this PhD study attempts were made to confirm that the patients were allergic to the suspected allergen, by history and specific IgE or skin test. However, even this falls short of the ideal which would be a positive double blind challenge to kiwi fruit and the other allergen. No study to date has embraced these stringent criteria.

The finding that cross-reactivity to pollen allergens is not important was supported in this study by the allergy profiles of the patients. Reported allergy to grass pollen (29%) and tree pollen (23%) on the questionnaires (3.3.3) is similar to the prevalence of pollen allergy expected for an atopic population (Boulet, 1997; Kaleyias, 2002), but if there was a causal link the prevalence would be higher in the study population

A higher prevalence of cross-reactive allergy between foods and pollens in continental Europe is supported by prevalence data. Eriksson *et al (Eriksson, 1984)* reported 600 pollen allergic patients from Sweden, 380 of whom were birch pollen allergic. Fifty three percent of the birch allergic patients reported that they had reactions to hazelnut, 47% to apple, 34% to peach and 27% to cherry.

Further evidence to support the genuine existence of cross-allergy in some individuals with birch pollinosis and food allergy, is the apparent beneficial effect of pollen immunotherapy on related food allergy (Asero, 1998; Asero, 2000a). Since pollen allergy is common in the UK, it is not clear why pollen related food allergy is less common than in continental Europe. Perhaps different allergens are important in different populations with different genotypes or different dietary habits. Perhaps specific pollen allergens are important. For example, in Switzerland hazel nut allergy is usually a benign condition as a consequence of cross reactivity between Cor a 1 and Bet v 1. However, in the UK where Bet v 1 allergy is less common, hazel nut allergy is often severe. (Hansen, 2003).

In summary, cross-reactivity to seeds is clinically relevant and requires further evaluation to determine the prevalence of this problem in the seed allergic population. Immunological cross reactivity with grass and peanut allergens was demonstrated, but is unlikely to be of major importance because the major kiwi allergens were not involved. No cross reactivity was demonstrated with Der p 1 or birch pollen. Most previous studies have not distinguished cross-immunogenicity from symptom elicitation. Only some cross-reactive antibodies

give rise to clinical food allergy, and future studies should attempt to confirm clinical relevance of *in vitro* findings.

7 Effect of digestion and pH on allergenicity of kiwi fruit proteins

7.1 Introduction

Foods contain a wide variety of proteins yet only a few are allergens. The reason why some proteins are highly allergenic and others are not remains poorly understood, but certain chemico-physical properties appear to be associated with allergenicity. Most allergens are said to have a molecular weight of 10-70 kDa and are typically stable to changes in heat and pH, and to digestion. They generally have an acid pI and are soluble for absorption across the GI tract. However, many non-allergenic proteins also show these properties.

Several studies have suggested that food allergens are resistant to pepsin digestion, whereas non-allergenic proteins are more likely to be rapidly and completely digested (Asero, 2000b; Tanaka, 2002). However a number of studies have shown some food allergens to be rapidly digested (Fu, 2002; Rodriguez-Perez, 2003; Yagami, 2000). Furthermore, analysis of the avocado allergen Prs a 1, shows it to be extensively digested, but the resulting peptides remain reactive *in vivo* and *in vitro* (Diaz-Perales, 2003).

With the introduction of novel genetically modified (GM) foods it has become necessary to devise methods to identify potential allergens and various assessment strategies have been developed. The FAO/ WHO decision pathway for pre-marketing assessment (1.2.4) of the allergenicity of GM foods focuses on the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunochemical binding of the newly introduced protein with IgE from the serum of individuals with known allergies to the transferred genetic material, and the physicochemical properties of the newly introduced protein including digestion. Thus evaluation of protein digestibility using pepsin is currently a factor in predicting the allergenic potential of a novel protein.

A study by Yagami showed that all allergens in kiwi fruit were readily digested by simulated gastric fluid (SGF) when assessed by means of SDS-PAGE and Western blotting (Yagami, 2000) using sera from patients with latex allergy, some of whom had fruit allergy. Until now no study has used sera from patients recruited because they have kiwi fruit allergy, and no study has directly compared IgE reactivity of patients with systemic and OAS symptoms to any digested food.

Recent studies have highlighted that allergen digestibility is influenced by the pH of gastric juices (Untersmayr, 2005b), a hypoacidic environment potentially increasing the absorption of undigested proteins and the risk of sensitization (Untersmayr, 2005a). This study examined the effect of pH in SGF on digestion of kiwi fruit proteins.

A recent study from Italy investigated the effect of heat treatment on kiwi fruit and found the allergens in the fruit to be heat labile (Fiocchi, 2004). The work regarding heat stability was not repeated because it is of little relevance in a food that is predominantly consumed in a raw form.

The aim of this study was to assess the digestibility of kiwi proteins to SGF, and to assess the immunogenicity of the digested products by Western blotting and ELISA. In particular, it was designed to address the hypothesis 'The characteristics of the responsible allergens, including lability to pH and digestion, determine the severity of symptoms experienced by the allergic patient' (1.4).

7.2 Methods

Digestion of kiwi fruit extracts using simulated gastric fluid (SGF) was investigated as described in 2.3.12. Additionally to act as control conditions, kiwi fruit protein extract was treated in ultra-pure water and in SGF without pepsin.

To determine whether pH conditions influence digestion of kiwi extracts and thereby its immunogenicity, in addition to the SGF with pH 1.5, SGF solution were prepared with pH 2, 2.5, 3, 5 and 7. SDS-PAGE of the treated extracts and control extracts (2.3.12.4) was performed under reduced conditions (2.3.12.5). Western blotting to the digested extract was performed using pooled sera from a group of patients with isolated oral symptoms (n=12), and a pool of sera from patients with systemic reactions (n=12). The characteristics of the patients for the two pools are shown in *Table 7.1*.

Figure 7.1 Characteristics of patient's sera used for *A.* **systemic pool and** *B.* **OAS pool.**

Age given at time of bleeding (years); SPT= prick to prick test with fresh kiwi; sIgE= specific IgE to kiwi; ND=not done.

A.	Age	SPT(mm)	sIgE
	54	ND	0
	20	ND	4.6
	12	11	0
	10	ND	4.1
	43	6	0.8
	24	11	0
	72	ND	0
	35	ND	3.9
	5	ND	17.9
	27	ND	0
	43	6	0
	57	ND	0.5

B.	Age	SPT(mm)	sIgE
	38	4	0
	43	3	0
	51	ND	0
	60	ND	2.9
	53	ND	0
	49	9	6.80
	53	5	0
	42	8	0.5
	63	ND	2.4
	49	6	0.5
	53	7	0
	29	4	0

7.3 Results

7.3.1 SDS-PAGE of digested proteins

Visualisation of the SDS gel showed rapid and complete digestion of the kiwi proteins by SGF with pH 1.5 (*Figure 7.2*). Original full-length proteins were undetectable 0.5 minutes after the start of digestion but a digestion fragment, or possible protein aggregate (approximately 70 kDa), was observed up to 2 minutes. In comparison, the control protein β-lactoglobulin was resistant to digestion and remained visually unchanged after 60 minutes of digestion (not shown). Kiwi fruit extract incubated at 37°C in ultra-pure water showed no significant degradation over 1 hour (*Figure 7.3*) and kiwi extract incubated with SGF without pepsin (i.e. pH1.5, 35mM NaCl) also showed no significant loss of protein bands over one hour (data not shown). This suggests that protein breakdown was predominantly due to pepsin

The pH of the SGF had a significant effect on pepsin activity. Rapid digestion of proteins occurred at pH≤2, but by 10 minutes digestion of extract by SGF with a pH of 2.5 remained minimal, and even after an hour protein bands were clearly visible in extracts treated with SGF with a pH≥3 (*Figure 7.4*)

Figure 7.2 Kiwi extract digested in SGF at different time points

0.5-60 minutes (S0.5, S60). S0 shows kiwi extract added to inactivated pepsin. 10μg of extract per lane were run on 12% NuPAGE gels under reduced conditions. MW= molecular weight (Magic Mark, Invitrogen).
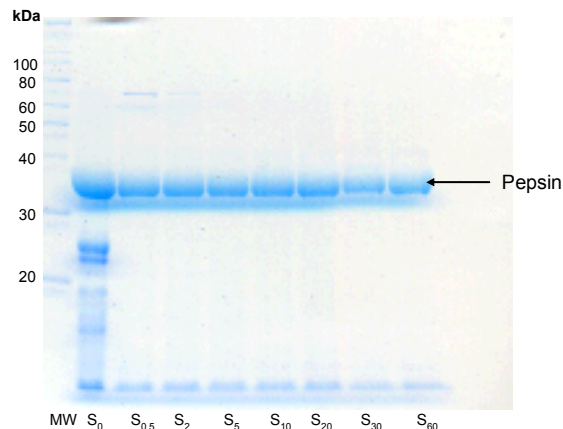
Figure 7.3. Kiwi extract incubated at 37˚C in ultra pure water (pH7.1).

0.5-60 minutes ($W_{0.5}$- W_{60}). W_0 ice cold kiwi extract added directly to the sample buffer and sodium bicarbonate solution with immediate heating to 70˚C. 10µg of extracts per lane were run on 12% NuPAGE gels under reduced conditions. MW= molecular weight (Magic Mark) standards.
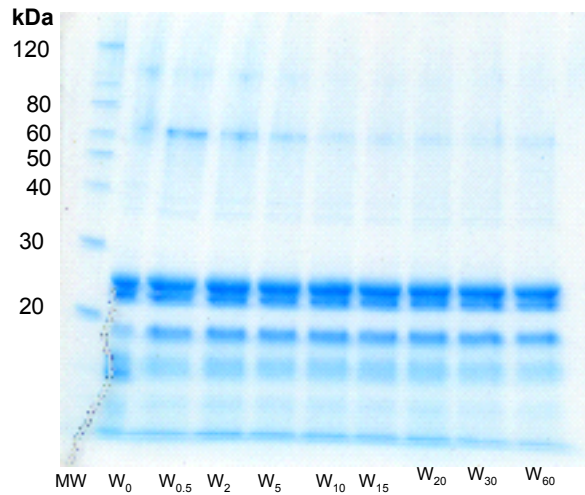
Figure 7.4. SDS-PAGE of kiwi extract treated with SGF with PHS 1.5, 2, 2.5, 3, 5 and 7.

A. shows extracts added to inactivated pepsin. B. shows the proteins after 10 minutes in SGF and C shows the extracts after an hour in SGF. P= pepsin. MW= molecular weight markers (SeeBlue Plus2, Invitrogen).
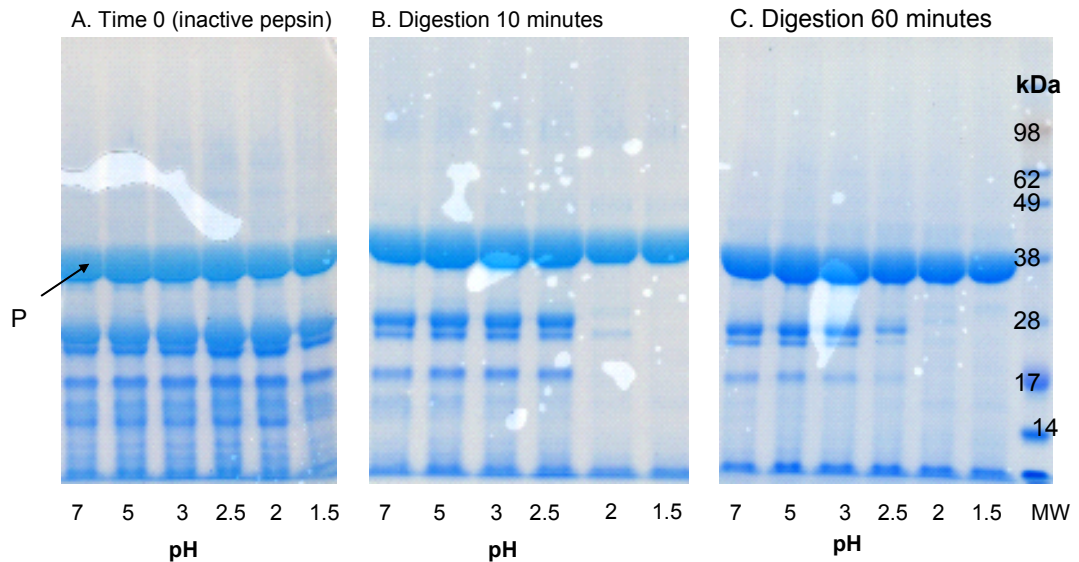
7.3.2 Western blotting

Western blotting using pooled sera from patients with symptoms of OAS to kiwi showed IgE binding to proteins in untreated kiwi fruit extract with molecular weights of 30, 38, 40, 42, 60 and 80 kDa (*Figure 7.5*, lane K). Unfortunately the molecular weight of pepsin (39 kDa) is similar to some of the allergens in kiwi fruit, and IgE binding to allergens of 38,40 and 42 kDa was 'blocked' by this protein as demonstrated in lane S_0 where the extract was added to inactivated pepsin so that digestion has not occurred. After 0.5 minutes in SGF the allergens of 30 and 80 kDa had disappeared, and by 10 minutes it appeared that all allergens had been digested. The extracts treated with water, instead of SGF demonstrated no difference in immunogenicity after 1 hour (W_{60}) in comparison to time zero (W_0) confirming that the changes seen over time in the digested extracts were due to to effects of SGF.

Figure 7.5: Western blot using sera from patients with symptoms of OAS.

Kiwi fruit extract was digested using SGF with pH 1.5 at times ranging from 0.5 minutes ($S_{0.5}$) to 1 hour (S_{60}). Lane S_0 has kiwi extract added to inactivated pepsin. Lane K: kiwi extract with no treatment (ie. As for Western blotting 1.3.8.2). Lanes W_0 and W_{60}: the extract was incubated with water instead of SGF as a control.
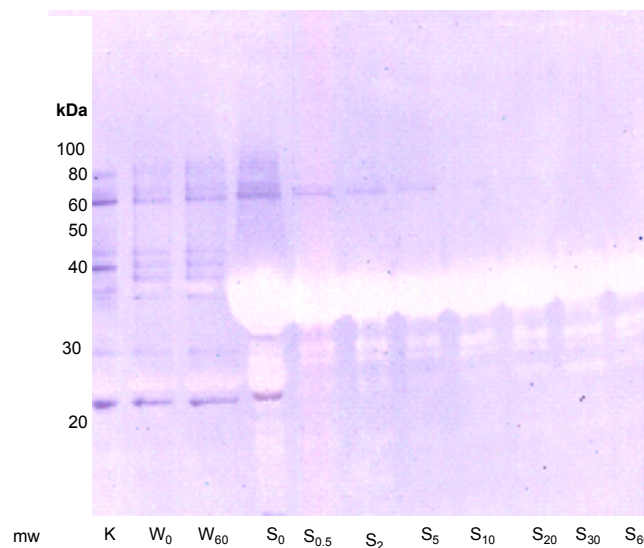
Figure 7.6 Western blot using sera from patients with systemic symptoms on eating kiwi fruit.

Kiwi fruit extract was digested using SGF with pH 1.5 at times ranging from 0.5 minutes ($S_{0.5}$) to 1 hour (S_{60}). Lane S_0 has kiwi extract added to inactivated pepsin. Lane K: kiwi extract with no
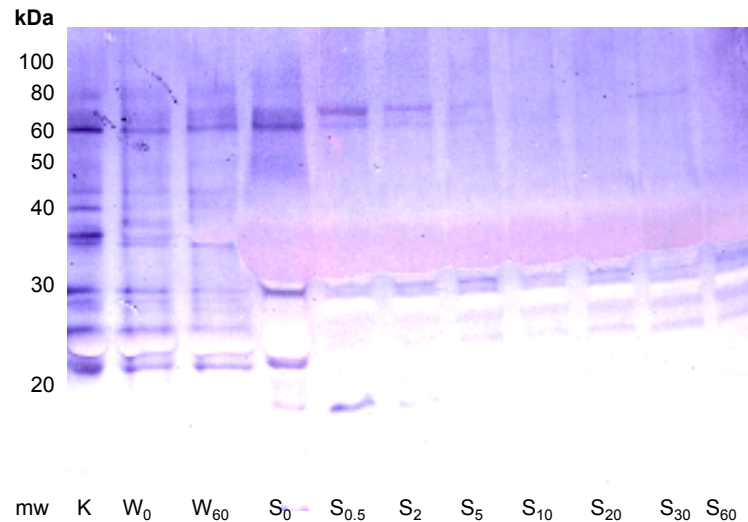
Western blotting using pooled sera from patients with systemic symptoms to kiwi showed IgE binding to proteins in untreated kiwi fruit extract with molecular weights of 25, 28, 30, 38, 40, 42, 60 and 80 kDa (*Figure 7.6*, lane K). Again the pepsin caused a physical barrier to IgE bindinng to allergens with molecular weights 38-42 kDa. As with the sera from patients with OAS, after 0.5 seconds the allergens with molecular weights of 25, 28 and 80 kDa has been digested, and binding to a band at 62 kDa persisted for 10 minutes.

However, in marked contrast to the blotting results using sera from patients with OAS, binding to the 30 kDa allergen continued, and was still evident after 1 hours digestion. Also, there was evidence of new epitopes after 10 minutes digestion, with new bands of molecular weights of 22 and 25 kDa. These new epitopes remained present after 1 hour of digestion. A band also appeared at 70 kDa after 0.5 minutes digestion, that then reduced in intensity before disappearing. This binding could be to the 70 kDa band noted during SDS-PAGE of digested proteins (*Figure 7.2*). Therefore there were significant differences between IgE binding to digested proteins by systemic reactors and patients with OAS, with systemic reactors not just recognising digestion stable epitopes, but also binding to new epitopes which developed during digestion.

7.4 Discussion

Resistance to digestion is generally regarded as a characteristic of food allergens (Astwood, 1996; van Ree, 2002b), and pepsin stability is currently a factor that is considered in deciding the probability that a novel protein is allergenic (Joint FAO/ WHO Consultation, 2001). However, the stability of most allergens has not yet been determined, and some studies have questioned the association between allergenicity and digestive stability (Fu, 2002; Rodriguez-Perez, 2003; Yagami, 2000). Astwood *et al* (Astwood, 1996) investigated the stability of common allergenic foods including peanut, soy, egg and milk relative to non-allergens. They reported that all non-allergenic proteins were rapidly digested, lasting 15 seconds in SGF, in comparison to allergenic foods which lasted 8-60 minutes. Some subsequent studies have supported their findings (Besler, 2001), but with some quantitative differences in the times of stability, presumably due to variation in methodology. However, other studies have failed to find an association between digestive stability and allergenicity (Fu, 2002; Yagami, 2000). At least some of these discrepancies are due to methodological issues (Bannon, 2003), for example standardisation of assays and no agreed time frame for stability. If digestibility is to be considered part of the safety assessment of novel proteins, digestion assay protocols clearly need to be standardised. A multi-centre study from 9 laboratories used a common protocol for evaluating the *in vitro* digestibility of common allergens and found it to be reproducible, yielding consistent results when performed using the same proteins at different laboratories (Thomas, 2004). Their protocol was followed for this study. A limitation of the method is the 'blocking' of proteins by pepsin (39kDa) on SDS-PAGE and Western blots. This was particularly relevant because the major allergen of the study population had a molecular weight of 38 kDa. Future studies using ELISA will overcome this problem, and will also determine whether binding to small digestion fragments, not visualised on Western blots, are capable of IgE binding (Diaz-Perales, 2003).

Most kiwi proteins were rapidly digested, with only one faint protein bands visible with Coomassie staining after 0.5 minutes digestion. This supports the findings of Yagami *et al*, who showed that all allergens in kiwi fruit were readily digested by simulated gastric fluid (SGF) when assessed by means of SDS-PAGE (Yagami, 2000).

Until now no study has directly compared IgE reactivity of patients with systemic and OAS symptoms to digested food. Despite no protein bands visible on staining, the pool of sera from our patients with systemic reactions demonstrated IgE binding by Western blotting to proteins persisting after an hour of simulated digestion. Interestingly, new IgE binding epitopes appeared after 20 minutes suggesting that digestion may even increase allergenicity. These findings contrasted with those using sera from the pool of patients with OAS, who failed to show IgE binding to extract after it had undergone simulated gastric digestion for 10 minutes. Likewise Yagami's patients (Yagami, 2000) with latex allergy, some of whom had fruit allergy, failed to show IgE binding to kiwi fruit after several minutes of digestion. It is generally believed that 'complete' food allergens are stable to gastric digestion, and absorption of the intact allergens allows sensitization, and on subsequent ingestions, systemic reactions to the food. Sensitisation to pollens and latex is likely to occur non-enterally, with fruit allergy occurring as a cross-reaction to homologous proteins, and symptoms to the digestion-labile fruit proteins restricted to the oral mucosa (OAS). Although it has long been hypothesized that the digestibility of the responsible allergens determines whether a person develops systemic symptoms or OAS, this is the first study to confirm a difference in the lability of allergens recognised by patients with systemic reactions and those with OAS. These unique findings, if confirmed in different populations and to different food types, may provide the basis for the first test to predict which patients will proceed to have severe reactions.

Pepsin digestion of kiwi fruit proteins was effected by hypoacidic conditions. Reducing the acidity of the SGF marginally (pH 2.5 compared to 1.5) strongly reduced pepsin digestion, presumably increasing the sensitization capacity of

the protein. Similar findings were found by a group looking at parvalbumin from fish (Untersmayr, 2005b). They went on to compare IgE responses to labile food allergens in gastroenterology patients prior to, and 3 month after starting H2-blockers (Untersmayr, 2005a), and reported a significant increase in food sensitisation. Their findings are perhaps surprising because many patients receive antacid treatment, and there have not been reports of these patients developing new food allergies. However, my data pertaining to protein digestion supports their *in vitro* findings and if confirmed will not only be relevant for patients receiving antacids, but will have implications for other groups with gastric hypoacidity. Newborns have a gastric pH≈4 and perhaps this hypoacidic state is responsible for the increased sensitisation observed in infants who are weaned early.

This use of 'test tube' simulation for gastric digestion does not provide a good physiological model. The stomach stores and mechanically churns foods, and the effects of gastric motility and gastric emptying on protein digestion are unknown. Additionally, factors such as the presence of other foods, hunger or anxiety may affect pH as well as gastric motility. The use of animal models may provide clearer insights to the effect of digestion on allergenicity of foods.

In summary, this study highlights several important findings. Lability of all kiwi fruit proteins to pepsin digestion was suggested by SDS-PAGE and Coomassie staining. However, although this is the method advocated to assess lability of novel proteins by the WHO (Joint FAO/ WHO Consultation, 2001), it is clear that simply using an approach of pepsin digestion followed by SDS-PAGE and Coomassie staining to assess digestibility in relation to allergenicity is flawed. As illustrated by the findings here, staining with Coomassie was not sufficiently sensitive and IgE binding occurred to allergens not visible on the stained gels. It is therefore extremely important to address the stability of allergens in relation to IgE reactivity. However, for novel foods suitable sera may not be available.

Until now no study has confirmed that the lability of proteins to which a person demonstrates IgE binding may predict whether their symptoms will always be

localised to the oral mucosa, or whether they may develop systemic symptoms. This provides supportive evidence to support the hypothesis that patients with oral allergy syndrome react to labile allergens, whilst those with systemic reactions react to proteins stable to gastric digestion. Further studies using sera from individual patients, are required, and then it will be necessary to confirm the findings to different foods. If confirmed, studying IgE reactivity to digested food extracts may provide a tool to predict whether an allergic patient's symptoms will remain mild, or whether they are at risk of developing systemic reactions.

8 Immunological and allergenic comparison of two species of kiwi

8.1 Introduction

The genus *Actinidia* contains about 60 species (Ferguson AR, 1999), but until recently only one has been eaten regularly in the Western world. Green kiwi fruit, *Actinidia deliciosa,* has become a common food throughout the world over the last 30 years. *Actinidia chinensis* is a species very similar to *Actinidia deliciosa, and* a gold variety is now grown and marketed under the name Zespri^{TM} Gold (1.3.2). Exports were first made to USA and Europe in 2000, and this novel food is increasingly available in many parts of the world.

The aims of this study were to identify the allergenicity and immunogenicity of *Actinidia chinensis* in patients with kiwi fruit allergy.

8.2 Methods

8.2.1 Clinical investigations

Five patients with allergy to green kiwi fruit confirmed by double-blind, placebo controlled food challenge (DBPCFC) were investigated by skin test and DBPCFC to gold kiwi fruit using recipe 2 (2.2.3). They included adults and children with systemic and isolated oral symptoms (Table 8-1). Prick to prick skin testing was performed using fresh pulp of green and gold kiwi on the volar aspect of the distal forearm (2.2.1).

Extracts of gold and green kiwi fruit were made (2.3.1). Size fractionation of the proteins was performed by SDS-PAGE, and stained with Coomassie stain (2.3). For 2-D gel electrophoresis, green and gold extracts were cleaned, and 400µg (for MS analysis) or 200µg (for staining) of the protein extracts incubated with a 3-10 pH gradient strip. Isoelectric focusing was followed by second dimension electrophoresis (2.3.4). The gel was stained with Coomassie (EZBlue, Sigma).

MALDI-TOF MS was used to measure the masses of the peptides derived from tryptic digestion of the 2-D gel-separated proteins (2.3.7). Peptide mass fingerprinting was used in an attempt to identify proteins, but no positive matches were identified. ESI NanoLC tandem MS was then used to obtain fragmentation data for each peptide, and *de novo* sequence information obtained. Sequence homology between proteins in the two fruits was sought.

IgE binding to green and gold fruit was compared by Western blotting (2.3.8) to green and gold kiwi protein extracts using a pool of sera from 9 patients with green kiwi fruit allergy and individual sera from a further 5 patients who had undergone DBPCFC to green and gold fruit. A pool of sera from 5 non-atopic patients was used as controls.

Inhibition immunoblots were studied (2.3.9). Green kiwi proteins were blotted onto a PVDF membrane, and doubling dilutions (0.1mg/ml to 0.00313mg/ml) of gold kiwi extract were pre-incubated with pooled sera for 1 hour. TBS without additives, and 0.1mg/ml BSA in TBS were incubated with the sera as controls. Strips of the PVDF were then incubated with each of the mixtures overnight. Immuno-detection of IgE binding was performed (2.3.8). The procedure was repeated by pre-incubating the sera with extract of green kiwi fruit as a control.

Gold kiwi extract proteins were similarly separated by SDS-PAGE, and immunoblotting performed using the pooled sera incubated with green and gold kiwi extract. The competitive inhibition of gold kiwi was only at a 'maximal blocking dose' of 0.1mg/ml of extract, because of limited sera.

ELISA inhibition studies were also performed (2.3.10). ELISA plates were coated with green kiwi extract. Inhibition experiments were conducted by pre-incubation of pooled sera with serial dilutions of the green or gold inhibitor (inhibitor concentrations: 0.2, 0.1, 0.025, 0.00625, 0.00156 and 0.0004mg/ml). The inhibition mixtures (including sera with no inhibitor as a positive control) and standard curves were dispensed in the wells. Immunodetection was conducted as described in 2.3.10.

8.3 Results

8.3.1 Clinical Investigations
All five patients reacted adversely to green kiwi (entry requirement to study), and four of the five patients allergic to green fruit reacted adversely to gold kiwi during a DBPCFC (Table 8.1). 2.5mg was the lowest dose for green kiwi and gold kiwi DBPCFCs. The first dose of each challenge elicited symptoms in some patients.

Patients 4 and 5 had oral pruritus corresponding to active but not placebo doses during DBPCFC with green and gold fruit. These two patients expressed the subjective feeling that the intensity of itching was less throughout the gold kiwi challenge than the DBPCFC using green fruit (the gold kiwi challenge occurred some months after the green kiwi challenge). Patient 1's green challenge was stopped at dose 1 (2.5g kiwi fruit) because she developed lip swelling and facial urticaria. She completed all doses of the gold challenge (cumulative dose 92.5g kiwi fruit) but complained of oral tingling and soreness that occurred during active, but not placebo doses. The reactions for patient 2 were identical during the two DBPCFCs. On the first dose she developed marked lip swelling, complained of abdominal pain and became withdrawn. Patient 3 developed abdominal pain and an urticarial rash with green kiwi but completed the whole gold challenge without symptoms.

Prick to prick skin testing with gold kiwi correctly predicted the patient who had a negative food challenge. Two of the patients were birch pollen allergic, but no patients had latex allergy. Two of the patients had mono-allergy to kiwi.

Table 8-1 *Characteristics and clinical investigations results of the patients who underwent DBPCFCs to green and gold kiwi .*

Symptoms were as reported by the patient during a reaction in the community. All allergies to allergens other than fruit were confirmed by SPT; patients who reported seasonal rhinitis were skin tested to a mix of grass pollens and birch pollen. (BP=birch pollen, TP=tree pollen; HDM=house dust mite; GI= abdominal pain plus vomiting and/ or diarrohoea)

Patient	Age	Reported reactions to green	Other allergies (symptoms AND SPT)	Green SPT (mm)	Gold SPT (mm)	Specific IgE Green	Green challenge	Gold challenge	Binding bands to green (kDa)	Binding bands to gold (kDa)
1	10 year	Pharyngeal oedema, urticaria, angioedema	BP/ GP/ cat HDM	7	3	3.6	Lip swelling, urticaria	Oral tingling and soreness	30,38.	40, 42, 60
2	10 year	GI, facial and oral swelling	BP/ peanut/ HDM	8	8	2.38	Lip swelling, abdo pain	Lip swelling, abdo pain	24, 30, 38, 42	40, 42, 45, 60
3	9 year	Rash, GI	Peanut/ walnut	7	0	1.9	Rash and abdo pain	Negative	38	6, 20, 34
4	52 year	OAS	Nil	13	12.5	6.8	Intense oral itching	Intensity of itching milder	30, 38	6, 20, 34
5	44 year	OAS	Nil	6	2	0.5	Intense oral itching	intensity of itching milder	38	6, 20, 34

8.3.2 1D and 2D SDS PAGE electrophoresis

SDS PAGE separation of proteins provided distinctly different protein profiles in the two fruit (*Figure 8.1*). Both extracts showed an abundant band of approximately 25 kDa. In the green fruit this corresponds visually on gel analysis to the protein reported as actinidin by previous studies (Pastorello, 1998). There were more bands and greater intensity of bands with a molecular mass >30kDa in the gold kiwi extract compared with the green kiwi extract.

On 2D gels (*Figure 8.2*), an abundant protein in green kiwi fruit with molecular weight ≈30KDa and pI ≈3 was confirmed to be the major allergen actinidin using

ESI-MS and a BLAST search of the NICBnr data base. There was no corresponding 30 kDa/ pI 3 protein in the gold fruit. However, a less abundant protein with similar molecular weight but basic pI in gold kiwi fruit (*Figure 8.2, B*) had significant homology with Act c 1 and is likely to be a basic form of actinidin.

Figure 8.1 Green and gold kiwi protein extracts separated by 1D-SDS PAGE on a 12% gel with MOPS running buffer.

MW=Molecular weight standards (Magic Marker, Invitrogen)..

kDa

120 —
100 _
80 —
60 —
50 —
40 —
30 —
20 —

Gold   Green   MW
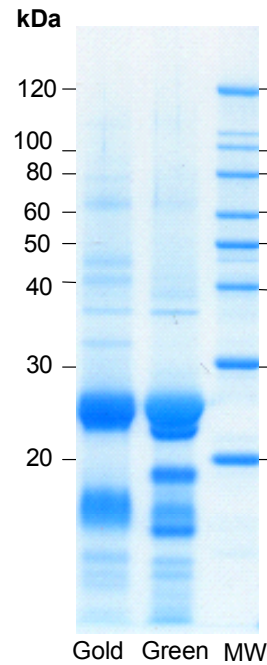
Figure 8.2: 2D SDS-PAGE a) green kiwi extract, b) gold kiwi extract.

200 µg extract per gel. Protein spot A was confirmed to be actinidin by tandem MS and a BLAST search. Protein spot B has significant sequence alignment with actinidin

a)   b)
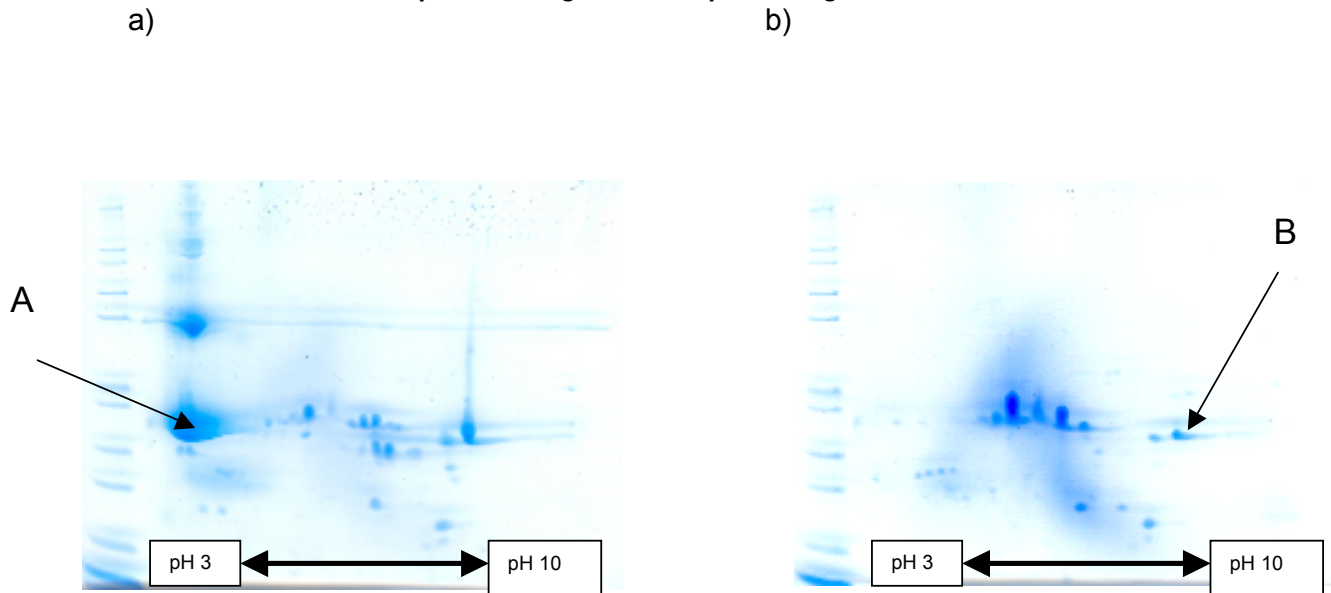
8.3.3 Western Blotting

Non-specific bands at 20 and 36 kDa were present in both green and gold extracts. These bands were present when the protein strips were incubated with sera from non-atopic volunteers (*Figure 8.3*, NA). Corresponding bands in kiwi allergic patients were therefore not considered significant.

The IgE binding patterns to green and gold kiwi extracts by pooled green-allergic sera are shown in figure 8.3, lanes KA. IgE binding bands recognised by individual sera from patients who had DBPCFC to green and gold kiwi are shown in figure 8.3 lanes 1-5, and described in table 8.1. The most common green kiwi bands recognised by the five patients were at 38 kDa and 30 kDa, and these bands were also present on the kiwi sera pool blots. Two patients had IgE binding gold kiwi proteins of 40, 42 and 60 kDa, and three patients, including patient 3 who did not react to the gold DBPCFC, reacted to proteins of 6, 20 and 34 kDa.

Figure 8.3 Western blots using green (left) and gold kiwi fruit extract. Lanes were incubated with pooled or individual sera.

NA=non-atopic pool; KA= kiwi allergic pool; patients=lanes 1-5 corresponding to patient numbers in Table 8.1..
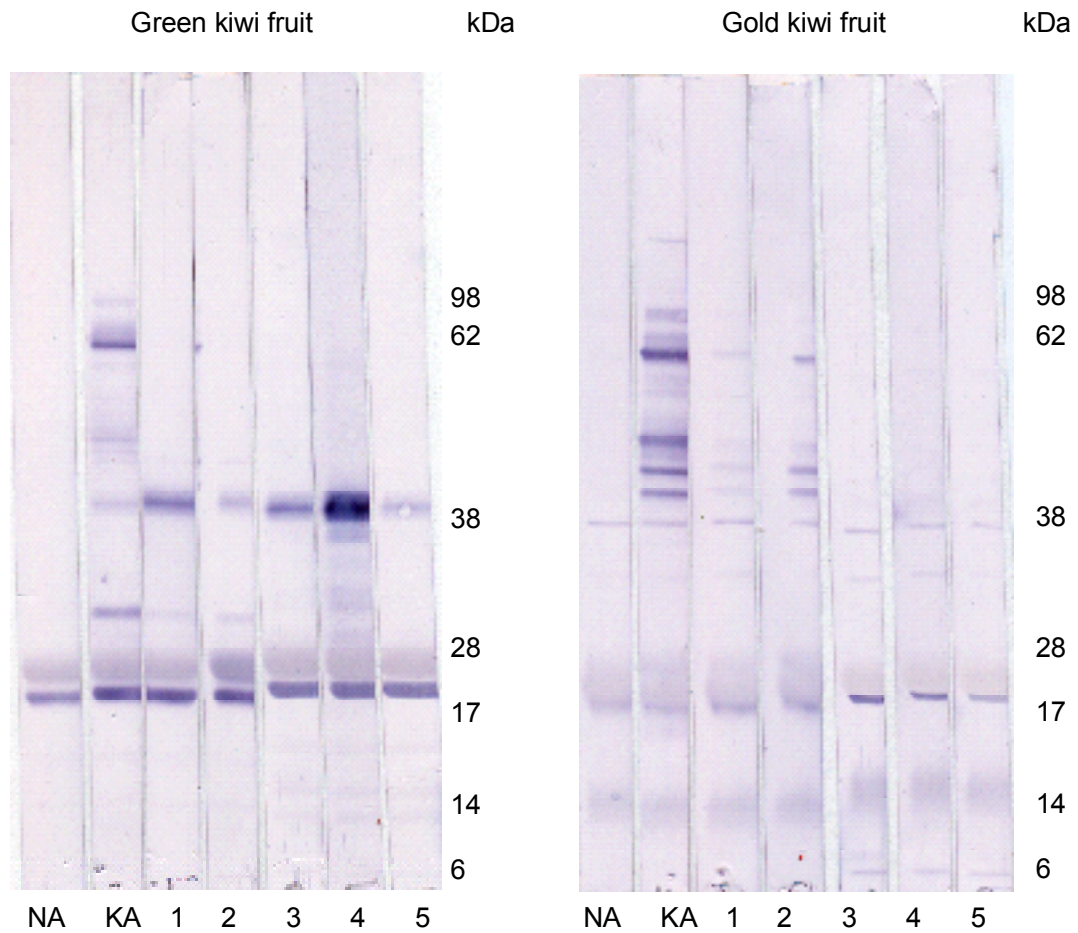
8.3.4 Immunoblotting inhibition
Immunoblotting inhibition

Complete inhibition of IgE binding to green kiwi extract occurred when the kiwi pool was pre-incubated with gold extract at 0.05mg/ml and some bands were inhibited at concentrations as low as 0.00625mg/ml (*Figure 8.4*). There was no inhibition of IgE binding when the sera were incubated with 0.1mg/ml of BSA, thus excluding non-specific protein binding of the IgE. IgE binding to gold kiwi extract was likewise inhibited by green extracts using pooled sera.

Figure 8.4 Inhibition of green kiwi immunoblots by gold and green kiwi extract (at doubling dilutions of inhibitor (0.1-0.00313 mg/ml) using pooled kiwi allergic sera.

TBS= control lane with no inhibitor

kDa

98
62

38

28

17

14

0.1mg/ml
0.05
0.025
0.0125
0.00625
0.00313
TBS
By Green Extract

0.1mg/ml
0.05
0.025
0.0125
0.00625
0.00313
TBS
By Gold extract
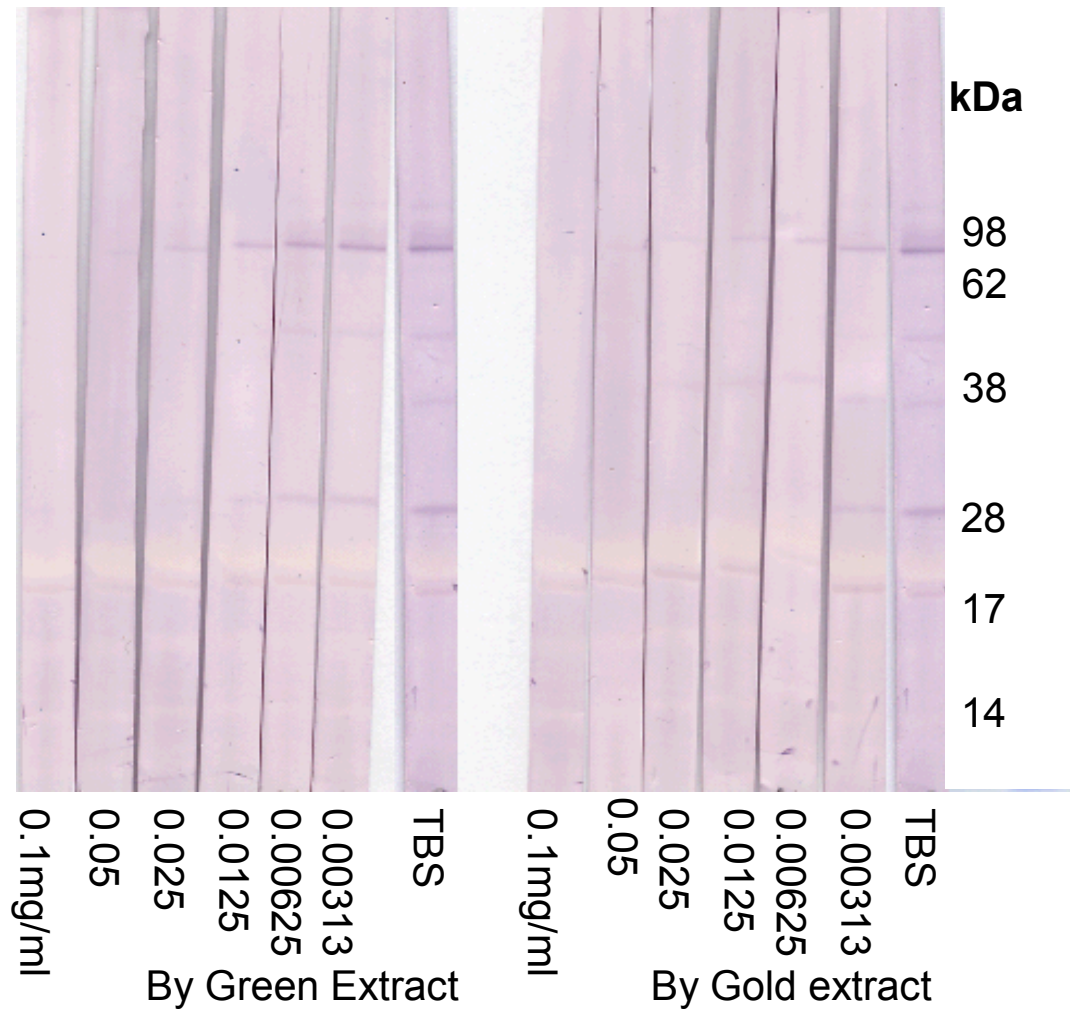
8.3.5 ELISA inhibition

ELISA inhibition with green kiwi extract as a positive control showed strong inhibition which was maintained even at low dilutions. Inhibition with gold extract showed inhibition equivalent to green kiwi at higher dilutions of inhibitor, but the inhibition was not maintained with dilution (*Figure 8.5*). This confirms significant cross-reactivity between epitopes in green and gold extracts.

Figure 8.5 Inhibition of IgE binding to green kiwi extract in ELISA by gold extract and green extract (control) in logarithmic concentrations. A pool of kiwi allergic sera was used.
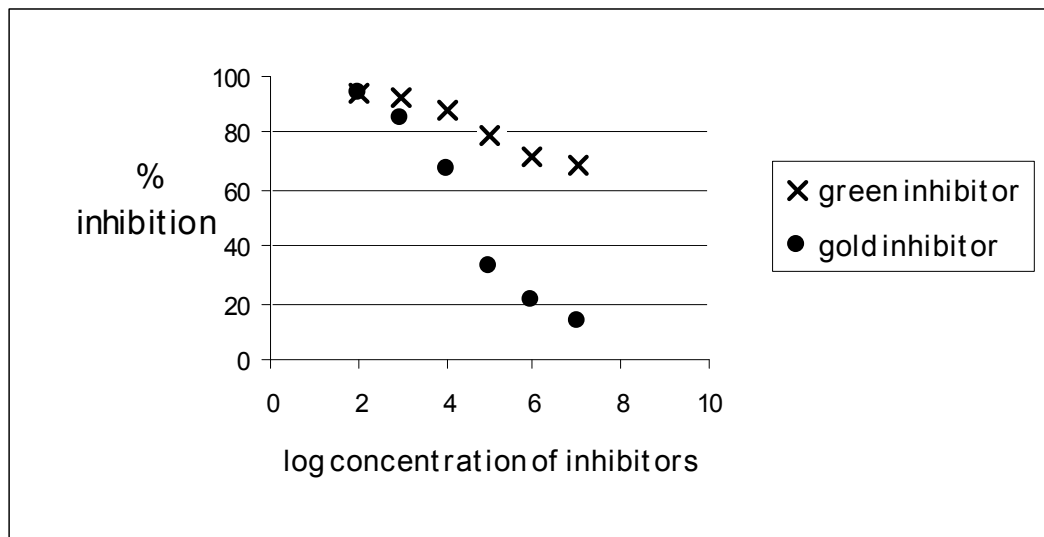
8.4 Discussion

This is the first study to confirm clinical reactivity to gold kiwi fruit. A recent paper has demonstrated the *in vitro* immunogenicity of gold kiwi (Bublin, 2004), but did not examine the clinical relevance. The European study used sera from patients with kiwi fruit allergy and performed Western blots to extracts of green and gold fruits. Serological cross-reactivity does not necessarily correlate with clinical allergy as demonstrated by patient 3 who tolerated gold kiwi showed inhibition in immunoblotting experiments. This highlights the need to confirm the results of *in vitro* work with clinical investigations.

The introduction of gold kiwi onto the UK market in 2000 prompted this part of the study to examine its allergenicity in patients with allergy to kiwi fruit. With the increasing prevalence of allergies, the issues of allergenicity of foods has become a major consideration for the food industry and its regulators. All new foods should be scrutinized prior to entry to the market for safety. The UK has a well defined pre-market assessment system for novel GM and non-GM foods. The Advisory Committee on Novel Foods and Processes (ACNFP) is a non-statutory, independent body of scientific experts that advises the Food Standards Agency on any matters relating to novel foods and novel processes (eg. irradiating food). It carries out safety assessments on any novel food or process submitted for approval under the EC Novel Food Regulation (EC 258/97). This regulation introduced a statutory pre-market approval system for GM and non-GM novel foods that do not have a history of significant consumption in the UK, using various strategies for assessing allergenicity including the WHO/ FAO Decision Tree (2.4). For reasons that are not clear, gold kiwi was not assessed by the ACNFP. However, this study clearly demonstrates that if the proper assessments had been made prior to the fruit coming to the market, it would have been identified as an allergen source by the WHO/ FAO Decision Tree (*Figure 8.6*).

The first question in the Decision Tree is 'Is the source of the gene allergenic?' Gold kiwi is not a GM food and consequently does not have any gene insertions. However it is closely linked genetically to green kiwi, with considerable overlap of genes and expressed proteins (Personal communication Elspeth McRae, Plant Geneticist, HortResearch, NZ). A basic form of actinidin was identified in the gold kiwi protein extract. Actinidin is not an allergen in the UK population, but if this Decision Tree had been used prior to marketing in 2000, most studies had reported actinidin as a major allergen, and the food would have been ranked as highly likely to be allergenic.

Figure 8.6: The WHO/FAO Decision Tree. If used before marketing, the Decision Tree would have identified gold kiwi fruit as likely to contain allergenic proteins.

1. Is the source of the gene allergenic?
• Not really- this is not a GM food
• **But** this is a novel food that is closely related to kiwi fruit, a known allergenic food.
• Therefore for the purpose of this study, YES

WHO 2001 Decision Tree

Is the source of gene allergenic?

Yes — No

Is the sequence homologous to known allergens? — Is the sequence homologous to known allergens?

Yes / No

Yes / No

Specific serum screen — No — Targeted serum screen

Yes

Yes / No

Protein likely to be be allergenic

Pepsin resistance and Animal models

No

Probability of protein being allergenic

HIGH ▬▬▬▬ LOW

2. Is the sequence homologous to know allergens?
•MS analysis of spot A (mw≈30; pI≈3) confirmed it as actinidin, previously reported as the major allergen in kiwi (figure 8.2).
•Visual inspection suggests that this major kiwi allergen is missing from gold kiwi.
•**BUT** *de novo* sequencing of spot B shows homology with actinidin.
•Therefore there is significant sequence homology

3. Specific serum screen
• A pool of serum from 9 subjects allergic to kiwi fruit and individual sera from 5 patients was used to investigate:
  a. **IgE binding against proteins in gold kiwi:**
   • IgE from kiwi allergic subjects binds to proteins in gold kiwi (Figure 8.3)
  b. **Cross reactivity by Western Blot Inhibition**
   • Green and gold kiwi extensively cross react (Figure 8.5)

The Decision Tree then recommends a serum screen. The protocol for specific serum screening suggests using sera from 8 patients with allergy to the source food who have a specific IgE of >10 IU/l (Joint FAO/ WHO Consultation, 2001). Sera from >100 patients with symptoms suggestive of allergy to green kiwi fruit were donated to this study, but only two have a specific IgE >10 IU/l. The practicality of obtaining sufficient quantities of appropriate sera for testing will need to be addressed. This was highlighted by the ACNFP assessment of Chia seed, *Salva hispanica* (ACNFP 64/13). The applicant was unable to satisfy the request of the panel to investigate cross-reactivity in patients with sesame and mustard seed allergy. However, in our study, a screen using serum from patients with allergy to green kiwi fruit clearly confirmed the immunogenicity of the novel fruit in this population despite low specific IgE. Finally the allergenicity of *Actinidia chinensis* was confirmed *in vivo*, four of our five patients reacting during a DBPCFC. Using the gold standard investigation for food allergy, this demonstrated that patients allergic to green kiwi fruit are at high risk of allergy to gold kiwi.

SDS-PAGE confirmed distinct differences between green and gold extracts as described by Bublin *et al* (Bublin, 2004), including the increased intensity of higher molecular weight proteins in gold extract. The lack of abundant actinidin in the gold fruit allows other proteins to be relatively more prominent in SDS-PAGE. The absence of actinidin in gold kiwi fruit has been previously described (Bublin, 2004; Nishiyama I, 2002). Selecting proteins from a 2D gel, we have used electrospray ionization NanoLC tandem mass spectrometry to obtain fragmentation data for peptides and obtained *de novo* sequence information. An abundant protein in the green fruit with molecular weight ≈30 kDa and pI≈3 was confirmed to be actinidin using a BLAST search of the NICBnr database. There was no corresponding 30 kDa/ pI 3 protein in the gold fruit. However, a much less abundant protein, with similar molecular weight but basic pI in gold fruit had significant sequence alignment with actinidin, and is likely to be the same protein with post-translational modification.

The Western blots suggest that IgE in sera from patients with allergy to green kiwi fruit recognise differing proteins in the two fruit, but the inhibition studies indicate that these proteins have extensive cross-reactivity. The DBPCFC results support the conclusion that the *in vitro* cross reactivity has clinical relevance.

To date there have not been any confirmed reports of allergy to gold kiwi in the community, perhaps because individuals with kiwi allergy are successfully avoiding this fruit. However, in a fruit salad, chopped gold kiwi looks like melon rather than kiwi fruit. It is important for consumers to be aware that the fruit is marketed as Zespri[TM] Gold and labels do not refer to kiwi. If the popularity of this novel food increases in a similar fashion to the traditional kiwi fruit, it is likely that reports of allergic reactions to *Actinidia chinensis* will increase accordingly.

9   Conclusions and plans for future works

9.1   Summary of the contribution of this study to knowledge concerning kiwi fruit allergy and food allergies in general

At the start of this project, kiwi fruit allergy was not considered important by most health professionals, including allergists. The questionnaire responses highlighted that kiwi fruit allergy is increasingly common, that anaphylaxis is not uncommon particularly in the very young, and that many patients have been unable to access appropriate care and advice. During the course of the research a paper from France confirmed the high prevalence of kiwi allergy, which was reported more commonly than peanut allergy in French school children (Rance, 2005).

The original aims of the study (1.4; aims 1 & 2) were to describe the characteristics of kiwi fruit allergy and to investigate the use of clinical investigations in the diagnosis of kiwi fruit allergy. These two aims have been accomplished, the data published (Lucas, 2004) and the results discussed in chapters 3 and 4. The findings of the descriptive study were used to derive hypotheses which formed the basis of the rest of the thesis (chapters 5-8).

9.1.1   Description of the characteristics of kiwi fruit allergy in the UK

This was the first survey of kiwi fruit allergy in the UK. The characteristics of the allergy were very different from those gleaned from previous reports mostly of small groups of patients from central and Southern Europe, who were often selected because of their cross-reactivity with birch pollen and latex allergens. Kiwi fruit was a pertinent allergen to study, because of its relatively recent introduction onto the UK market. It could therefore be used as a model of how allergy to a new food might develop in a previously unexposed population. For example, the pattern of consumption of a food reflects its novelty. In the 1970's kiwi fruit was considered exotic and was predominantly eaten on an occasional basis by adults. It is now readily available and is eaten regularly by children (Mori School Survey 2001). The most striking results from the questionnaire

study (Chapter 3) suggest that kiwi allergy is increasingly common, particularly amongst children, often resulting in systemic reactions including anaphylaxis. This probably reflects the increase in kiwi consumption, and highlights the fact that a food might not be recognised as a potential allergen whilst its consumption remains relatively low in a population. Post marketing surveillance of new foods therefore needs to be long term.

The severity of reactions experienced by young children may simply reflect that they represent a population with a strong and increasing allergic predisposition, and as such are more likely to have severe reactions. The *in vitro* studies showed no difference between the protein bands that bound IgE from adults and children, or by severe reactors and those with mild reactions to account for the different symptoms. However, an interesting finding was discovered by the *in vitro* digestion work. Pepsin digestion of kiwi proteins was strongly inhibited at high pH, > 2.5. Several of the children on this study who reacted with anaphylaxis, were initially fed kiwi as an early weaning food when gastric pH may still be higher than in adults. This could account for sensitisation to pepsin sensitive proteins, as they will get to the GI tract intact in infants. Another explanation for sensitisation to pepsin sensitive allergens may be sensitization by non-enteral exposure. Numerous skin preparations containing kiwi extract are available, potentially causing sensitisation to pepsin labile and stable epitopes (Lack, 2003; Strid, 2005). One would anticipate that sensitisation to labile proteins would not result in subsequent systemic reactions, but if gastric acidity was high or absorption of allergens occurred before reaching the stomach (Walzer M, 1942), we could postulate mechanisms by which systemic reactions could occur.

9.1.2 The Use of Clinical Investigations

This study has highlighted some of the problems associated with investigating allergy to a food that contains many labile allergens. Commercially available skin test solutions (Alyostal) and measurement of specific IgE antibodies (FEIA-CAP, Pharmacia) had poor sensitivity rendering them inappropriate for clinical use. Prick-to-prick testing with fresh fruit had excellent sensitivity, but less

specificity, perhaps because of CCDs or other clinically irrelevant cross-reactive epitopes. To overcome this there is increasing interest to investigate the use of recombinant allergens in the diagnosis of allergic diseases (Nowak-Wegrzyn, 2003). Their use in food allergy diagnosis should offer improved specificity, particularly in patients with allergy to pollens, although diagnostic sensitivity will be lower than with natural allergen extracts because minor allergens will not be detected. An Austrian group is currently developing a recombinant form of actinidin, for the diagnosis of kiwi fruit allergy. Based on the reports of Pastorello (Pastorello, 1996; Pastorello, 1998) suggesting that actinidin is an allergen in 100% of patients with kiwi fruit allergy, this development appeared sensible. However, I now believe that actinidin is not an allergen, and am certain that patients in the UK would not react to recombinant actinidin skin test solution.

The DBPCFC is the gold standard for diagnosing food allergy. However, this study highlights various issues including interpretation of subjective symptoms particularly when using the same protocol for patient with systemic reactions and OAS. Symptom diaries were used for each challenge, allowing interpretation of signs and symptoms by two researchers blinded to the sequence of active and placebo doses. Subsequent to the clinical studies, a report from Sweden stated that symptom diaries provide good inter-observer reliability when interpreting subjective symptoms during DBPCFCs (Gellerstedt, 2004). However, interpretation was often not straight-forward with the protocols reported in this thesis, and using strict criteria, many of the challenges were labelled as 'inconclusive'. The DBPCFC was designed to be uniform for all patients, and patients will OAS therefore had the same protocol as those with a history of systemic symptoms. In retrospect, protocols designed individually for patients according to their previous history would be safe, informative and easier to interpret. In particular, a wider dose interval should be used for patients with OAS eg 1 hour. Further work to investigate objective measures of oral reactions, eg. by measuring different mediators in the saliva should also be conducted.

9.1.3 Hypothesis 1:
Allergy to kiwi as a result of cross reactivity produces only mild symptoms such as oral allergy syndrome, while de novo sensitization leads to a full range of reactions including anaphylaxis.

This hypothesis was formulated in an attempt to explain why a significant proportion of patients in this study, had systemic reactions, but most previous reports, mostly of patients with pollinosis, reported predominantly localized oral symptoms.

The finding that subjects with seasonal rhinitis or allergy to pollens reported similar rates of systemic symptoms to those without pollen allergy refutes the hypothesis. There was no difference in IgE binding patterns between patients with pollinosis and those without, suggesting that there is no difference in the protein profiles to which these two groups are sensitized. To complete the picture, it would be interesting to examine whether epitope binding to the various proteins is different in patients with and without pollen allergy. However the inhibition Western blots (chapter 6) indicate that cross-reactivity of kiwi fruit allergens with pollen allergens, including CCDs, is not clinically relevant in the UK.

These data are at odds with all previously reports (Fahlbusch, 1998; Moller, 1997b; Pastorello, 1996; Voitenko, 1997). In part, this may be due to selection criteria of patients and inappropriately defining allergy by immuno-reactivity (see 6.3), resulting in reports of clinically irrelevant *in vitro* cross reactivity by some studies. However, a genuine difference between patients in the UK and those from continental Europe is supported by prevalence data (6.3). The difference is difficult to explain, but possible reasons could be differences in the pollen load, pollen diversity or genetic differences.

9.1.4 Hypothesis 2:
The characteristics of the responsible allergens including lability to pH and digestion, determine the severity of symptoms.

It has previously been hypothesised that that clinical severity of symptoms is due to lability of allergens, but this is the first published work to confirm the association by comparing IgE binding to digested food using sera from patients with OAS and with systemic symptoms. The pooled IgE from patients with systemic symptoms recognised epitopes that were digestion resistant, and interestingly, new epitopes which bound IgE by systemic reactors were revealed during digestion (7.3.2). This is important because it highlights that digestion does not just result in loss of allergenicity by breakdown to smaller fragments. The process is dynamic and it is possible that as larger proteins are broken down, the peptides can then aggregate or dissociate. There is therefore the potential for formation or unmasking of new epitopes as evidenced by these results.

Pepsin digestion of kiwi fruit proteins was effected by the acidity of SGF. Reducing the acidity to pH 2.5 from 1.5 strongly inhibited pepsin activity, presumably increasing the sensitization capacity of the protein. These data support the *in vitro* findings of a study investigating digestion of the allergen parvalbumin (Untersmayr, 2005b). This is not only relevant for patients receiving antacids, but will have implications for other groups with gastric hypoacidity. Newborns have a gastric pH≈4 and perhaps this hypoacidic state is responsible for the increased sensitisation observed in infants who are weaned early.

SDS-PAGE analysis showed that the majority of kiwi fruit proteins were pH stable (chapter 7).

SDS-PAGE of food extracts, stained with Coomassie is advocated to assess lability of novel proteins by the WHO (Joint FAO/ WHO Consultation, 2001), as

a predictor of allergenicity. However this study highlights that staining with Coomassie is not sufficiently sensitive as demonstrated by IgE binding to allergens not visible on the stained gels. It is therefore important to address the stability of allergens in relation to IgE reactivity. However, for novel foods suitable sera may not be available.

The *in vitro* techniques used have limitations. When using Western blots, pepsin obscures IgE binding to allergens with molecular weights ≈32-45 kDa. The digestion technique itself is far from physiological. Under *in vivo* conditions mechanical churning by the stomach, the presence of other foods and physiological changes in pH are all likely to effect protein digestion. Animal models might provide better insights. For example, with a pig model, gastric pH, concurrent foods, and protein load could all be controlled and ileal sampling compared to blood levels of allergens.

9.1.5 Hypothesis 3:
 Gold kiwi fruit is not allergenic in subjects with kiwi fruit allergy.

This hypothesis was proposed because gold kiwi fruit, a novel fruit to the UK, contains very low amounts of actinidin (Nishiyama I, 2002). However, as reported in chapter 7, it subsequently transpired that actinidin is not an allergen in the UK, and it is therefore not surprising that patients with kiwi fruit allergy reacted to gold kiwi.

This was the first study to report the immunogenicity of gold kiwi in patients with kiwi fruit allergy (Lewis SA, 2003), and the findings were later confirmed in a study from three centres in continental Europe (Bublin, 2004). Importantly the clinical relevance of the *in vitro* findings were confirmed by DBPCFC (Lucas JSA, 2005).

9.1.6 Summary of relevant findings not addressed by the original hypotheses of the study

In addition to the contribution to medical science addressed by the hypotheses, the study has added considerably to the current knowledge base regarding kiwi fruit allergens, and in the wider food allergy context, the findings highlight inadequacies in our marketing surveillance of novel foods and question our definition of a major allergen.

9.1.6.1   Kiwi fruit allergens

Actinidin is not a kiwi fruit allergen in the UK. Having performed Western blots and ELISAs using kiwi fruit extract, native actinidin and purified actinidin, followed by inhibition studies, there is no evidence of IgE-binding to actinidin in 76 patients with reported kiwi fruit allergy. Actinidin was first described as the major allergen in kiwi fruit by Pastorello (Pastorello, 1998), reportedly recognized by IgE from 100% of her study population. As discussed in chapter 5, there are methodological issues to explain incorrect reporting of this protein as an allergen. I have been unable to acquire sera from the Italian study population or from others where actinidin has been reported as a major allergen (Bublin, 2004) to investigate actinidin reactivity using my methods.

A 38 kDa protein was the major allergen in this population and on going studies in collaboration with HortResearch will identify this protein.

9.1.6.2   Lessons for pre- and post-marketing surveillance of novel foods for allergenicity

It is a requirement that novel foods, both GM and non-GM should be assessed by the Advisory Committee on Novel Foods and Processes (ACNFP) prior to acceptance for UK marketing under the EC Novel Food Regulation (EC 258/97). Gold kiwi fruit (Zespri Gold- *Actinidia chiensis*) was introduced into the UK in 2000. Gold kiwi fruit is a non-GM novel food but it was not presented to the ACNFP for consideration.

The WHO/FAO Decision Tree (Joint FAO/ WHO Consultation, 2001) (Figure 1.1) has been recommended as a tool for assessing the potential allergenicity of novel GM-foods. Kiwi fruit are not GM, but since they are a relatively new food in the UK, they can be used as a model to determine whether the decision tree would have identified kiwi fruit as a possible allergen. Proteins in kiwi fruit have homology with known allergens (5.3.1), and using the suggested criteria (Joint FAO/ WHO Consultation, 2001), kiwi fruit would therefore be correctly predicted to contain allergens. Using sera from patients with kiwi fruit allergy in a targeted screen, IgE bound to various proteins in gold kiwi. This step in the decision tree would have correctly identified gold kiwi as a potential allergen. However, the methods accepted for testing allergen stability to pepsin, using SDS-PAGE and Coomassie staining of digested extracts, would predict that all proteins in kiwi are digestion labile, and are therefore low risk for causing systemic reactions. Only by using appropriate sera for Western blotting did it become clear that epitopes recognised by patients with systemic symptoms persisted for at least an hour in SGF. Also, modification of the protocols are required to incorporate digestion studies at a range of different pH. This is particularly important for any food that might be used during weaning.

Therefore the current recommendations for predicting allergenicity would have identified both green and gold kiwi fruit as likely sources of allergens, but the recommendations for digestion studies are not adequately sensitive. In the case of novel foods, sera for Western blotting may not be available, and other methods of addressing pepsin stability may be necessary eg. staining with more sensitive stains.

There is currently no post-marketing surveillance (PMS) for food allergy in the UK. Kiwi fruit allergy was first reported in the medical literature in 1981, but 14 study patients reported that their first reaction was prior to this. In the absence of a national PMS strategy it is not possible to identify allergy to a new food as a significant problem until it a large number of people have developed the problem. Even a large allergy clinic will not register it as a problem at an early stage. A PMS programme would need to be long term. Cases of allergy may

remain rare until the food is regularly consumed by a significant proportion of the population.

This study also highlights the inadequate resources available to people with allergy in the UK. Only 50% of subjects who had a life threatening reaction had been seen by an allergist. This is likely to underestimate the problem, since the allergy clinics in Southampton were used as a source of recruits.

9.1.6.3 Identifying major allergens

This thesis highlights the need for improved monitoring of the naming of major allergens. The definition of a major allergen is that it is recognised by the IgE antibodies of sera from more than 50% of patients who have an allergy to the whole product. Individual authors can then claim to have identified a major allergen when they have done a study on their own population of patients but there is no minimum number of patients to be studied before the claim can be made, and the findings can be restricted to one study population. Inadequate blocking (Pastorello, 1996), lack of negative control sera, or ignoring non-specific binding by negative control sera will result in proteins being incorrectly defined as allergens. Therefore stricter peer review is required of the methods used to identify the allergen.

Furthermore, the numbering goes in order of publication and as in the case of actinidin, the first allergen may in the long run not turn out to be the most important and this in itself creates confusion and problems.   The consequence is that there is room for major error as demonstrated in this thesis.

Actinidin cannot be claimed to be a major allergen given that none of our patients react to it. This is irrespective of whether the other groups that have identified it as a major allergen can be shown to have valid data.   Therefore actinidin should not be called Act c 1.

On-going studies will soon identify the 38kDa allergen. Other groups will then need to determine whether it is relevant in their population.

It is clear there has to be some form of exchange of sera between groups in order to validate observations. Attempts have been made to collaborate with other groups studying kiwi fruit allergy, but exchange of sera has not occurred to date.

9.2 Future work

1. If consumer confidence is to be maintained the least that should be expected is that all new foods are assessed and a prediction of their allergenicity made. On the basis of the assessment a decision regarding appropriate labeling should be established for each food. Post marketing surveillance (PMS) should then be used to confirm the findings of pre-marketing assessment and to identify unexpected late health events. PMS is needed to communicate information to the food industry and the public and to improve labelling. The findings of this study highlight the need for long term surveillance. Consumer habits may change with time resulting in food being eaten in different forms, quantities or by different groups (eg. Children).

    A PMS strategy is required in the UK. It should not be restricted to new foods because allergy may become more prevalent to an established food due to changes in its preparation/ consumption frequency etc. There are various options as to which food allergens are reported, but novel foods would be highlighted. The clinician would provide data concerning patient details, food type, allergic symptoms and confirmatory tests. This would be a long term surveillance program. A pilot scheme for reporting of new allergies is therefore required.

2. The care of patients with food allergies must improve. Over 50% of patients on this study who had suffered severe reactions had not been seen by an allergist. 'Allergy: the unmet need' (Report of the Royal College of Physicians Working Party on the provision of allergy services

in the UK, 2003) has highlighted the dismal failing to meet minimal standards of care for people with allergies. The Department of Health has still not started to act on the recommendations of the report. Also, evidence base for treating patients with food allergies remains poor, and the current management continues to be avoidance of the index food with appropriate rescue treatment incase of accidental exposure.

It is imperative that political pressure is maintained to improve allergy services in the UK. In addition, clinicians and scientists have responsibility to continue researching the use of new therapies that may cure food allergies. For example, a recent clinical trial showed that monthly injections of humanized recombinant anti-IgE antibodies increased the threshold for allergic responses of peanut-sensitive individuals (Leung, 2003). Safety and efficacy trials are currently underway for the use of anti-IgE in adults and children with peanut allergy. However, this treatment cannot cure food allergy, and continuous monthly injections are necessary to maintain protection.

3. The 38 kDa kiwi fruit allergen needs to be identified. To increase the relative amount of 38 kDa in the protein extract, actinidin will be removed by immunoprecipitation using anti-actinidin antibody. It should them be possible to elute the 38 kDa from a SDS-PAGE gel for analysis using proteomic techniques. Ideally, sera from kiwi allergic patients in different populations then needs to be tested for IgE to the 38 kDa allergen.

4. Differences in IgE binding to digested kiwi by systemic and oral reactors needs to be confirmed using sera from individual patients. The work will then need to be repeated with other food allergens, comparing sera from patients with oral and systemic reactions. If the preliminary findings reported in this thesis are confirmed in different populations and to different foods, it may provide the basis for the first test to predict which patients may proceed to have severe reactions.

Information concerning gastric pH in different ages and disease states is sparse. Normal data referring to physiological changes in pH depending on diet etc is also lacking. For example, are undigested allergens more likely to reach the gut if kiwi is eaten following an alkaline glass of milk? Therefore basic gut physiology needs to be clearly defined before *in vitro* results can be interpreted.

The methods for assessment of lability as outlined in the WHO Decision Tree need to be standardized. Also, more sensitive methods of detecting potential allergens in the digested food need to be established. I was able to identify digestion stable epitopes by using allergic sera despite no proteins being visible by Coomassie staining. For novel proteins appropriate sera may not be available, and alternative sensitive methods therefore need to be established.

5. This study has identified many problems with the clinical investigation of food allergy. In particular, food challenges for patients with subjective symptoms are difficult to interpret, but in patients with severe symptoms challenges may provoke life-threatening reactions. Development of the pilot study involving measurement of oral mediators following a buccal challenge may prove fruitful. Measurement of histamine was not helpful, but other mast cell mediators such as tryptase and prostaglandins need to be investigated.

9.3 Summary

This project has been exciting and productive. At the start, information concerning kiwi allergy was sparse, but with increasing reports of reactions the field has become highly competitive and fast moving.

The study has changed attitudes to kiwi allergy, which is now recognized as potentially life-threatening. It has also highlighted some of the difficulties with diagnosing food allergies. Not only does the work question the role of actinidin

as an allergen, but it exposes the faults with guideline for labeling of major allergens. The *in vitro* studies using digested kiwi fruit are exciting because they provide the first evidence to confirm long held hypotheses concerning the differences between people with OAS and systemic reactions, and perhaps provide us with theories to explain why young children become sensitized to allergens.

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