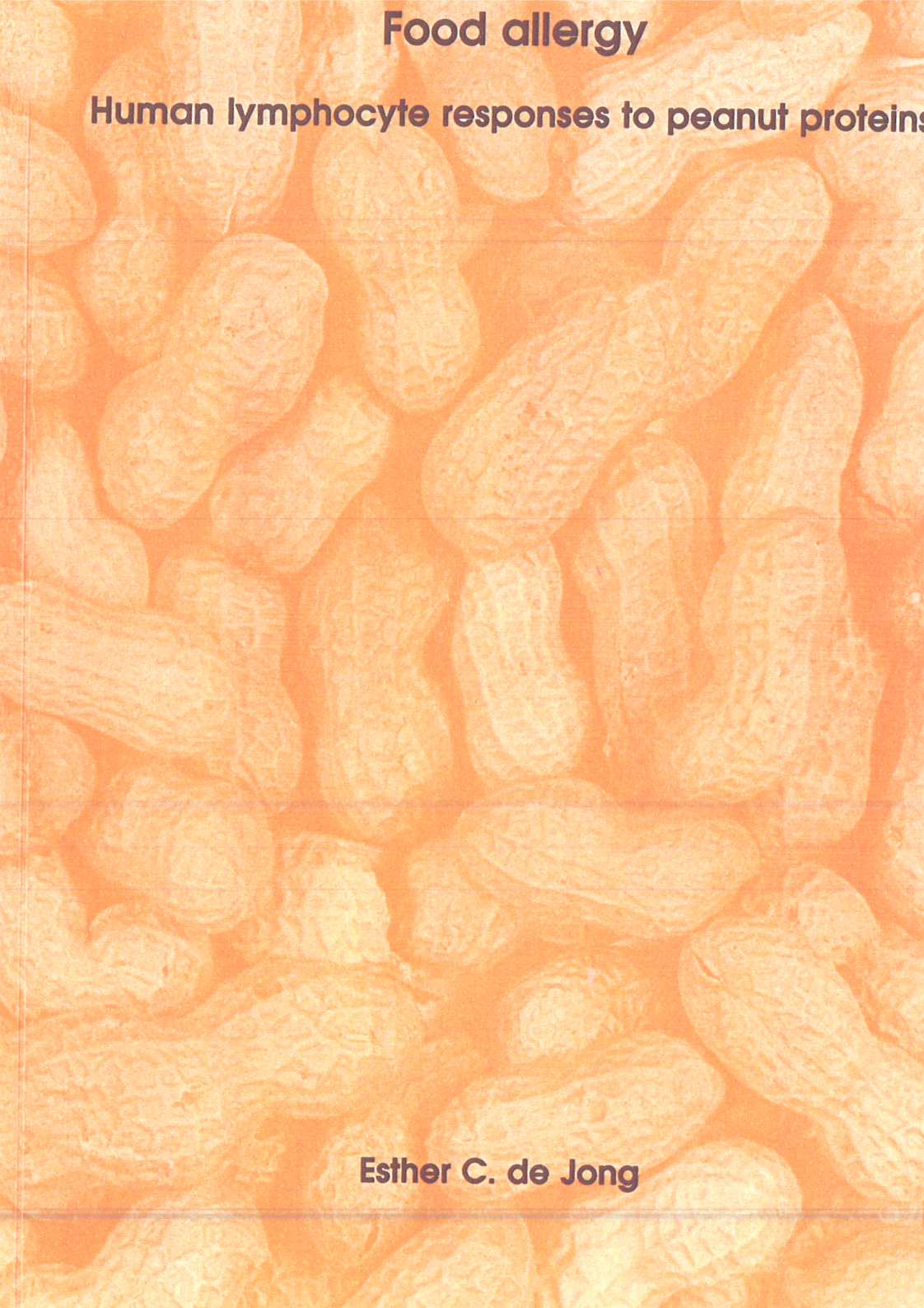
Food allergy

Human lymphocyte responses to peanut proteins



Esther C. de Jong

Food allergy
Human lymphocyte responses to peanut proteins

Voedselallergie
Responsen van humane lymfocyten op pinda-eiwitten
(met een samenvatting in het Nederlands)

No reprints available.

Proefschrift ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel
ingevolge het besluit van het College van Dekanen
in het openbaar te verdedigen
op vrijdag 19 januari 1996 des morgens om 10.30 uur

door

Esther Christina de Jong

geboren 26 februari 1965, te Amsterdam

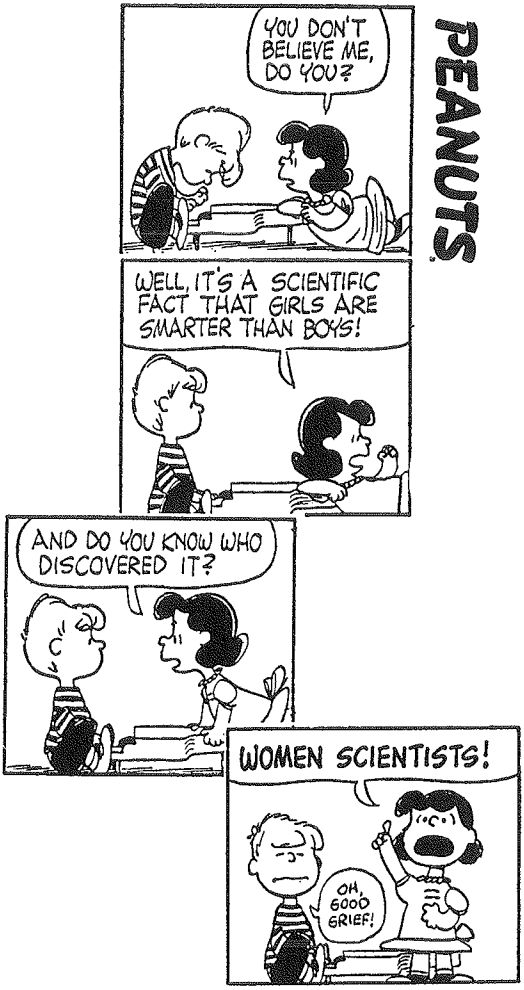
Promotor: Prof.Dr. W. Seinen (RITOX, Universiteit Utrecht)
Co-promotores: Dr. A.H. Penninks (TNO Voeding, Divisie Toxicologie)
Dr. M.L. Kapsenberg (Laboratorium voor Celbiologie en Histologie, Universiteit van Amsterdam)

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Jong, Esther Christina de

Food allergy : human lymphocyte responses to peanut proteins / Esther Christina de Jong. - Utrecht : Universiteit Utrecht, Faculteit Diergeneeskunde
Thesis Universiteit Utrecht. - With ref. - With summary in Dutch.
ISBN 90-393-1182-X
Subjects headings: food allergy / peanut ; human lymphocytes responses

Omslag-foto: M. van der Vaart
Drukwerk: Ponsen & Looijen, Wageningen



Aan mijn moeder en T.E.

Referents

Prof.Dr. R.C. Aalberse, Afdeling Allergie, Centraal Laboratorium voor Bloedtransfusie Dienst (CLB)

Prof.Dr. C.A.M.M. Bruijnzeel-Koomen, Afdeling Dermatologie, Academisch Ziekenhuis Utrecht

Prof.Dr. P.J.A. Capel, Vakgroep Immunologie, faculteit geneeskunde, Universiteit Utrecht

Prof.Dr. J.G. Vos, Laboratorium voor Pathologie, RijksInstituut voor Volksgezondheid en Millieuhygiëne, Bilthoven

Dr. H.F.J. Savelkoul, Afdeling Immunologie, Erasmus Universiteit Rotterdam

The studies described in this thesis were carried out at TNO Nutrition and Food Research Institute, Division of Toxicology and Division of Occupational Toxicology and Nutrition (Zeist, the Netherlands). Financial support for the publication of this thesis was provided by TNO Nutrition and Food Research Institute, Utrecht Toxicology Center (UTOX), van den Bergh Foods, van der Laar stichting and HAL Allergenen Laboratorium B.V..

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CONTENTS

ANPA:	allergic but not peanut-allergic
APC:	antigen presenting cells
BSA:	bovine serum albumin
CD:	cluster of determination
CPE:	crude peanut extract
cpm:	count per minute
DAB:	diaminobenzidine
DBPCFC:	double blind, placebo controlled food challenge
DEAE:	diethylaminoethyl
DNA:	deoxyribonucleic acid
DTT:	dithiotreitol
EBV-B:	Epstein Barr virus transformed B cells
ELISA:	enzyme linked immuno-sorbent assay
FCS:	fetal calf serum
FITC:	fluorescein thiocyanate
HDM:	house dust mite
HLA:	human leucocyte antigens
HPLC:	high performance liquid chromatogrphy
HS:	human serum
(^{3}H)-TdR:	tritiated thymidine deoxyribose
IMDM:	Iscove's Dulbecco's modified medium
IFN:	interferon
Ig:	immunoglobulin
IL:	interleukin
LST:	lymphocyte stimulation test
MHC:	major histocompatibility complex
NA:	not-allergic
o/n:	over night
PA:	peanut-allergic
PBMC:	peripheral blood mononuclear cells
PHA:	phytohaemagglutinin
PMA:	phorbol myristate acetate
PNA:	Peanut agglutinin
PO:	peroxidase
PVDF:	polyvinylidenedifluoride
r:	recombinant
RAST:	radio allergo-sorbent test
RT:	room temperature
SDS-PAGE:	sodium dodecyl sulphate-polyacryl gelelectrophoresis
SEM:	standard error of mean
SI:	stimulation index
SPT:	skin-prick-test
Th0, Th1, Th2:	T helper cells type 0, 1, 2
TMB:	3,3',5,5'-tetramethyl-benzidine
USF:	unheated soy flour

Chapter 1

General Introduction

Adverse reactions to foods

Adverse reactions to foods are increasingly being recognized as important medical problems and are the subject of confusion and controversy. Foods or foodstuffs may consist of three major groups of components: the food itself, either native or processed, additives such as dyes, preservatives, stiffeners etc, and contaminants. All these groups of components can cause adverse reactions. These adverse reactions to foods can be classified in several types of reactions (1-3). As shown in Figure 1, adverse reactions can be divided in toxic, non-toxic reactions and aversions. The non-toxic reactions (hypersensitivity) to foods are classified either as immune-mediated (food allergy) or non-immune-mediated (food intolerance). Several types of food intolerance reactions have been recognized based on the knowledge of their working mechanism. According to the classification of the European Academy of Allergy and Clinical Immunology (EAACI) subcommittee on food hypersensitivity, food allergic reactions can be divided into IgE-mediated and non-IgE-mediated reactions. With respect to the non-IgE-mediated reactions, no real evidence is available for a causative role of these type of reaction in food allergy.

The majority of hypersensitivity reactions to foods are mediated by non-immunologic mechanisms. However, most of these reactions are very mild and considered to be normal, and as such should not be considered a hypersensitivity reaction, as for example the diuretic effect of coffee-derived caffeine.

The definitions of adverse reactions to foods are given in Table 1.

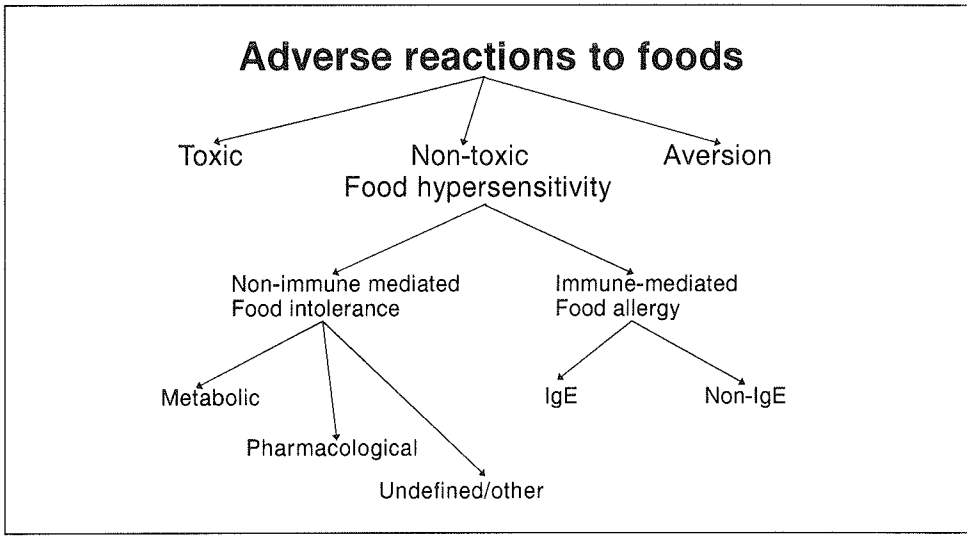


Figure 1: Schematic representation of adverse reactions to food.

Toxic reactions to food

Foods may contain many toxic substances which can induce a wi variety of symptoms. Food toxicity may affect all organa and tissu especially the liver and kidneys and can be caused by naturally occurri toxins, contaminants and additives or by toxins induced during fo processing (4, 5). A toxic reaction to food is discriminated from a hyperse sitivity by the fact in case of food toxicity every exposed individual show symtoms if the dose is high enough whereas a food hypersensitivity host-dependent. Toxic reactions may be acute but may also becor apparent after some time as for instance toxin-induced tumors. A w known example of a contaminating toxin in foods is aflatoxin, a to produced by microorganisms in peanuts and meats, which can cau gastro-intestinal problems but also severe effects such as liver tumors.
Since toxic reactions to food are not part of this thesis they will not discussed further.

Aversions

If a person reacts to a certain food only when that person is aware suspicious of its presence, then this reaction is probably not based on

Table 1: Definitions of adverse reactions to food (adapted from Bruinzeel-Koomen et al. (3)).

Term	Definition
Adverse reaction	An undesired response to ingested food
Food toxicity/poisoning	An adverse reaction following food ingestion that is due to a natural component or additive of the food, toxins induced by food processing or contamination of the food by microorganisms or their toxins. In general, toxic reactions will occur in any exposed individual if the dose is high enough
Food aversion	An adverse reaction to a food which is due to a psychologic cause
Food hypersensitivity	An adverse reaction to a food which is not so much caused by the food itself, but rather by a specific trait and susceptability of the individual. Food hypersensitivity includes both food allergy and food intolerance. However, the term is often used as a synonym for food allergy
Food allergy	A hypersensitivity to the ingestion of a food in which the immune system plays the primary role
Food intolerance (FI)	A hypersensitivity to an ingested food in which the immune system does not play a primary role. This category includes pharmacologic, metabolic and other or undefined reactions
Pharmacologic FI	A druglike or pharmacologic hypersensitivity in the host recipient as a result of compounds in the ingested food
Metabolic FI	A hypersensitivity to the ingested food caused by a metabolic disorder of the host
Undefined or other FI	An intolerance with an unknown or other mechanism

physiological mechanism but rather has a psychologic origin, that may still result in a physiological reaction. This form of adverse reactions to foods

can be distinguished from a hypersensitivity to food in a so called doub blind, placebo controlled food challenge. In such a test, the complaints the patient suffering from a psychologic based adverse reaction to fo will not be confirmed. A patient suffering from a food aversion may th that he or she is allergic or that the food is wrong for him or her because is fattening (14). This kind of adverse reactions can be treated with medi care in combination with psychiatric help (15). However, in many cas the occurence of an aversion is due to insufficient knowledge and awa ness of safety assessment of food products.

Food intolerance

Many of the adverse reactions to food do not involve the immune syste Food intolerance, defined as a hypersensitivity in which the immune-syste does not play a primary role, is characteristically described by a positi history of adverse reactions to the offending food and/or a provocati test that clearly shows a causative role with the food but without a evidence that the immune system plays a primary role (6). Food intol ance is divided into metabolic, pharmacological and undefined or oth intolerance reactions.

In metabolic food intolerance the adverse reaction to foods is caused instance by an enzyme deficiency or defect (genetically) of the patie which affects the ability to metabolize a food component or enhances t sensitivity to a food-born chemical. An example is lactose intoleran observed in the Western population and which is due to a deficiency intestinal β-galactosidase (7).

Pharmacological food intolerance can be caused by different types substances. Vasoactive amines, as for instance histamine which can found as a nutural constituent of foods, may cause allergy-like sympto (8). Moreover, histamine releasing factors, which can be found in strawb ries, shellfish, tomatoes, citrus fruit, chocolate and pork, can cause ph macological reactions (9).

Intolerances of which the mechanism is not clear are categorized unc undefined reactions. Many of the reported adverse reactions to foods c placed in this category because their modes of actions are not und stood, such as the suggested causative role of chocolate in migraine (or the role of food colouring agents suspected of adverse behaviour rec tions (11, 12). However, in some cases specific IgE to food colouri

agents has been documented (13), yet the relevance of these findings is still unclear.

Food allergy

The term "food allergy" is used when a hypersensitivity reaction that occurs after the consumption of certain foods is primarily immune-mediated. This reaction is mediated by immunocompetent cells and antibodies reacting specifically to foods which may cause functional and/or inflammatory reactions in target tissues which may become chronic and induce tissue damage. These immunological hypersensitivity reactions can be categorized in type I, II, III and IV reactions as described by Coombs and Gell. The majority of food allergic reactions are classified as type I, IgE-mediated allergies. However, it has been suggested that other types of hypersensitivity reactions may also occur in food allergy.

Non-IgE mediated immunopathological mechanisms

Several food-related diseases were suggested to be food allergic reactions which are not characterized by IgE but belong to one of the other 3 categories. However, in most cases the causative role of these reactions is not clear and the mechanism of the disease not well-understood. Because these types of reactions are not the subject of this thesis, they are discussed only very briefly.

A type II reaction is characterized by antibody (IgG) dependent cytotoxicity of target cells. This reaction has been demonstrated in children with cow's milk hypersensitivity, as well as milk-induced thrombocytopenia (16, 17).

Damage caused by immunecomplexes is the main feature of a type III allergic reaction. The circulation of immune complexes containing food antigens and specific antibodies is a common and normal phenomenon. These complexes occur approximately 30 minutes after food ingestion. In healthy individuals these complexes reach a relatively low level and are cleared from the blood fairly fast. In food hypersensitive patients, however, these immunecomplexes reach higher levels and circulate longer. Moreover, they can contain IgE in association with anti-IgE antibodies, instead of

IgG or IgA as seen in healthy individuals. An example of this phenomeno occurs in coeliac colitis (18, 19).
Cell-mediated food allergic reaction or delayed type hypersensitivity (Typ IV) to gliadin, found in gluten, could also be a mechanism involved i coeliac disease (20-22), although this is not clear. Delayed type hyperser sitivity has also been suggested to play a role in cow's milk allergy an other food-allergies (23) because delayed type hypersensitivity reactior have been observed to occur in combination with an IgE-mediated foo allergy.
Food-specific IgG4 antibodies are frequently detected in food-allergi patients (18, 24). It has been demonstrated that human IgG antibodie directed to milk proteins can also be anaphylactic as proven by passiv cutaneous anaphylaxis with monkeys as recipient suggesting that IgG antibodies may also be anaphylactic in humans (25) although this has n been confirmed by others (26).

IgE-mediated food allergy

The Type I or immediate type hypersensitivity reaction to food is characte rized by high serum levels of food-specific IgE and eosinophilea (27-29). Th food-specific IgE can bind to mast cells and basophils via the high affini IgE receptor. At a subsequent contact with the specific food, these Ig molecules will be cross-linked by the allergen which signals the mast cell basophil to degranulate. Degranulation results in a release of mediato such as histamine and other vasoactive amines which may induce symp toms such as swollen lips, vomiting, diarrhea, itching, urticaria and anaph lactic reactions (30, 31).

Food allergens

In theory, every product that is eaten can contain food allergens. Mar foods or food ingredients that can be responsible for a food allergy hav been described. The most observed food allergies are those to cow's mil hen's egg, peanut, soy bean, nuts, fish, seafood and fruits and vegetable (27, 32-37). An allergy to fruit or vegetables is usually not observed until th subject is a few years of age (37). The prevalence of allergies to cow's m and hen's egg tend to decrease with increasing age: 44% of the positiv food challenges in children to cow's milk or hen's egg, were negativ when tested 1-7 years later (32).

Food allergens in general are glycoproteins with a molecular weight between 10 and 50 kD (3, 38). Several studies have shown that cooking or heating a specific food did not result in reduced binding of specific IgE using immunoblotting techniques with serum from food allergic patients. Most food allergens are also relatively acid-stable and resistant to digestive enzymatic break-down (39-42). This indicates that despite cooking and digestion in the gastro-intestinal tract, B cell epitopes of food allergens may enter the body.

Prevalence

Reliable epidemiological data on the prevalence of food allergy are limited. The majority of the studies have been performed in the paediatric population. In a study of Bock et al. (43) in which 480 children were followed from birth upto the third year of life, 28% of the parents thought that their child suffered from a food hypersensitivity, but a food allergy could only be confirmed in 8% of the 480 children. Kajosaari estimated the same incidence of food allergy in young children but the prevalence reduced with age of the children (44). The decreased incidence of food allergy with age, suggests that immaturity of the immune system may be an important factor in the pathophysiology of the disease (32, 45). The incidence of the most common food allergy in young children, cows milk-allergy, has been estimated at 2.5% (46, 47).

Two recent studies (48, 49) have estimated that the prevalence of food allergy in adults is between 1 and 2%. The overassessment by parents of having food allergic children is simularly observed in studies on food allergy prevalence among adults. Several epidemiological studies indicated that approximately 20% of the responders state that they suffer from adverse reactions to foods but only in 20% of these persons a food allergy could be confirmed (46-52). This may indicate that knowledge about adverse reactions to foods is poor and that there is a need for education in this field.

Heredity is considered to be the most significant factor in predisposing individuals to food allergies. Almost 65% of the patients with a clinically proven food allergy has an atopic first degree relative (53, 54). However, in the prevalence of a specific food allergy dietary habits in a geographical region play a role as well. For example, allergic reactions to fish are often seen in Scandinavian countries (55), whereas soy bean allergy is more common in Japan (56).

Clinical symptoms

Food products, which are mainly absorbed in the gastro-intestinal trac can give rise to symptoms throughout the whole the body both in adu and in young children. The clinical manifestations that occur after a patie has eaten the offending food may vary from one patient to the other. Al the time-interval between eating the offending food and the onset symptoms can be extremely different varying from several minutes to da (57, 58). Some symptoms only appear under certain conditions such as e exercise-induced anaphylaxis (59). Table 2 gives an overview of sympton observed in food allergic patients after eating the offending food.

The oral allergy syndrome (OAS), which is the swelling and itching of lip mouth and throat, occurs within 30 min after consuming the offendi food (60). Although OAS has also been observed in response to fish, eg and milk (61, 62), it is mainly observed in response to fruits and vegetable such as apple, peach, hazelnut, peanut and apricot (63).

Gastro-intestinal problems are often observed in a patient with foo allergy. Nausea and vomiting can occur within 30 min after eating th food and is followed by diarrhea (64, 65). This is also often seen in youn children with food allergy and may even lead to malnutrition and structur damage of the intestinal mucosa (66). The skin is one of the most freque target organs of IgE-mediated food reactions mostly expressed as atop dermatitis and urticaria (67, 68). Respiratory symptoms such as wheezin rhinitis, bronchospasm and asthma are also frequently seen in patients wi food allergy, and are often associated with atopic dermatitis (69-71). Thes symptoms usually occur within minutes to a few hours. In some case especially in food allergic reactions to peanuts or nuts, anaphylacti reactions are observed, sometimes leading to a shock. Several patien have died from an anaphaylactic shock due to a food allergy. Initi symptoms may be swelling and itching of the lips which may be followe by a rapid loss of consciousness (72, 73). In young children the symptom can start very mild and just as the symptoms are considered to be impr ving, severe anaphylactic symptoms may develope (74).

Food allergic patients may suffer from one or a combination of the her described symtoms, but also other symptoms can occur such as conjunct vitis or sinusitis.

Table 2: Symptoms that may occur in food allergic patients.

Cardio-vascular:	Anaphylactic (fainting, cardiac arrest)
Oral:	Oral allergy syndrome (itching and swelling of mouth and pharynx)
Gastro-intestinal:	Vomiting, diarrhea, cramps, abdominal pains, bloody stools
Skin:	Atopic dermatitis, urticaria, angioedema
Respiratory:	Wheezing, rhinitis, bronchospasm, asthma
Other:	Cough, conjunctivitis, sinusitis, otitis media

Diagnostic tests

A correct diagnosis of food allergy is often difficult. Although the patient may be confident that he or she is suffering from a food allergy, often the diagnosis can not be confirmed in the laboratory (49-51). This is probably due to the observation that many of these hypersensitivities are caused by an intolerance rather than an allergy, or by an aversion.

For clinical purposes the most used diagnostic tests are the radio-allergo-sorbent test (RAST) and the skin-prick test (SPT). However, the results of such tests may differ from one and other. The problem with these test systems for food allergy lies within the used allergen extracts. Due to limited knowledge of food allergens, food extracts are not well-defined or standardized. For SPT also fresh foods are being used which, especially for fruit and vegetables, give better results than the commercially available extracts (62, 75). Other tests, such as specific IgG measurements, histamine releasing assays and lymphocyte proliferation assays, are also used for the diagnosis of food allergy. However, none of these tests were demonstrated to be highly predictive (76, 77).

The "golden standard" to diagnose a food hypersensitivity is still the double-blind, placebo-controlled, food challenge (DBPCFC) (78). This procedure is time-consuming which has to be performed in a clinical environment for an objective observation of the symptoms and subtle effects, and for the possibility of an anaphylactic reaction. This procedure may be a burden for the patient. Different protocols may be used for this test, but in general the patient is given in unmarked capsules the suspected allergen in several concentrations and a placebo at different time-intervals depending on the

supposed symptoms. The symptoms occurring after consuming the cap sules have to be evaluated carefully. Although the DBPCFC will giv substantial information about the symptoms of the patient, the time c onset of the symptoms and the maximum tolerated dose of the food, does not distinguish between a food intolerance and a food allergy.

The cellular mechanism of IgE-mediated allergy

B and T cells play an important role in the pathophysiology of IgE-mediate allergic disease and differ in antigen recognition. The B cell recognizes large conformational peptide of the antigen, by membrane bound im munoglobulins, whereas T cells recognizes a protein-derived linear peptid of 8-25 amino acids presented in association with a molecule of the maj histocompatibility complex (MHC).

T cells and their cytokines

T cells recognize antigen with the T cell receptor (TCR) present on the ce surface. The TCR is a complex molecule consisting of 2 transmembran poly-peptide chains called α and β (or alternatively γ and δ). Each cha consists of a constant (C) and a variable (V) region. The TCR is constructe after DNA rearrangements leading to a selection of V, J (joining), D (dive sity) and C segments creating a combination product of the α/β comple of more than 10^{16} different sequences, providing an enormous repertoire c receptor specificities (79, 80). After antigen uptake and processing by th antigen presenting cells (APC) the antigen, or rather antigen-derived pep tide, is presented to the T cell in the groove of a MHC molecule which present at the surface APC. $CD8^+$ T cells in general recognize peptide presented in context of MHC class I molecules, while $CD4^+$ T cells recogniz peptides in context of MHC class II molecules (81).

To activate T cells, several complicated interactions between APC and cells are necessary (82, 83). The peptide is recognized in the context of th MHC molecule by the TCR associated with CD3, the latter molecule bein responsible for the signal transduction into the cytoplasm (84). Binding c the MHC molecule to the TCR is augmented strongly by CD4 or CD present on T cells (85). A second, costimulatory signal for T cells can b

delivered by CD28, which is expressed on the majority of T cells, or CTLA-4 which is expressed only on activated T cells (86-88). The ligand for CD28 is B7.1 (CD80) or B7.2 (CD86), which are constitutively expressed on APC types such as dendritic cells, and activated T and B cells (89, 90). Triggering of CD28 stabalizes the mRNA encoding for cytokines and increases the cytokine gene transcription rate (91, 92).

In 1986 Mosmann and Coffman described 2 extreme types of $CD4^+$ T helper (Th) cells in the mouse which are phenotypically and functionally different (93). These T helper subsets have also been found in man (94-96). Both Th types express CD3, CD4, and CD45-RO but they differ in cytokine production after activation by antigen as is shown in Figure 2. Th1 cells exclusively produce interleukin (IL)-2 and interferon-γ (IFN-γ), while Th2 cells produce IL-4, IL-5 and IL-6. IL-10 and IL-13 are produced by both subsets, although in higher concentrations by the Th2 subset. The majority of T helper cells, however, are Th0 cells and produce the Th1 (IL-2 and IFN-γ) and the Th2 (IL-4, IL-5 and IL-6) related cytokines (97). Th1 or Th2 cells may maturate from naive T cells or Th0 cells under the influence of various microenvironmental factors such as cytokines or costimulatory molecules. The presence of IL-4 selectively skews to Th2 cells, while IL-12 and IFN-γ promote Th1 development (98-100). Recently it has been demonstrated that the most potent costimulatory molecules present on APC, B7-1 and B7-2, influence the Th1/Th2 ratio. Stimulation of B7-1 leads to increased production of IL-4 whereas stimulation of B7-2 induces IFN-γ (101). The different cytokine profile of Th cells results in diverse effects on other cells. Th1 cells induce cytotoxicity and delayed type hypersensitivity reaction. On the other hand, Th2 cells promote Ig production, including IgE, by B cells and induce eosinophilea (102, 103).

Antigen presentation.

After uptake of exogenous antigens, such as allergens, by the APC, they are degraded into peptides by intracellular enzymes. The peptide containing lysosomes fuse with vesicles derived from the endoplasmatic reticulum containing MHC class II molecules. The MHC molecules loaded with a peptide are subsequently expressed on the surface of the APC (104, 105). As the CD4 molecule can interact with the MHC class II molecule, exogenous antigens are presented to $CD4^+$ T cells.

The MHC class II peptide can be up to 25 amino acids due to open ends

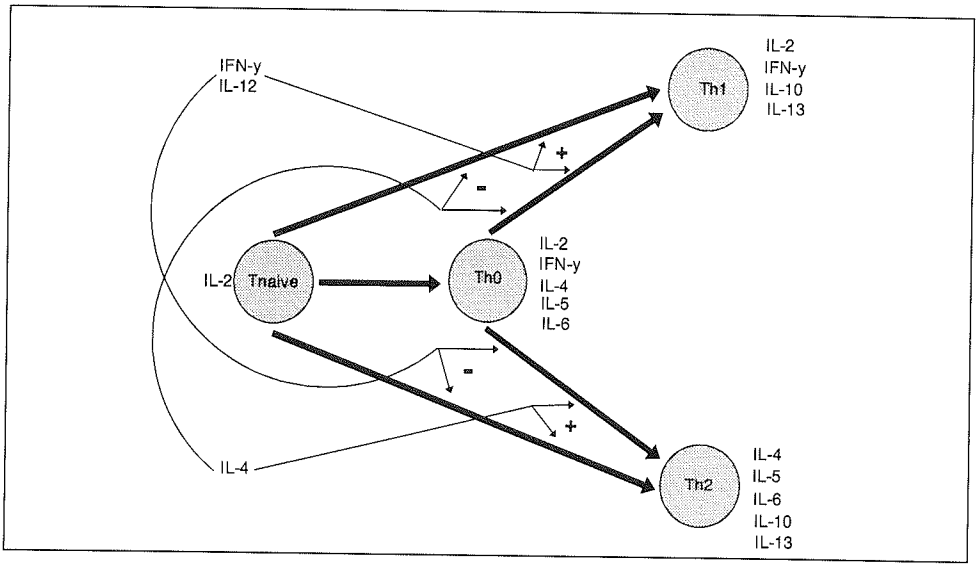


Figure 2: Schematic respresentation of different T helper subsets and the cytokine production.

at both sides of the MHC class II groove (105, 106). Whereas MHC class molecules are expressed by all cell types, the expression of MHC class molecules is restricted to dendritic cells, monocytes, macrophages and activated B-cells. MHC class II molecules are constitutively expressed a high levels by dendritic cells and B cells, but are inducible on other ce types such as monocytes, macrophages, epithelial and endothelial cells.

Role of T cells in IgE-mediated allergy

The last decade it has become clear that T cells play an important role in the pathophysiology of allergic disease. Several studies have revealed tha T cell clones specific for allergens derived from house dust mites (HDM) various pollen and domestic animals, generated from peripheral blood (97 103, 107, 108) or skin (109, 110) of atopic donors, are Th2 cells producing high levels of IL-4 and IL-5 but little or no IFN-γ. IL-4 plays an important role in the regulation of the production of IgE antibodies. Both *in vitro* (111-115) and *in vivo* (116) IL-4 induces an immunoglobulin switch to IgE in B cells which is inhibited by IFN-γ (117, 118). Consequently, allergen-specific Th2 cells are efficient helper cells for IgE synthesis (94, 119-121). Th2 cell-derived IL-5 has been shown to induce eosinophil proliferation and activation (122,

123). IgE can bind to the high-affinity Fcε receptor on basophils and mast cells. When these membrane-bound IgE molecules are cross-linked by allergens after a subsequent contact, a degranulation of basophils and mast cells will occur resulting in a release of mediators such as histamine. This immediate response is followed by a late phase, inflammatory response with an onset after 6-24 hours. This inflammation is characterized by infiltration of activated T cells, eosinophils, basophils and neutrophils in the target organs. The release of toxic mediators by eosinophils and basophils can cause severe tissue damage (124-126).
Also $CD8^+$ T cells play a role in the mechanism of IgE-mediated responses. Allergen-specific $CD8^+$ T cells inhibit the production of IgE although the mechanism is not clear (127, 128). Recently it has been shown that IL-4 can induce a Th2-like cytokine production profile in $CD8^+$ T cells, which can induce IgE-production in the presence of anti-CD40 costimulation. But their role in the IgE-mediated disease is not yet clear. However, these cells are thought to play a role in the protection against parasitic infections but may also play a role as "suppressor" cells or anti-inflammatory cells through the production of Th2-like cytokines (129, 130).

Immunoglobulin switch to IgE in B cells

Naive B cells express membrane-bound IgM or IgD and after recognition of the antigen and secondary signals, these B cells mature into plasma cells and will be able to produce immunoglobulins of other classes. The immunoglobulin switch to IgE is T cell dependent (131). Several studies have shown that the T cell-derived cytokine IL-4 is required for the induction of the synthesis of IgE in humans (112-114, 119, 120). IL-4 has the ability to activate transcription of the ε locus (132) although for the expression of mRNA and IgE synthesis additional stimuli are required. B cells process the allergen after uptake with surface immunoglobulin molecules, and present it in association with MHC class II molecules. The MHC class II complex is recognized by T cells leading to an antigen-specific T-B cell interaction which results in both T and B cell activation (133). Non-cognate T-B cell interaction can deliver the second signal for IgE production. It has been demonstrated that activated $CD4^+$ or $CD8^+$ T cell clones can induce IgE synthesis in B cells from randomly selected donors if exogenous IL-4 was added to the culture (134). This contact-dependent signal is delivered after binding of CD40 on B cells by its ligand (CD40L) on T cells (135).

Peanut allergy

Peanut, *Arachis hypogaea* L. which belongs to the legume family, can b the cause of very severe allergic reactions. Accidental consumption by peanut allergic individual of a few milligrams of peanuts can induce c anaphylactic reaction. Death due to an anaphylactic shock by peanuts not uncommon (136-142). At this moment, avoidance of peanuts peanut-containing food products is the best "therapy" for peanut allergi patients. But this proves to be very difficult because peanut proteins mc unexpectively be present in products such as soups and sauces (143 Moreover, if the patient will not prepare the food, as for instance in restaurant, it is very difficult to know exactly what specific ingredients wer used for the preparation of the food.
The prevalence of peanut allergy is difficult to estimate. Burks et al. (142 showed that approximately 30% of all adverse reaction to food in patien with atopic dermatitis is due to peanut.

Peanut proteins

Peanut kernels contain 45 to 50% oil, 25-30% protein, 5-12% carbohydrate 5% moisture, 3% fibre and 2.5% ash (144, 145). Peanut proteins can b separated in albumins and globulins. Major globulins are arachin an conarachin, both storage proteins, which account for 87% of the protei contents of peanuts (146, 147). Arachin and conarachin can be separate on the basis of precipitation in ammonium sulphate or on the basis of ioni strength (148, 149). By sodium dodecyl sulphate polyacrylamide gelelec trophoresis (SDS-PAGE) both arachin and conarachin can be seperated i a variable number of subunits with molecular weights between 10 and 7 kD (151, 150).

Peanut allergens

Several peanut allergens have been described (149, 151-157). Sachs et a described the first allergen, Peanut-1 (151). This is a protein consisting of subunits of approximately 20 and 30 kD. Barnett et al. (149, 152) showec the occurence of 16 IgE-binding proteins in peanut extract with a major IgE binding glyco-protein the so called Concanavaline A-reactive protein o approximately 66 kD. Two major allergens have been described by Burks e

al. (153, 154) *Ara h* I and *Ara h* II with molecular weights of 63.5 and 17 kD, respectively. Whether the Con A-reactive protein and/or Peanut-1 is one of these major allergens is unknown. Furthermore contain peanuts a lectin, peanut agglutinin (PNA) which has been demonstrated to bind IgE (155) and pocesses the ability to induce proliferation in bovine lymphocytes (156).
As most food allergens, also peanut allergens are heat stable. Roasting of peanuts does not result in loss of IgE binding (149). Moreover, it has been demonstrated that the treatment of peanut proteins with human digestive enzymes and acid, a condition that mimics the human digestion in the gastro-intestinal tract, did not destroy the IgE binding sites (42).

Scope of this thesis

Although the recognition of inhalation allergens, such as proteins from house dust mites, various pollen and domestic animals, by IgE and T cells has been studied extensively, little is known about recognition of food allergens. Food allergies are increasingly seen as difficult medical problems. Knowledge of recognition of food allergens by both B and T cells could lead to more insight in the mechanism of food allergy and improvement of specific immunotherapy and hypoallergenic food products.
In this thesis, peanut allergy was studied as a model for IgE-mediated food allergy. Peanut allergy was selected not only because it is a food allergy with very severe symptoms, but also because it is quite often seen in adults in contrast to cow's milk and henn's egg allergy. The cooperation of adult volunteers was essential for the experiments described in this thesis, for which large amounts of peripheral blood were used which can not be obtained from young children.
In this thesis several aspects of peanut allergy and peanut-allergic patients have been studied and described. Therefore, the clinical symptoms of peanut-allergic patients and the difficulties encountered when diagnosing peanut-allergic patients using the various available test methodswere studied and described in chapter 2. Another purpose of this study was to clinically test the allergenicity of crude peanut extract (CPE) prepared in our laboratory.

In chapter 3 the major IgE-binding peanut proteins were determined usir plasma samples of peanut-allergic patients, in relation to the IgE bindir properties of plasma samples of allergic, but not peanut allergic and no allergic subjects. The levels of peanut-specific immunoglobulins of all groups were determined as well.
Whether food allergen-specific T cells have a Th2 phenotype is unknow To investigate this, several peanut-specific T cell clones were generate from the peripheral blood of a peanut-allergic patient. The phenotype ar cytokine secretion profile of these clones have been determined and a described in chapter 4.
To obtain more insight in the protein specificity of the generated T ce clones, a peanut protein extract was seperated using different technique The obtained protein fractions were analyzed for their ability to induc proliferation of the T cell clones. Several peanut proteins expressing T ce epitopes have been identified and are described in chapter 5.
Chapter 6 describes a study in which several aspects of the PBMC-respor se of peanut-allergic, allergic but not peanut allergic and non-allergi subjects to peanut proteins as well as several other allergens were invest gated. This study was performed in order to obtain insight in the response of PBMC from allergic donors to a non-relevant allergen, peanut proteins.

References

1 **Anderson JA and Sogn DD.** (1984) Committee on adverse reactions to foods of th American Academy of Allergy and Infectious Disease. Adverse reactions to food US Department of Health and Human Services. NIH publications no 84-244 Bethesda: National Institute of Health.

2 **Metcalfe DD.** (1985) Food allergens. *Clin Rev Allergy* **3**:331-349.

3 **Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Bjorksten B, Monere Vautrin D and Wutrich B.** (1995) Adverse reactions to food. *Allergy* **50**:623-635.

4 **May CD.** (1983) Immunologic versus toxic reactions to foodstuffs. *Ann Allergy* **51**:26 268.

5 **Morton ID.** (1977) Naturally occurring toxins in foods. *Proc Nutr Soc* **36**:101-105.

6 **May CD and Bock SA.** (1987) Adverse reactions to foods due to hypersensitivity. IN Allergy, Principles and Practice. Eds: Middleton J, Reed CE and Ellis EF. St Louis Mosby. pp 1159-1171.

7 **Kocian J.** (1988) Lactose intolerance. *Int J Biochem* **20**:1-5.

8 **Taylor SL, Nordlee JA and Rupnow JH.** (1989) Food allergies and sensitivities. IN: Food Toxicology. A perspective on the relative risks. Eds: Taylor SL and Scanlan RA. New York. pp 255-295.

9 **Maher TJ.** (1986) Natural food constituents and food additives: the pharmacologic connection. *J Allergy Clin Immunol* **79**:413-422.

10 **Perkin JE and Hartje J.** (1983) Diet and migraine: a review of the literature. *J Am Diet Assoc* **83**:459-463.

11 **Harper AE and Gans DA.** (1986) Diet and behaviour - an assessment of reports of aggressive, antisocial behaviour from consumption of sugar. *Food Technol* **40**:142-149.

12 **Ortolani C, Mirone A, Fontana A, Folco GL, Miadonna A, Montalbetti N, Rinaldi M, Salsa A, Tedeschi A and Valenti D.** (1987) Study of mediators of anaphylaxis in nasal wash fluids after aspirin and sodium metabisulfite nasal provocation in intolerant rhinitis patients. *Ann Allergy* **59**:106-112.

13 **Kägi MK, Wütrich B and Johansson SGO.** (1994) Campari-orange anaphylaxis due to carmine allergy. *Lancet* **344**:60-61.

14 **Pearson DJ, Rix KJB and Bently SJ.** (1983) Food allergy: How much is in the mind? A clinical and psychiatric study of suspected food hypersensitivity. *Lancet* **i**:259-261.

15 **Furukawa CT.** (1991) Nonimmunological food reactions that can be confused with food allergy. *Immunol Allergy Clinics North Amer* **11**:815-829.

16 **Cafney EA, Sladen GE, Isaacs PET and Clark KGA** (1981) Thrombocytopenia caused by cow's milk. *Lancet* **2**:316.

17 **Saalman R, Carlsson B, Fällström SP, Hanson LA and Ahlstedt S.** (1991) Antibody-dependent cell-mediated cytotoxicity to β-lactoglobulin-coated cells with sera from children with intolerance of cow's milk protein. *Clin Exp Immunol* **85**:446-452.

18 **Paganelli R, Quinti I, D'Offizi P, Papetti C, Carini C and Aiuti F.** (1987) Immune complexes in food allergy: a critical reappraisal. *Ann Allergy* **59**:157-161.

19 **Carini C, Brostoff J and Wraith DG.** (1987) IgE complexes in food allergy. *Ann Allergy* **59**:110-117.

20 **Marsh MN.** (1988) Studies of intestinal lymphoid tissue. XI. The immunopathology of cell-mediated reactions in gluten sensitivity and other enteropathies. *Scanning Micros* **2**:1663-1684.

21 **Furguson A, Arranza E and O'Mahony S.** (1993) Spectrum of expression of intestinal cellular immunity: proposal for a change in diagnostic criteria of coeliac disease. *Ann Allergy* **71**:29-32.

22 **Goldman H and Proujansky R.** (1986) Allergic proctitis and gastroenteritis in children. *Am J Surg Path* **10**:75-86.

23 **Phaff RAS, Nierop G, Rijntjes E and Douwes AC.** (1990) Clinical aspects. IN: Consensus food hypersensitivity. Eds: Dutch Information Centre For Food Hypersensitivity (LIVO) pp 23-34.

24 **El-Rafei A, Peters SM, Harris N and Bellanti JA.** (1989) Diagnostic vale of IgG measurements in patients with food allergy. *Ann allergy* **62**:94-99.
25 **Parish WE.** Short-term anaphylactic IgG antibodies in human sera. (1970) *Lance* **2**:591.
26 **Aalberse RC, van Milligen F, Tan KY and Stapel SO.** (1993) Allergen-specific IgG4 i atopic disease. *Allergy* **48**:559-569.
27 **Sampson HA and Metcalf DD.** (1992) Food allergies. *J Am Med Assoc* **20**:2840-2844.
28 **Businco L, Meglio P and Ferrara M.** (1993) The role of food allergy and eosinophils i atopic dermatitis. *Pediatr Allergy Immunol* **4**:33-37.
29 **Jaffe JS and Metcalf DD.** (1993) Cytokines and their role in the pathogenesis severe food hypersensitivity reactions. *Ann Allergy* **71**:362-364.
30 **Sampson AS.** (1991) Immunological mechanisms in adverse reactions to food *Immunol Allergy Clinics North Amer* **11**:701-716.
31 **Roitt I.** (1988) Essential Immunology. Blackwell Scientific Publications, Oxford.
32 **Bock SA.** (1982) The natural history of food sensitivity. *J Allergy Clin Immunol* **69**:17 177.
33 **Dannaeus A and Inganäs M.** (1981) A follow-up study of children with food allergy Clinical course in relation to serum IgE- and IgG-antibody levels to milk, egg an fish. *Clin Allergy* **11**:533-539.
34 **Sampson HA and McCaskill CC.** (1985) Food hypersensitivity and atopic dermatiti evaluation of 113 patients. *J Pediatr* **107**:669-675.
35 **Bock SA and Atkins FM.** (1990) Patterns of food hypersensitivity during sixteen yea of double-blind, placebo-controlled food challenges. *J Pediatr* **117**:561-567.
36 **Esteban MM.** (1992) Adverse reactions in childhood: concept, importance and pre ent problems. *J Pediatr* 121:51-53.
37 **Hattvig G, Kjellman NIM, Johansson SGO and Bjorksten B.** (1984) Clinical symptom and IgE responses to common food proteins in atopic and healthy children. *Cli Allergy* **14**:551-559.
38 **Taylor SL, Lemansky RF, Bush RK and Busse WM.** (1987) Food allergens: structure an immunologic properties. *Ann Allergy* **59**:93-99.
39 **Crumpton MJD.** (1974) Protein antigens: the molecular basis of antigenicity an immunogenicity. IN: The antigens. Ed: Sela M. New York, academic press, pp 1.
40 **Hoffman DR, Day ED and Miller JS.** (1981) The major heat-stable allergen of shrimp *Ann Allergy* **47**:17-22.
41 **Taylor SL.** (1986) Immunologic and allergic properties of cow's milk proteins i humans. *J Food Prot* **49**:239-243.
42 **Burks WA, Williams LW, Thresher W, Connaughton C, Cockrell G and Helm RM.** (1992 Allergenicity of peanut and soybean extracts altered by chemical or therm denaturation in patients with atopic dermatitis and positive food challenge. *Allergy Clin Immunol* **90**:889-897.
43 **Bock SA.** (1987) Prospective appraisal of complaints of adverse reactions to foods i children during the first 3 years of life. *Paediatrics* **79**:683-688.

44 **Kajosaari M.** (1982) Food allergy in Finnish children aged 1 to 6 years. *Acta Paediatr Scand* **71**:815-819.

45 **Walker WA.** (1986) Antigen handling by the small intestine. *Baillieres Clin Gastroenterol* **15**:1-20.

46 **Host A and Halken S.** (1990) A prospective study of cow milk allergy in Danish infants during the first three years of life. *Allergy* **455**:587-596.

47 **Schrander JJP, van den Bogart JPH, Forget PP et al.** (1993) Cow's milk intolerance in infants under 1 year of age: A prospective epidemiology study. *Eur J Pediatr* **152**:6-40-644.

48 **Young E, Stoneham MD, Petruckevich A, Barton J and Rona R.** (1994) A population study of food intolerance. *Lancet* **343**:1127-1130.

49 **Niestijl Jansen JJ, Kardinaal AFM, Huijbers G, Vlieg-Boerstra BJ, Martens BPM and Ockhuizen Th.** (1994) Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* **93**:446-456.

50 **Bender AE and Mathews DR.** (1981) Adverse reactions to foods. *Br J Nutr* **46**:403-407.

51 **Burr ML and Merrett TG.** (1983) Food intolerance: a community survey. *Br J Nutr* **49**:217-219.

52 **Young E, Patel S and Stoneham M.** (1987) The prevalence of reactions to food additives in a survey population. *J R Coll Physicians Lond* **21**:241-247.

53 **Chandra RJ.** (1992) Food allergy: 1992 and beyond. *Nutr Res* **12**:93-99.

54 **Roundtree S, Cogswell JJ, Platts-Mills TAE and Mitchell EB.** (1985) Development of IgE and IgG antibodies to foods and inhalant allergens in children at risk of allergic disease. *Arch Dis Child* **60**:727-735.

55 **Aas KA.** (1966) Studies on hypersensitivity to fish. A clinical study. *Int Arch Allergy Appl Immunol* **29**:346-363.

56 **Moroz LA and Yang WH.** (1980) Kunitz soybean trypsin inhibitor. A specific allergen in food anaphylaxis. *N Engl J Med* **302**:1126-1128.

57 **Fukutomi O, Kondo N, Agata H, Shinoda S, Kuwabara N, Shinbara M and Orii T.** (1994) Timing of onset of allergic symptoms as a response to a double-blind, placebo-controlled food challenge in patients with food allergy combined with a radioallergosorbent test and the evaluation of proliferative lymphocyte responses. *Int Arch Allergy Immunol* **104**:352-357.

58 **Bock SA, Lee WY, Remigio LK and May CD.** (1978) Studies on hypersensitivity reactions to foods in infants and children. *J Allergy Clin Immunol* **62**:327-334.

59 **Kidd JM, Cohen SH, Sosman AJ and Fink JN.** (1983) Food-dependent exercise-induced anaphylaxis. *J Allergy Clin Immunol* **109**:270-276.

60 **Enberg RN.** (1991) Food-induced oropharyngeal symptoms. The oral allergy syndrome. *Immunol Allergy Clin North Am* **11**:767-772.

61 **Daul CB, Morgan JE, Hughes J et al.** (1988) Provocation studies in shrimp-sensitive individuals. *J Allergy Clin Immunol* **81**:1180-1186.

62 **Norgaard A and Bindslev-Jensen C.** (1992) Egg and milk allergy in adults. Diagnosis and characterization. *Allergy* **47**:503-509.

63 **Ortolani C, Ispano M, Pastorello EA, Ansoloni R and Magri GC.** (1989) Comparison c results of skin prick test (with fresh food and commercial extracts) and RAST in 10 patients with oral allergy syndrome. *J Allergy Clin Immunol* **83**:683-690.

64 **Schreiber RA and Walker WA.** (1989) Food allergy: facts and fiction. *Mayo Clin Pro* **64**:1381-1391.

65 **Sampson HA and Metcalfe DD.** (1992) Food allergies. *JAMA* **268**:2840-2844.

66 **Walker-Smith JA.** (1992) Gastrointestinal food allergy in childhood: current problem *Nutr Res* **12**:123-135.

67 **Sampson HA.** (1992) Immunopathogenic role of food hypersensitivity in atopic de matitis. *ACTA Dermato-Venereologia* **176**:34-37.

68 **Ortolani C, and Ispano M.** (1992) Foods and chronic urticaria. *Clin Reviews Allerg* **10:** 325-344.

69 **Onorato J, Merland N, Terral C, Michel FB and Bousquet L.** (1986) Placebo-controlled double-blind food challenge in asthma. *J Allergy Clin Immunol* **78**:1139-1146.

70 **Novembre E, De Martino M and Vierucci A.** (1988) Foods and respiratory allergy. *Allergy Clin Immunol* **81:** 1059-1065.

71 **Bindslev-Jensen C.** (1992) Respiratory reactions induced by food challenges i adults. *Pediatr Allergy Immunol* **3**:201-205.

72 **Yunginger JW, Sweeney KG, Sturner WQ, Giannandrea LA, Teigland JD, Bray M, Ben son PA, York JA, Biedrzycki L, Squillance DL and Helm RM.** (1988) Fatal food-induce anaphylaxis. *JAMA* **260**:1450-1452.

73 **Kalliel JN, Klein DE and Settipani GA.** (1989) Anaphylaxis to peanuts: clinical correlc tion to skin tests. *Allergy Proc* **10**:259-260.

74 **Sampson HA, Mendelson L and Rosen JP.** (1992) Fatal and near-fatal anaphylacti reactions to foods and adolescents. *N Engl J Med* **327**:380-384.

75 **Rosen JP, Selcow JE, Mendelson LM, Grodofsky M, Factor JM and Sampson HA** (1994) Skin testing with natural foods in patients suspected of having food allergies Is it a necessity? *J Allergy Clin Immunol* **93**:1068-1070.

76 **de Weck AL.** (1988) Immunologic diagnostic tests in food allergy. IN: Food allergy Ed: Schmidt E. Nestlé Nutrition Workshop Series, Vol 17. New York, Vevey/Raver Press, pp 177-186.

77 **Ownby DR.** (1991) In vitro assays for the evaluation of immunologic reactions t foods. *Immunol Allergy Clin North Amer* **11**:851-862.

78 **Bock SA, Sampson HA, Atkins FJ Zeiger RS, Lehrer S. Sacks M, Bush RK and Metcalfe DD.** (1988) Double-blind, placebo controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* **82**:986-997.

79 **Siu G, Clark SP, Yoshikai Y, Malissen M, Yanagi Y, Strauss E, Mak TK and Hood L** (1984) The human T cell antigen receptor is encoded by variable, diversity, and joining gene segments that rearrange to generate a complete V gene. *Cell* **37**:393 401.

80 **Davis MM and Bjorkman PJ.** (1988) T cell antigen receptor genes and T cell recogni tion. *Nature* **334**:395-402.

81 **Rothbard JB and Gefter ML.** (1991) Interactions between immunogenic peptides and MHC proteins. *Annu Rev Immunol* **9**:527-565.

82 **Weaver CT and Unanue ER.** (1990) The costimulatory function of antigen-presenting cells. *Immunol Today* **11**:49-55.

83 **Jenkins M and Johnson JG.** (1993) Molecules involved in T-cell costimulation. *Curr Opin Immunol* **5**:361-367.

84 **Weiss A. Imboden J, Hardy K, Manger B, Terhorst C and Stobo J.** (1986) The role of T3/antigen receptor complex in T cell activation. *Annu Rev Immunol* **4**:593-619.

85 **Collins TL, Hahn WC, Bierer BB and Burakoff SJ.** (1993) CD4, CD8 and CD2 in T cell adhesion and signalling. *Curr Opin Microbiol Immunol* **6**: 385-393.

86 **June CH, Ledbetter JA, Linsley PS and Thompson CD.** (1990) Role of the CD28 receptor in T cell activation. *Immunol Today* **11**:211-216.

87 **Koulova L, Clark EA, Shu G and Dupont B.** (1991) The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. *J Exp Med* **173**:759-762

88 **Linsley PS, Green JL, Tan P, Bradshaw J, Ledbetter JA, Anasetti C and Damle NK.** (1992) Coexpression and functional cooperation of CTLA-4 and CD28 on activated T cells. *J Exp Med* **176**:1595-1604.

89 **Norton SD, Zuckerman L, Urdahl KB, Shefner R, Miller J and Jenkins MK.** (1992) The CD28 ligand B7, enhances IL-2 production by providing a costimulatory signal to T cells. *J Immunol* **149**:1556-1561.

90 **Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL and Somoza C.** (1993) B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* **188**:259-273.

91 **Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK and Ledbetter JA.** (1991) Binding of B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin-2 mRNA accumulation. *J Exp Med* **173**:721-730.

92 **Schwarz RH.** (1992) Costimulation of T lymphocytes: The role of CD28, CTLA-4 and B7/BB1 in IL-2 production and immunotherapy. *Cell* **71**:1065-1068.

93 **Mossman TR, Cherwinsky MW, Bond MW, Giedlin MA and Coffman RL.** (1986) Two types of murine helper T cell clones. I. Definition according to profiles of lymphokines activities and secreted properties. *J Immunol* **136**:2348-2357.

94 **Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM and Kapsenberg ML.** (1990) Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes in atopic patients. *J Immnunol* **144**:4651-4656.

95 **Romagnani S.** (1991) Human Th1 and Th2 subset: Doubt no more. *Immunol Today* **12**:256-257.

96 **Del Prete G.** (1992) Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy;* **47**: 450-455.

97 **Firestein GS, Roeder WD, Laxer JA, Townsend KS, Weaver CT, Hom JT Linton J, Torbett BE and Glasebrook AL.** (1989) A new murine CD4+ T cell subset with an unrestricted cytokine profile. *J Immunol* **143**:518-525.

98 **Maggi E, Parronchi P, Manetti R, Simonelli C, Piccini MP, Rugiu FS, De Carli M, Ricci and Romagnani S.** (1992) Reciprocal regulatory effect of IFN-γ and IL-4 on the in vi development of human Th1 and Th2 clones. *J Immunol* **148**:2124-2147.

99 **Manneti R, Parronchi P, Giudizi MG, Piccini MP, Maggi E, Trinchieri G and Romagn S.** (1993) Natural killer cell stimulatory factor (interleukin-12) induces T helper type (Th1)-specific immune responses and inhibits the development of IL-4 producing T cells. *J Exp Med* **177**:1199-1204.

100 **Hsieh CS, Heimberger AB, Gold, JS, O'Garra A and Murphy KM.** (1992) Different regulation of T helper phenotype development by interleukin-4 and -10 in an alt beta T cell transgenic system. *Proc Natl Acad Sci USA* **89**:6065-6069.

101 **Kuchroo VK, Prabhu Das M, Brown JA, Ranger AM, Zamvil SS, Sobel RA, Weiner I Nbavi N and Glimcher LH.** (1995) B7-1 and B7-2 costimulatory molecules activo differentially the Th1/Th2 developmental pathways: Application to autimmu disease therapy. *Cell* **80**:707-718.

102 **Mosmann TR and Coffman RL.** (1989) Th1 and Th2 cells: Different patterns of cytoki production may lead to different functional properties. *Annu Rev Immunol* **7**:145-17

103 **Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RAW and Kapsenberg ML.** (19 Human atopen-specific types 1 and 2 T helper clones. *J Immunol* **147**:2942-2949.

104 **Neefjes JJ and Momburg F.** (1993) Cell biology of antigen presentation. *Curr Op Immunol* **5**:27-34.

105 **Bjorkman PJ, Saper MA, Samraoui B, Bennet WS, Strominger JL and Wiley DC.** (19 Structure of the human class I histocompatobilty antigen, HLA-A2. *Nature* **329**:5 512.

106 **Brown JH, Jardetzki TS, Corga JC, Stren LJ, Urban RG, Strominger JL and Wiley D** (1993) Three-dimensional structure of the human class II histocompatibility antig HLA-DR1. *Nature* **364**:33-39.

107 **Ebner E, Széphalusi Z, Ferreira F,** et al. (1993) Identification of multiple T cell epitop on *Bet v* I, the major birch pollen allergen, using specific T cell clones and overlap ing peptides. *J Immunol* **150**:1047-1054.

108 **van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RC a Kapsenberg ML.** (1994) Characterization of cat dander-specific T lymphocytes fro atopic patients. *J Immunol* **152**:4203-4210.

109 **van der Heijden FL, Wierenga EA, Bos JD and Kapsenberg ML.** (1991) High frequen of IL-4 producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesion skin. *J Invest Dermatol* **97**:389-394.

110 **Ramb-Lindhauer Ch, Feldmann A, Rotte M and Neumann Ch.** (1991) Characteriz tion of grass pollen reactive T cell lines derived from lesional atopic skin. *Arch De matol Res* **183**:71-76.

111 **Snapper CM, Finkelman FD and Paul WE.** (1988) Differential regulation of IgG1 a IgE synthesis by interleukin-4. *J Exp Med* **167**:183-196.

112 **Romagnani S, Del Prete G and Maggi E.** (1989) Role of interleukins in induction a regulation of human IgE synthesis. *Clin Immunol Immunopathol* **50**:S13-S23.

113 **Schultz CL and Coffman RL.** (1991) Control of isotype switching by T cells and cytokines. *Curr Opin Immunol* **3**:350-354.

114 **Vercelli D and Geha RS.** (1991) Regulation of IgE synthesis in humans: a tale of two signals. *J Allergy Clin Immunol* **88**:285-285.

115 **Heusser ChH, Bews J, Brinkmann V, Delespesse G, Kilchherr E, Ledermann F, Le Gros G and Wagner K.** (1991) New concepts in IgE regulation. *Int Arch Allergy Appl Immunol* **94**:87-90.

116 **Lebedin YS, Raudla LA and Chuchalin AG.** (1991) Serum levels of interleukin 4, interleukin 6 and interferon-gamma following in vivo isotype-specific activation of IgE synthesis in humans. *Int Arch Allergy Appl Immunol* **96**:92-94.

117 **Coffman RL and Carty J.** (1986) T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J Immunol* **136**:949-954.

118 **Snapper CM and Paul WE.** (1987) Interferon-γ and B cell stimulatory factor-1 reciprocally regulated Ig isotype production. *Science* **236**:944-947.

119 **Pene J Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, Yokota T, Arai N, Arai K, Bancherau J and de Vries J.** (1988) IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2. *Proc Natl Acad Sci USA* **85**:6880-6884.

120 **Wierenga EA, Snoek M, Bos JD, Jansen HM and Kapsenberg ML.** (1990) Comparison of diversity and function of house dust mite-specific T lymphocyte clones from atopic and non-atopic donors. *Eur J Immunol* **20**:1519-1526.

121 **Parronchi P, Macchia D, Piccinni MP, Biswas P, Simonelli C, Maggi E, Ricci M, Ansari AA and Romagnani S.** (1991) Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. *Proc Natl Acad Sci USA* **88**:4538-4542.

122 **Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG and Vadas MA.** (1988) Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med* **167**:219-223.

123 **Clutterbuck EJ, Hirst EMA and Sanderson CJ.** (1989) Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: Comparison and interaction with IL-1, IL-3, IL-6 and GM-CSF. *Blood* **73**:1504-1512.

124 **Gleich GJ.** (1990) The eosinophil and bronchial asthma: current understanding. *J Allergy Clin Immunol;* **85**: 422-436.

125 **Weller PF.** (1991) The immunobiology of eosinophils. *N Engl J Med* **324**:1110-1118.

126 **Bradley BL, Azzawi M, Jacobson M, AssoufiB, Collins JV, Irani AMA, Schwartz LB, Durham SR, Jeffery PK and Kay AB.** (1991) Eosinophils, T lymphocytes, mast cells, neutrophils, and macrophages in bronchila biopsy specimens from atopic subjects with asthma: Comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* **88**:661-74.

127 **McMenamin C and Holt PG.** (1993) The natural immune response to inhale solubleantigens involves major histocompatibility complex (MHC) class I-restricte CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immur deviation resulting in sellective suppression of immunoglobulin E production. *J E Med* **178**:889-899.

128 **Kemeny DM and Diaz-Sanchez D.** (1993) The role of CD8+ T cells in the regulation IgE. *Clin Exp Allergy* **23**:466-470.

129 **Croft M, Carter L, Swain SL and Dutton RW.** (1994) Generation of polarized antige specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 promoting type 2 versus type 1 cytokine profiles. *J Exp Med* **180**:1802-1805.

130 **Seder RA and Le Gros GG.** (1995) The functional role of CD8+ T helper type 2 cells. *Exp Med* **181**:5-7.

131 **Del Prete GF, Maggi E, Parronchi P, Chretien I, Tiri A, Macchia D, Ricci M, Bancherc J, de Vries J and Romagnani S.** (1988) IL-4 is an essential factor for the IgE synthes induced in vitro by human T cell clones and their supernatants. *J Immunol* **140**:419 4198.

132 **Stavnezer J, Radcliff G, Lin Y-C, Nietupski J, Berggren L, Sitia R and Severinsin** (1988) Immunoglobulin heavy-chain switching may be directed by prior induction transcripts from constant-region genes. *Proc Natl Acad Sci USA* **85**:7704-7708.

133 **Vercelli D, Jabara HH, Arai K, Yokota T and Geha RS.** (1989) Onduction of human Ig requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD complex and MHC class II antigens. *J Exp Med* **169**:1295-1307.

134 **Parronchi P, Macchia D, Piccinni M-P, Biswas P, Simonella C, Maggi E, Del Prete G Ricci M and Romagnani S.** (1990) Noncognate contact-dependent B cell activatio can promote IL-4 dependent in vitro IgE synthesis. *J Immunol* **144**:2102-2108 .

135 **Hollenbaugh D, Grosmaire L, Kullas CD, Chalupny NJ, Noelle RJ, Stamenkovic Ledbetter JA and Aruffo A.** (1992) The human T cell antigen gp30, a member of th TNF gene family, is a ligand for the CD40 receptor. Expression of a soluble form gp39 with B cell co-stimulatory activity. *EMBO J* **11**:1111-1118.

136 **Fries JH.** (1982) Peanuts: allergic and other untoward reactions. *Ann Allergy* **48**:22 226.

137 **Kemp AS, Mellis CM, Barnett D, Sharota E and Simpson J.** (1985) Skin test, RAST an clinical reactions to peanut allergens in children. *Clin Allergy* **15**:73-78.

138 **Bock SA and Atkins FM.** (1989) The natural history of peanut allergy. *J Allergy Cl Immunol* **93**:900-904.

139 **Yunginger JW, Squillance DL, Jones RT and Helm RM.** (1989) Fatal anaphylacti reactions induced by peanuts. *Allergy Proc* **10**:249-253.

140 **Burks AW, Williams LW, Mallory SB, Shirell MA and Williams C.** (1989) Peanut prote as a major cause of adverse food reactions in patients with atopic dermatiti *Allergy Proc* **10**:265-269.

141 **Bush RL, Taylor SL and Nordlee JA.** (1989) Peanut sensitivity. *Allergy Proc* **10**:261-264.

142 **Assem ESK, Gelder CM, Spiro SG, Baderman H and Armstrong RF.** (1990) Anaphylaxis induced by peanuts. *Br Med J* **300**:1377-1378.

143 **Dutch Food Intolerance Data Bank (ALBA)** (1995) List of peanut-containing brand products. TNO Nutrition and Food Research Institute.

144 **Pancholy SK, Deshpande AS and Krall S.** (1987) Amino acids, oil and protein content of some selected peanut cultivars. *Proc Amer Peanut Res Educ Assoc* **10**:30-37.

145 **Ahmed EM and Young CT.** (1982) Composition, nutrition, and flavor of peanuts. IN: Peanut science and technology. Eds: Pattel HE and Young CT. American Peanut Research and Education Society, Inc, pp 655-688.

146 **Johns CO and Jones DB.** (1916) The proteins of the peanut, Arachis hypogaea. I The globulins arachin and conarachin. *J Biol Chem* **XXVIII**:77-87.

147 **Basha SMM and Pancholy SK.** (1981) Polypeptide composition of arachin and non-arachin proteins from early bunch peanuts (*Arachis hypogaea* L.) seeds. *Peanut Science* **8**:82-88.

148 **Neucere NJ.** (1969) Isolation of α-Arachin, the major peanut globulin. *Anal Biochem* **27**:15-24.

149 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts. *J Allergy Clin Immunol* **72**:61-68.

150 **Bushan R, Reddy GP and Reddy KRN.** (1988) Chemistry of peanut proteins: a review. *Adv Protein Chem* **39**:235-238.

151 **Sachs MI, Jones RT and Yunginger JW.** (1981) Isolation and partial characterization of a major peanut allergen. *J Allergy Clin Immunol* **67**:27-34.

152 **Barnett D and Howden MEH.** (1986) Partial characterization of an allergenic glycoprotein from peanut (*Arachis hypogaea* L.). *Biochim Biophys Acta* **882**:97-105.

153 **Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G and O'Brien T.** (1991) Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* **88**:172-179.

154 **Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien T and Helm RM.** (1992) Identification and characterization of a second major peanut allergen, *Ara h* II, with the use of sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* **90**:962-969.

155 **Burks AW, Cockrell, Connaughton C, Guin J, Allen W. and Helm RM.** (1994) Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. *Int Arch Allergy Immunol* **105**:143-149.

156 **Nagi AM and Babiuk LA.** (1989) Peanut agglutinin (PNA): binding and stimulation of bovine intestinal and peripheral blood leucocytes. *Veterinary Immunol Immunopathol* **22**:67-79.

Chapter 2

Comparison of *in vivo* and *in vitro* reactivity to peanut extracts in peanut-allergic patients

Esther C. de Jong[1,2], Steven Spanhaak[1],
Ben P.M. Martens[3] and André H. Penninks[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[2] Utrecht Toxicology Center (UTOX), Utrecht, The Netherlands
[3] Allergy Center Utrecht (ACU), Utrecht, The Netherlands

Submitted for publication

Abstract

Peanut allergy is one of the most common food allergies, not only in infan but also in adults. Although the double-blind, placebo-controlled foo challenge (DBPCFC) is still regarded as the "golden standard" to diagnos food hypersensitivity, several other tests are being used to diagnose a foo allergy. In the present study, exclusively performed in adult patients, th predictive value of the skin-prick-test (SPT), RAST scores, the minimu threshold dose in the DBPCFC and the lymphocyte stimulation test (LS were compared.

The protein composition of the various commercially available, tested SP solutions, showed a different profile on SDS-PAGE with only 3 protei present in 2 extracts. Furthermore, poor correlations were observed cor paring the SPT, RAST, minimum threshold in oral provocation and LST.

The results of this study indicate that there is a wide variety in both th response between different patients as between different diagnostic tes and test extracts. Even the DBPCFC does not always result in the sam response in patients when tested a second time. Better characterizatio and standardization of peanut extracts could improve the diagnostic valu of the different test systems. In addition, to reduce the diagnosis of fals negative results, a combination of test methods is recommended. Horeve based on the results presented in this study, the LST seems not to be good diagnostic tool.

Introduction

Food allergies do occur in 1 to 10% of the Western population (1, 2). One of the most common food allergies is peanut allergy. Approximately 30% of all adverse reactions to foods in children is caused by peanut (3). The symptoms observed in peanut-allergic patients after consumption of peanuts can vary from oral allergy syndrome, atopic dermatitis, gastro-intestinal symptoms, airway symptoms to an anaphylactic shock (4). Death caused by anaphylactic shock induced by peanuts is not uncommon (5). Once sensitized for peanuts, this allergy is persistent for life (6).
To diagnose a patient with food hypersensitivity, the double blind, placebo controlled, food challenge (DBPCFC) is still seen as the "golden standard" (7). But due to the possible severe reaction of a peanut-allergic patient, this has to be performed very carefully and under controlled clinical conditions. In addition, skin prick test (SPT) and radio-allergo-sorbent-test (RAST) scores are important screening tools for the diagnosis of IgE-mediated food allergy (8, 9). As the commercially available peanut extracts for SPT can be different in composition, this could lead to wrong diagnosis of patients and creating a life-threatening situation. Therefore, in this study, the results of the SPT, conducted with several commercially available peanut extracts in The Netherlands, coded X, Y and Z, were compared with a crude peanut extract (CPE) prepared in our own laboratory. Subsequently, the SPT scores were correlated to the RAST scores and the minimum threshold dose of peanut-protein in a DBPCFC.
In addition, the lymphocyte stimulation test (LST), a controversial laboratory tool to diagnose allergic reactions (10-12) by measuring the proliferative response of peripheral blood mononuclear cells (PBMC) to allergens (CPE), was studied as a possible diagnostic marker for food allergy.

Materials and methods

Peanut-allergic patients

In this study adult patients (n=16) were used with a previously proven IgE-mediated peanut allergy as determined by a positive history, a positive peanut-specific RAST, SPT and oral provocation. Patients' characteristics

are presented in Table 1. All patients suffered from atopic allergy wi atopic dermatitis, allergic rhinitis, allergic asthma or a combination of thes 3 symptoms. Approval for this study was obtained from an independe Medical-Ethical Committee. An informed consent was obtained from subjects.

Table 1: Characteristics of the peanut allergic patients.

Patient	Sex	Age (years)	Total IgE (IU/ml)	Clinical Characteristics[1]	Peanut RAST[2]
A	F	43	591	R, OAS, GI	2
B	F	35	2800	U, OAS, A, Ana	4
C	F	34	17900	AD, R, A, Ana	4
D	F	27	957	AD, R, A	3
E	F	28	6200	AD, R, OAS	5
F	F	22	895	AD, R, A	4
G	F	17	1000	AD, R, A, Ana	5
H	F	29	343	AE, R, A, Ctd	3
I	F	37	112	AD, R, OAS, Cte	1
K	F	27	710	AD, R, U, A	3
L	M	31	28800	AD, R, A	4
M	F	30	665	OAS, R, A, GI	5
N	M	41	108	OAS, R, GI	4
0	M	37	16600	AD, R, OAS, GI	4
P	F	30	836	AD, R, A	5
T	M	32	1600	SD, R, GI	4

[1] A: asthma; AD: atopic dermatitis; Ana: anaphylactic shock; AE: Angio-edem Ctd: contact dermatitis; Ctu: contact uticaria; GI: gastro-intestinal OAS: or allergy syndrome; R: allergic rhinitis; SD: seborrhoeic dermatitis.

[2] RAST scores as determined on a scale of 1 to 5.

Peanut protein purification

Crude peanut extract (CPE) was prepared from raw, unshelled peanuts described before (13). In short, peanuts were ground and fat w extracted by Soxhlet using petroleum ether at 40-60^{0}C. The defatted flo

was ground again and suspended in ammoniumbicarbonate (0.1M). This suspension was stirred for 4 h and insoluble particles were removed by centrifugation for 30 min at 10,000g at 4°C. The supernatant was dialysed over night against distilled water using a 3.5 kD cut-off membrane (Spectrum Medical Industries, Inc, Houston, Tx), lyophilized and stored at -20°C.

Protein assay

The concentration of proteins in the different extracts was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and following the manufacturers instructions.

Gelelectrophoresis (SDS-PAGE) and immunoblotting

After dilution of the protein samples (1:1) with sample buffer consisting of 1% DTT (Sigma Chemicals Co., St. Louis, MO), 63 mM-Tris-HCl, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8, they were reduced by incubation for 15 min at 100°C. Of the CPE solution of 2,5 mg/ml 5 µl was loaded on gel while of the commercially available extract solutions of 5 mg/ml 20 µl was loaded. SDS-PAGE was performed essentially according to Laemmli (14) using precasted 15% Tris-HCl polyacrylamide gels (Bio-Rad Laboratories). To visualize the protein bands, the gels were stained with Coomassie brilliant blue R-250. For the determination of the molecular weights of the protein bands, pre-stained molecular weight markers (Bio-Rad) with molecular weights of 200, 97.4, 69, 46, 30, 21.5 and 14.3 kD were used.

To detect IgE antibodies by immunoblotting, the separated proteins were transferred to a polyvinylidenedifluoride (PVDF, Immobilon-P Transfer membrane; Millipore Corporation, Bedford, MA) membrane using a semi-dry electrophoretic transfer apparatus as described by Towbin (15). The membranes were blocked with 3% (w/v) milkpowder (Provitar; Nutricia, Zoetermeer, The Netherlands) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. After 1.5 h of blocking at room temperature (RT), the membrane was incubated overnight with pooled plasma (1:10 v/v diluted) of the 16 peanut allergic patients. After washing the membrane was incubated with anti-human IgE-peroxidase conjugated monoclonal antibodies (1:1000 v/v) for 2 h at 37° C. Subsequently, the blots were washed thor-

oughly and developed for peroxidase activity using chloronaftol/DA staining.

Skin-prick-test (SPT)

With CPE and the commercially available peanut extracts coded X, Y an Z, SPT have been performed. In order to do so, a drop of the extract in titrated concentrations, was placed on unaffected skin and a prick with lancet was given in the middle of the drop. After 20 min, the wheals wer measured and the scores are expressed as area of the wheal in squar mm. All commercial extracts were tested in their own test solution and cor centration as recommended by the manufacturer as well as severe additional dilution. The SPT with raw peanut was performed by covering prick in the skin with scrapes of raw peanut.

Radio-allergo-sorbent test (RAST)

Standard RAST determinations were performed in serum samples of th patients at the Central Laboratory of Blood Transfusion Services (CL Amsterdam, The Netherlands) as described previously (16) using a wate soluble total peanut extract and were scored on a scale of 0 to 5.

Double-blind, placebo-controlled food challenge (DBPCFC)

The DBPCFC was performed under clinical condition at the Utrecht Allerg Centre in the presence of a physician. The patient was given blinde capsules (Lofarma, Milan, Italy) containing an increasing dose (0.5, 2, 10, 5 and 200 mg) of peanut flour containing 0.2, 0.8, 4, 20 and 80 mg protein. the patient did not yet show any symptoms at 200 mg of peanut flou either a combination of capsules was used to increase the dose or whol peanuts. The dose was increased after time intervals of 2 h. After the dos to which the patient showed clear clinical symptoms, an open provocatio with peanuts was performed. Clinical observation of the patient wa conducted for 2 h after open provocation at the clinic and late phas symptoms during the next 48 h were reported by the patient.

Lymphocyte stimulation test (LST)

Before SPT were performed, heparinized, venous blood was collected c the peanut-allergic patients for the isolation of PBMC using density gradien separation on Ficoll-Paque (Pharmacia LKB) essentially as described b

Böyum (17). Recovered cells were cultured (2×10^5/200 µl) in triplicate at 37°C and 5% CO_2 for 7 days in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Paisley, UK) supplemented with 10% pooled human serum (HS; BioWhittaker, Walkersville, ML) in the absence or presence of increasing concentrations of CPE (1, 10, 50, 100, 150, 200 and 250 µg/ml) in 96-well flat-bottom culture plates (NUNC, Roskilde, Denmark). Proliferation was measured using ^{3}H-thymidine (^{3}H)-TdR) incorporation. After 7 days, (^{3}H)-TdR (0.4 µCi/well; Amersham, Aylesbury, UK) was added and the cells were incubated for another 18 h and subsequently harvested (Harvester 96, Tomtec, Orange, USA). (^{3}H)-TdR incorporation was measured using a 1450 Microbeta-counter (Wallac, Turku, Finnland). Proliferation of the PBMC is expressed as stimulation index (SI) which is the counted cpm in presence of CPE divided by the counted cpm in absence of CPE. To compare the LST to other diagnostic markers, the highest observed SI was used for every patient.

Results

Protein contents and gelelectrophoresis of the extracts used for SPT

The protein content of the various extracts was determined and as is shown in Table 2, the commercially available extracts did contain very little protein (6 to 18%).

Table 2: Characteristics of the used peanut SPT extracts.

Manufacturer	Variety[1]	Material	Protein percentage[2]
CPE	Jumbo Runners	Raw	90
X	North Carolina 17, 11	Raw	6
Y	Unknown	Roasted	18
Z	Unknown	Roasted	8

[1] As provided by the manufacturer, [2] as determined by protein assay.

On SDS-PAGE (Figure 1) very faint bands were visible for the 3 commercially available extracts. The profile of protein bands as visualized by gelelec-

trophoresis (Figure 1, left panel) was also found to be different for th tested peanut extracts which are normally used in SPT to diagnose pean allergic patients. Based on the detected proteins, lane 3 (X) and lane 5 (are almost comparable. Lane 4 (Y) shows a remarkable resemblance wi the proteins present in the CPE (lane 2) prepared at our own laborator From the results it is obvious that 3 bands of approximately 20 kD a present in every extract. The Western blot incubated with pooled plasm of 16 peanut allergic patients, (Figure 1, right panel) shows IgE-binding the three bands, although binding can also be seen to other bands as shown by CPE and Y.

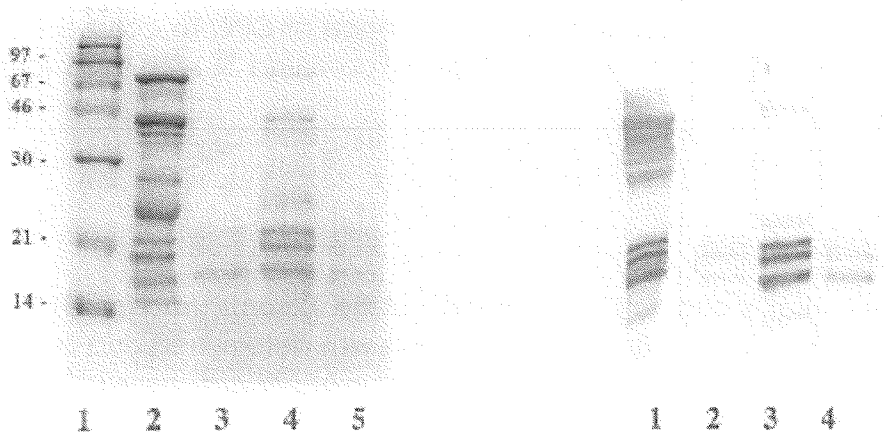


Figure 1: Gelelectrophoresis and IgE immunoblotting of the used SPT-extrac from X, Y, Z and CPE.
Left panel: SDS-PAGE (15% gel). Lane 1: MW markers (5 µl); lane 2: CP (5 µl); lane 3: X (20 µl); lane 4: Y (20 µl); lane 5: Z (20 µl).
Right panel: IgE-immunoblot. Blots were incubated o/n with poole serum (1:10 v/v) of peanut allergic patients (n=16), incubated wi anti-human IgE (2 h) and stained for 2 min.
Lane 1: CPE; lane 2: X; lane 3: Y; lane 4: Z.

SPT

SPT were performed with several commercially available extracts (X, Y ar Z), fresh, raw peanut and our own extract (CPE). Since the water solubili

of CPE was low, a solution of only 1 mg/ml was used instead of a solution of 5 mg/ml for the commercially available extracts. As is shown in Table 3, a wide variety in response was observed of the different peanut-allergic patients. This was not only apparent by comparing the individual reactions to the different extracts but also by the diverse responses of patients with a comparable RAST. For example, within the group of patients with a RAST score of 4 (n=7), the SPT scores varies between 0-198 for raw peanut, 0-144 for CPE, 0-315 for X, 0-952 for Y and 0-406 for Z. The most extreme variation was observed with the 3 commercially available extracts. Comparing the results within one patient, measured area's showed to differ up to 50 times between the lowest and highest score. Although the patients used in this study, all suffered from an earlier diagnosed peanut allergy, not everybody responded to the SPT. SPT have been performed in 3 concentration, which were for the 3 commercially available extracts of 5, 1 and 0.1 mg/ml and for CPE 1, 0.1 and 0.01 mg/ml. The patients who responded strongly in the SPT showed a nice dose-related increase in wheal area. In patient M, for instance, a wheal size of 72 mm^2 was observed at a dose of 0.1 mg Y/ml, 273 mm^2 at a dose of 1 mg y/ml and 600 mm^2 at a dose of 5 mg y/ml. None of the patients showed a decrease of wheal area in response to increasing concentration of the SPT extract.

DBPCFC

As oral provocation is still regarded as the golden standard for the diagnosis of a food allergy or hypersensitivity, we conducted this procedure for all 16 peanut-allergic patients. Patients were observed for clinical symptoms for 2 h after the last challenge at the Allergy Center and for the next 48 h they were assessed by the patient and reported to the physician. As is shown in Table 4, the observed symptoms were very diverse. The previously diagnosed peanut allergic patients A and B, did not show, any clinical symptoms, not even after a provocation with 5000 mg of peanuts. Two other patients (D and O) already showed symptoms after smelling the peanut flour. Not only the clinical symptoms but also the minimum threshold dose was observed to vary among patients between 0.5 and 5000 mg. The minimum threshold dose did not correlate with certain clinical symptoms.

Table 3: Results of the SPT conducted with raw peanut, CPE as well as sever commercially available extracts.

		Skin-prick-test results[1]				
PP	RAST	Peanut raw	CPE 1[2]	X 5[3]	Y 5[3]	Z 5[3]
A	2	0	4	0	16	4
B	4	4	0	0	9	0
C	4	49	99	81	144	196
D	3	9	9	0	9	4
E	5	36	49	144	144	121
F	4	64	144	49	289	169
G[4]	5	225	16	25	9	4
H	3	399	88	12	72	19
I	1	32	50	24	28	32
K	3	3	6	8	6	5
L	4	1	0	25	36	0
M	5	102	231	770	600	525
N	4	25	49.5	4	40	18
O	4	0	0	0	0	0
P	5	136	n.d.	147	338	525
T	4	198	n.d.	315	952	406

Wheals were measured 20 min after application of the allergen.

[1] Results are expressed as area of wheals in square mm.

[2] The CPE solution contained 1 mg defatted crude peanut protein extra per ml. Due to the low solubility in water, CPE was used in a lower co centration than the commercially available extracts, although the prote concentrations were comparable.

[3] Concentration of SPT extracts were used as provided by the manufa turer, which is 5 mg peanut flour in 1 ml SPT-buffer.

[4] Because of the severe anaphylactic reactions of this patient, only ve low concentrations were used: CPE: 0.01 mg/ml; X: 0.1 mg/ml; Y: 0 mg/ml; Z: 0.1 mg/ml.

n.d. Not done

Table 4: Results of the double-blind, placebo-controlled food challenge and the lymphocyte stimulation tests of the peanut-allergic patients.

PP	RAST score	Threshold dose DBPCFC[1]	Symptoms[2]	Stimulation Index (SI)[3]
A	2	5000 mg	none	1.8
B	4	5000 mg	none	10.1
C	4	2 mg	OAS, GI, D	3.1
D	3	<0.5 mg	AD, Prur, GI	0
E	5	300 mg	Prur, AE, GI	16.1
F	4	50 mg	OAS, D	9.1
G	5	0.5 mg	Ana	26.5
H	3	2 mg	D, AE	0
I	1	200 mg	D, Prur	0
K	3	200 mg	DT	2.5
L	4	5000 mg	AD	3.0
M	5	50 mg	GI, Coll	2.9
N	4	300 mg	GI	2.2
O	4	<0.2 mg	Prur, AD, GI	7.1
P	5	200 mg	Prur	2.5
T	4	150 mg	Ana, C, R, GI	36.4

[1] Threshold dose is given in mg peanut flour as indicated by the manufacturer of the capsules (Lofarma). If the patient did not show a response to the highest capsule dose of 200 mg peanut flour, a combination of capsules was used. The 5000 mg does was obtained by giving the patient an open challenge with whole peanuts.

[2] AD: flair-up of atopic dermatitis; AE: angio-edema; Ana: anaphylactic shock; Ctu: contact urticaria; D: dyspnea; GI: gastro-intestinal symptoms; OAS: oral allergy syndrome; R: allergic rhinitis; U: urticaria; Prur: generalized pruritus.

[3] SI is the cpm in the presence of CPE divided by the cpm in the absence of CPE. The highest observed response of every patient is presented here which was in most cases observed after stimulation with 200 μg/ml CPE.

LST

The results of the LST are expressed as stimulation index (SI) of the highest

observed response. In most patients the highest response was observe after stimulating the cells with 200 µg/ml CPE. As is shown Table 4, only 8 of the 16 patients showed a proliferative respons with an SI of 3 or higher which is considered a threshold for CP specific proliferation. These responses were only seen patients with a RAST score of 4 or 5 while some other patients with hig RAST scores did not show a proliferative response.

Correlations

Comparing the SPT scores of the different extracts with the RAST scores poor correlation was found for fresh peanut, X, Y and Z. Only CPE resulte in a higher correlation coefficient of 0.73 (Table 5).
Comparison of the threshold provocation dose needed for the appea ance of clinical symptoms with the RAST scores, the mean SPT scores and gave a very poor correlation (respectively r=0.20, 0.28 and 0.34). Also th correlation between the RAST score and the SI were very poor (r=0.34).

Table 5: Correlation coefficients of the comparison of the different extracts use in skin prick test and RAST score.

	RAST	Peanut	CPE	X	Y	Z
RAST		0.13	0.73	0.38	0.16	0.37
Peanut			0.81	0.22	0.51	0.34
CPE				0.85	0.96	0.92
X					0.97	0.79
Y						0.85
Z						-

Discussion

This study shows the variability of the *in vivo* and *in vitro* reactions of peanu allergic patients to peanut extracts. Hereto several commercially availab peanut extracts, coded X, Y and Z, as well as fresh raw peanut and o own extract, CPE, were compared in a SPT. Subsequently, these resul

were compared with those obtained by the RAST, the lymphocyte stimulation test with CPE and an oral provocation test with peanut flour. In summary, the results demonstrated that every patient showed a variable clinical response to peanuts. Differences were found in response to the 5 used SPT extracts as well as between the different used test methods, viz. SPT, RAST, LST and DBPCFC.

The observation that on SDS-PAGE the used extracts showed both different protein contents and profiles could contribute to the discrepancies observed within the SPT with the 5 used extracts. These differences in protein contents and profile can be explained in several ways. First, not all extracts were manufactured from the same variety of peanuts and therefore could result in different protein compositions. However, the two main storage proteins, arachin and conarachin, which make up for 87% of the protein content of peanuts are probably well-preserved in all varieties. Second, the different protein profiles and protein contents of the various extracts could also be due to differences in preparation method of the manufacturers which are unknown. This is supported by the observation that 2 of the extracts (X, Z) show only 3 proteins on SDS-PAGE which not only indicates a loss of total protein but also a loss of particular proteins. Roasting of peanuts is for instance known to reduce the protein composition. Therefore extracts from roasted peanuts may show less bands on SDS-PAGE when compared with an extract of raw peanuts (18). The extracts Y and Z were prepared from roasted peanuts and extract X and CPE from raw, unprocessed peanuts. The protein profile of the Y extract and CPE and those of the X and Z extracts were found to be almost comparable. Probably due to the limited protein contents of the extracts X and Z, only 3 proteins of approximately 20 kD were visible on SDS-PAGE. These protein bands were also present in the Y extracts although the protein bands were less sharp than in CPE. The 3 proteins of approximately 20 kD have been shown to be the most important allergens (19) and probably also contain the major allergen described by Burks (20), *Ara h* II. In a previous study we have demonstrated that more than 70% of our peanut allergic patients have IgE against all 3 proteins (19). The immunoblot incubated with pooled plasma showed IgE binding to these proteins in all extracts but also to other proteins as demonstrated in our own CPE but, although less clearly, also in the Y extract (Figure 1b). Recently, a difference in protein profiles of various peanut extracts commercially available in the United States, has also been

shown by Hefle et al. (20). They tested 6 different extracts for their ability t
bind IgE on immunoblot, to provoke a positive skin test and measured th
concentration of *Ara h* I and *Ara h* II. Although these extracts showe
different protein profiles, they observed, in contrast to our results, little d
ference in SPT scores. If in our study the wheal sizes resulting from SPT wit
different extracts are compared, several observations can be made. Apa
from a group of patients (A, B, D, K, L and N) that showed low wheal size
or even no response to some of the extracts, another group of patien
with medium or large sized wheals could be distinguished. In both group
however, wheal sizes were found to be extremely variable depending o
the used extract. This could indicate that different IgE epitopes are presen
in the extracts in different concentrations. In addition, one patient (O) wit
a RAST score of 4, did not respond to any of the used peanut extracts. Th
variability in SPT scores upon testing with different peanut extracts show
that the use of one single peanut extract can give false-negative result
No clear explanation can be given to explain the different results observe
in the studies of Hefle et al. (21) and those of our study. It can not b
excluded, however, that a difference in patient population might b
involved. In our study the patient population consisted of 16 patient wit
multiple characteristics of atopic allergy syndrome, while the patien
population in the studies of Hefle is not clearly defined.
Comparing the RAST scores with the SPT scores, a poor correlation wa
found in our study, except for CPE with a correlation coefficient of 0.73.
several other studies SPT results were compared with RAST or DBPCFC (2
28). The results do not agree with each other. A good correlation o
peanut-specific SPT and positive DBPCFC was found in majority of thes
studies (22-25). However, Kemp et al. (26) studied the SPT response, RAS
and clinical reactions to peanuts in 104 children and only found a goo
correlation when roasted peanuts were used. A very poor correlation wa
found between peanut-specific SPT and RAST scores in the studies o
Bahna et al. (27) and Adler et al. (28), which was explained by the diffe
ence in peanut sources from which the different extracts were prepared
Most of these studies compared the results in terms of positive RAST an
positive SPT and not in mm^2 wheal size with the exact RAST score. If w
transform our data in the same fashion, we still find discrepancies in 5 o
the 16 patients, which is relatively high. These patients showed a low or
not detectable SPT response depending on the used peanut extracts, i

combination with a RAST score of 2, 3 or 4 (Table 3). With the extract coded Y only one discrepancy was observed, while with our own CPE, which resembles the Y extract on SDS-PAGE, in 3 cases a poor correlation with RAST score was observed which might be due to a difference in protein concentration. The discrepancies between SPT and RAST scores with the extracts of X and Z, could be due to the fact that many protein bands are not present in these extracts compared to CPE or the Y extract.
Comparing the RAST score with the proliferative responses of PBMC, a poor correlation was found as well (r=0.34). Not all patients showed a proliferative response to peanuts, which could be due to the fact that these patients have a strict peanut-free diet. Agata et al. (29) showed already that an elimination diet reduces the proliferative response in food allergic patients. Although in the literature there is some controversy (10-12) with respect to whether the proliferation assay can be used as a diagnostic marker, the results of our study clearly show that at least for peanut allergy it cannot be used which is in agreement with the conclusions of May et al. (12) for other food proteins. Moreover, in a subsequent study (30) we have shown that also allergic patients with no peanut-allergy can show a high proliferative response to peanut protein. Moreover, recently it has been demonstrated that the use of a serum-free medium for the performance of a LST results in a strong proliferative response in both allergic and non-allergic individuals (31).
As the DBPCFC is still seen as the best diagnostic marker for food allergy, we compared the minimum threshold dose of peanut with the RAST and SPT scores. The correlation between the oral provocation dose and the RAST score was very poor (r=0.20). Three (A, B and L) of the 16 patients described here did not show any reaction after the consumption of peanuts while they did have peanut-specific IgE indicated by RAST scores of 2 and 4 respectively. Of these three patients the SPT scores are not very clear either. Comparison of the SPT scores with the provocation dose resulted in correlation coefficients between r=0.23 and r=0.61 (Table 4). Although several authors have suggested that whith using fresh materials in SPT, better results would be obtained (26, 32), we could not confirm this. Even with fresh, raw peanuts we observed false-negative SPT. Several possible explanations might be that the recognized epitope is not directly exposed in the protein but will become exposed after digestion, a difference between the local immune response in the gastro-intestinal tract, the

peripheral blood and the skin, and finally, that the patient suffers from food intolerance rather then a food allergy. This last explanation is not ver likely because these patients did show a high RAST score and, in an earlie test, also a reaction in the SPT. This confirms once more how complex foo allergy and its diagnosis is. The poor correlation between provocation dos and RAST scores or SPT scores questions the use of RAST and SPT other the to determine whether a patient suffers from a peanut-intolerance or peanut-allergy. The provocation dose indicates the amount of peanu that is tolerated by the patient and determines the strictness of the die This can be important because peanut is present in many food stuffs in very disguised manner, like for instance, in bouillon tablets. However, als this test seems not to be exclusive, since within one patient the results of provocation can differ when performed at different times, with no vali explanation.

From this study we conclude that to diagnose a peanut allergic patien one cannot rely on one method or one extract. This is mainly determine by the observation that the available extracts are not uniformly or clear characterized or standardized, which might result in a false-negativ diagnosis. For a severe food allergy, such as peanut allergy, this could b very dangerous due to the severe clinical symptoms. A DBPCFC may giv the most objective view whether a patient has symptoms after eating th offending food and the severity of the symptoms. However, also this te seems not to be conclusive since it can give different results within on patient when performed at different time points and it is not able t distinguish between a food intolerance and a food allergy. Moreover, wit peanuts a DBPCFC will never be without the risk of a severe reaction. Th most reliable manner to diagnose a possible peanut-allergic patient is combination of SPT with several extracts, RAST and a DBPCFC.

References

1 **Belanti JA.** (1991) Developmental aspects of food allergy in infancy and childhoo *Immunol Allergy Clin* **11**:885-891.

2 **Schreiber RA and Walker WA.** (1989) Food allergy: facts and fiction. *Mayo Clin Pro* **64**:1381-1391.

3 **Sampson HA.** (1983) Role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* **71**:473-480.
4 **Metcalfe DD.** (1991) Food allergy. *Curr Opin Immunol* **3**:881-886.
5 **Yunginger JW, Squillance DL, Jones RT and Helm RM.** (1989) Fatal anaphylactic reactions induced by peanuts. *Allergy Proc* **10**:249-253.
6 **Bock SA and Atkins FM.** (1989) The natural history of peanut allergy. *J Allergy Clin Immunol* **83**:900-904.
7 **Bock SA, Sampson HA and Atkins FM.** (1988) Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* **82**:986-997.
8 **Ebbeling WL and Bahna SL.** (1992) Food allergy diagnosis. *Nutrition Res* **12**:137-144.
9 **De Weck AL.** (1988) Immunologic diagnostic tests in food allergy. IN: SCHMIDT E, eds. Food allergy. Nestlé Nutrition Workshop Series. New York: Nestlé Ltd. Vevey/Raven Press. Ltd. **17**:177-186.
10 **Scheinmann P, Gendrel D, Charlas J and Paupe J.** (1976) Value of lymphoblast transformation test in cow's milk protein intestinal intolerance. *Clin Allergy* **6**:515-521.
11 **Kondo N, Agata H, Fukutomi O, Motoyoshi F and Orii T.** (1990) Lymphocyte responses to food antigens in patients with atopic dermatitis who are sensitive to foods. *J Allergy Clin Immunol* **86**:253-260.
12 **May CD and Alberto R.** (1972) *In vitro* responses of leukocytes to food proteins in allergic and normal children: lymphocyte stimulation histamine release. *Clin Allergy* **2**:335-340.
13 **de Jong EC, Spanhaak S, Martens BPM, Kapsenberg ML, Penninks AH and Wierenga EA.** (1995) Food allergen (peanut)-specific Th2 clones generated from the peripheral blood of a peanut-allergic patient. *J Allergy Clin Immunol* in press.
14 **Laemmli UK.** (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**:359-365.
15 **Towbin Hk, Staehelin TH and Gordon J.** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc Natl Acad Sci USA* **76**:4350-4354.
16 **Calkhoven Pg, Aalbers M, Ksohte VL and Allberse RC.** (1991) Relation between IgG_1 and IgG_4 antibodies to foods and the development of IgE antibodies to inYant allergens. I. Establishment of a scoring system for the overall food responsiveness and its application to 213 unselected children. *Clin Exp Allergy* 21:99-107.
17 **Böyum A.** (1968) Isolation of leucocytes from human blood. *Scan J Clin Lab Invest* **21**:9-14.
18 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts. *J Allergy Clin Immunol* **72**:61-68.
19 **de Jong EC, van Zijverden M, Spanhaak S, Pellegrom H and Penninks AH.** (1995) Identification of multiple allergens in peanuts. *Submitted for publication.*

20 **Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ and Helm RM.** (199 Identification and characterization of a second major peanut allergen, *Ara h* II, wit use of sera of patients with atopic dermatitis and positive peanut challenge. *J Allerg Clin Immunol* **90**:962-969.

21 **Hefle SL, Helm RM, Burks WA and Bush RK.** (1995) Comparison of commercial peanu skin test extracts. *J Allergy Clin Immunol* **95**:837-842.

22 **Aas K.** (1978) The diagnosis of hypersensitivity to ingested foods. *Clin Allergy* **8**:39-50.

23 **Bernstein M, Day JH and Welsh A.** (1982) Double-blind food challenge in the diagnos of food sensitivity in the adult. *J Allergy Clin Immunol* **70**:205-210.

24 **Sampson HA and Alberg R.** (1984) Comparison of results of skin tests, RAST, an double-blind, placebo-controlled food challenge in children with atopic dermatitis. *Allergy Clin Immunol* **74**:26-33.

25 **Atkins FM. Stainberg SS and Metcalfe DD.** (1985) Evaluation of immediate reactions t foods in adult patients I: Correlation of demographic, laboratory, and prick skin tes data with response to controlled oral food challenge. *J Allergy Clin Immunol* **75**:348 355.

26 **Kemp AS, Mellis CM, Barnett D, Sarota E and Simpson J.** (1985) Skin test, RAST an clinical reactions to peanut allergens in children. *Clin Allergy* **15**:73-78.

27 **Bahna SL and Gahndi MD.** (1987) Reliability of skin testing and RAST in diagnosis o food allergy. IN: Food allergy. Eds: Chandra RK. Newfoundland: Nutrition Researc Education Foundation St. John's.

28 **Adler BR, Assadullahi T, Warner JA and Warner JO.** (1991) Evaluation of a multipl food specific IgE antibody test compared to parental perception, allergy skin test and RAST. *Clin Exp Allergy* **21**:683-688.

29 **Agata H, Kondo N, Fukutomi O, Shinoda S and Orii T.** (1993) Effect of elimination diet on food-specific IgE antibodies and lymphocyte proliferative responses to foo antigens in atopic dermatitis patients exhibiting sensitivity to food allergens. *J Allerg Clin Immunol* **91**:668-679.

30 **de Jong EC, Spanhaak S, Pellegrom H, Bruyntjes JP, Penninks AH and Kapsenberg ML** (1995) Enhances proliferative responses of peripheral blood T cells of allergic patient to a non-relevant allergen, peanut protein. *Manuscript in preparation.*

31 **Upham JW, Holt BJ, Baron-hay MJ, Yabuhara A, Hales BJ, Thomas WR, Loh RKS O'Keefe PT, Palmer L, Le Souef WR. Sly PD, Burton PR, Robinson BWS and Holt PG** (1995) Inhalant allergen-specific T-cell reactivity is detectable in close to 100% o atopic and normal individuals: covert responses are unmasked by serum-free medium. *Clin Exp Allergy* **25**:634-642.

32 **Ortolani C, Ispano M, Pastorello V, Ansoloni R and Magri GC.** (1989) Comparison o results of skin prick test (with fresh foods and commercially food available extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* **83**:683-690

Chapter 3

Identification of multiple allergens in peanut proteins

Esther C. de Jong[1,2], Maaike van Zijverden[1], Steven Spanhaak[1], Hillie Pellegrom[1] and André H. Penninks[1]

[1] TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[2] Utrecht Toxicology Center, Utrecht, The Netherlands

Submitted for publication

Abstract

Peanuts are a major cause of food allergies both in children and adul which can induce an anaphylactic shock. The identification and charac terization of major peanut allergens will be of importance to increase th knowledge of the mechanism of food allergy and also contribute to th improvement of diagnostic tests for peanut allergy.

In the present study, the plasma concentrations and the binding to peanu proteins of peanut protein-specific immunoglobulin of peanut-allergic (PA allergic but not peanut-allergic (ANPA) and non-allergic (NA) individua were analyzed both by immunoblotting techniques and ELISA.

Only plasma of PA patients had IgE antibodies towards peanut protein. O these protein bands, 6 were recognized by more than 50% of the plasm samples with molecular weights of approximately 44, 40, 33, 21, 20 and 1 kD whereas to the last 3 protein bands more than 70% of the PA patien had IgE. The binding of peanut protein-specific IgA, IgM, IgG and IgG subclasses showed a more diverse recognition pattern of peanut-protei bands in the PA group compared to the ANPA and NA group. In the pla ma concentrations of peanut protein-specific immunoglobulins of th various classes, except IgE, no differences were found between the PA ANPA and NA group.

From the present study we conclude that peanuts contain multiple alle gens. The recognition of peanut proteins by immunoglobulins is mor diverse in PA individuals compared to ANPA or NA individuals which is no substantiated in the concentrations of peanut-specific immunoglobulins i plasma.

Introduction

The occurrence of food allergies is estimated between in 1-10% of the Western population (1, 2), especially in young children (3). Allergic reactions to foods involve the gastrointestinal tract (nausea, vomiting and diarrhoea), the skin (hives and angiodema), and the circulatory system (hypotension and ultimately systemic anaphylaxis) (4).

Peanuts, along with milk and eggs, account for approximately 80% of allergic reactions to foods in patients with atopic dermatitis (5, 6). Fatal anaphylactic reactions induced by peanuts are not uncommon (7). Allergic reactions to peanuts tend to persist for life, in contrast to foods like milk, eggs and soy. If children younger than 3 years with a positive food challenge for milk or egg are tested again 1-7 years later, 44% is negative, whereas the positive responses to peanut still exist in the majority of the children (3). Several food allergens have been identified that induce IgE-mediated disorders in humans, such as cod fish (8), shrimp (9-11) hen's egg (12, 13), cow's milk (14, 15), soybean (16) and peanut (17-22). Despite its name, peanut is not a nut, but an oil-legume (23), consisting of about 44% to 56% oil and 22% to 30% protein. Peanut proteins can be divided into the albumins, and the storage proteins arachin and conarachin which comprise about 87% of the total protein content (24). Sachs et al. described the first major allergen in peanuts, designated Peanut-I, which existed of two major bands with molecular weights of approximately 20-30 kD (17). Barnett et al. demonstrated IgE-binding to a Concanavaline A-reactive glycoprotein of approximately 65 kD and that the allergenicity of peanut is spread through both arachin and conarachin. Roasting does not seem to affect the allergenicity (18, 19). Burks et al. identified two major allergens, *Ara h* I (20) and *Ara h* II (21), with molecular weights of 63.5 and 17 kD, respectively. Whether either Peanut-1 or the concanavaline A-reactive glycoprotein and *Ara h* I or *Ara h* II are the same protein bands is unknown. The identification and characterization of allergens is essential for the understanding of the specific IgE-mediated immune response (25). Moreover, this identification could contribute to the improvement of diagnostic tests for peanut allergy.

In a previous study we showed that the T cell recognition by CPE-specific T cell clones from a peanut allergic donor is diverse (26). In this study, we

investigated the recognition of peanut proteins by immunoglobulins in th plasma of peanut allergic patients (PA) compared to allergic, but n peanut allergic (ANPA) and non-allergic (NA) individuals. For this, plasm samples from PA, ANPA, and NA subjects were analyzed by immuno-blotting and enzyme-linked immuno-sorbent assay (ELISA) for the presenc of peanut specific IgA, IgE, IgG, IgM and IgG subclasses antibodies.

Materials and methods

Subjects

The PA group consisted of 14 individuals with a peanut allergy as indicate by clinical symptoms, a positive skin-prick-test (SPT) and a positive radi allergo-sorbent test (RAST) score to peanut proteins. The ANPA grou consisted of 9 individuals with a positive SPT, RAST and clinical symptoms at least 2 of 7 tested common allergens: alternaria, birch, bird, cat, do grass and house dust mite. Their SPT and RAST to peanuts were negativ The NA group consisted of 10 individuals with no history of allergy and wi a negative RAST and SPT to the above mentioned allergens. Subject characteristics are summarized in Table 1.

Plasma was collected after centrifugation of heparinized venous blood 300g for 10 min at room temperature. The plasma was kept at -20^{0}C un used.

Crude peanut protein (CPE) preparation

Peanut proteins were extracted from raw, unshelled peanuts of the Runn Jumbo 38/42 variety (van Zijl, Hilversum, The Netherlands) as describe previously (27).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially according to Laemmli (28) using tw types of prepacked gels, 12% (2 wells prep comb, Bio-Rad Laboratorie Richmond, CA) and 15% (10 wells, Bio-Rad) Tris-HCl gels. Prior to loadin protein samples were reduced in extraction buffer, consisting of 1% (w/ dithiotreitol (DTT), 63 mM Tris-HCl, 2% (w/v) sodiumdodecylsulphate (SDS 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8, at 100^{0}C for 2

min. The 12% gel was loaded with 50 µl/well CPE (5 mg/ml) and the 15% gel with 10 µl/well. Pre-stained molecular weight markers (14.3-200 kD, Bio-Rad) were used as a reference. Electrophoresis was performed in 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS using a Bio-Rad Mini Protean II system (15 min at 80 V, followed by 1 h at 160 V). If not used for immunoblotting, gels were stained with Coomassie brilliant blue R-250.

Table 1: Characteristics of the PA, ANPA and NA subjects.

	PA	ANPA	NA
n	14	9	10
Mean age (years)	30.6	29	32.3
Sex (M/F)	3/11	2/7	2/8
Total IgE (IU/ml)	2334±4792	462±528	102±148
SPT[1]	positive	positive	negative
RAST[1]	positive	positive	negative
SPT[2]	positive	negative	negative
RAST[2]	positive	negative	negative

[1]: SPT/RAST total, [2]: SPT/RAST peanut specific

Immunoblotting

After separation of CPE by SDS-PAGE, proteins were transferred to polyvinylidenedifluoride membrane (PVDF-membrane, Immobilon-P Transfer membrane, Millipore Corporation, Bedford, MA) essentially according to Towbin et al. (29). Blotting was performed for 1 h at 100 V using a Mini Trans-Blot System and Power Supply Bio-Rad. After blotting, remaining binding sites were blocked for 1.5 h with 3% milkpowder (Protifar, Nutricia, The Netherlands) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH=7.4). Subsequently the PVDF-membranes were cut in either 2 (for the 12% gel) or 10 (for the 15% gel) identical strips. The strips were incubated overnight with plasma 1:10 (v/v) for 15% gel blots; 1:25 (v/v) for 12% gel blots at room temperature on a rocking facility.

For the detection of CPE-specific IgA, IgE, IgM, IgG and IgG subclasses, the 12% gel blots were placed in a Mini-protean II Multi Screen (Bio-Rad) con-

taining 20 lanes which were used to incubate the blot with variou monoclonal antibodies. Peroxidase (PO)-conjugated rabbit-anti-humar IgA, -IgE, -IgM and -IgG (DAKO, Glostrup, Denmark) were used at concentration of 1:1000 (v/v) with exception of IgG which was used at concentration of 1:500 (v/v). To detect IgG-subclasses, mouse-anti-humar IgG1, -IgG2, -IgG3 and -IgG4 (The Central Laboratory for Blood Transfusio Services, Amsterdam, The Netherlands) were used at a concentration 1:1000 (v/v). After incubation at 37°C for 2 h the lanes of the block contai ing anti-IgG-subclass antibodies, were washed and incubated for anothe hour with PO-conjugated rabbit-anti-mouse-IgG antibodies (DAKO Subsequently, the blots were washed thoroughly and developed f peroxidase activity using chloronaftol/DAB staining.

To determine in more detail the IgE binding in the plasma of PA subject the 15% gels were used. The blots were incubated and stained described above.

Specific binding to CPE on immunoblot was analyzed with a compute programme (Diversity One™ V1.1; IDF Inc., Huntington Station, NY) wit which the blots were scanned and stained proteins, indicating specifi binding, were detected.

CPE-specific ELISA

A CPE-specific ELISA was performed with 96-well plates (Maxisorp, NUNC Roskilde, Denmark) coated with CPE (1 mg/ml, 100 µl/well) in 0.1 M sodiur carbonate buffer, pH 9.6, overnight at 4°C. After incubation for 1 h at 37° with plasma, binding of CPE-specific immunoglobulins was detected wit the above described antibodies. PO-conjugated antibodies directe against IgA, IgM and IgG were used at a dilution of 1:2000, while ant bodies against the IgG-subclasses were diluted 1:1000. For the detection the IgG subclasses the secondary antibodies, rabbit-anti-mouse-IgG-P conjugated (1:1000) was used. Bound PO-conjugated antibodies wer detected using 3,3',5,5'-tetramethyl-benzidine (TMB) as substrate an absorbance was measured at 450 nm. One arbitrary unit was defined the amount of CPE-specific antibodies present in 1 ml of an appropriat dilution of pooled plasma. This pooled plasma consisted of plasma of subjects used in this study. The starting dilutions were as follows: for IgA 1:1 for IgM 1:20, for IgG 1:200, for IgG1 1:20, for IgG2 1:20, for IgG3 1:10 and f IgG4 1:10.

Statistical analysis

Statistical analysis was performed by Students' *t*-test taking $p<0.05$ as level of significance.

Results

Immunoblotting analysis of peanut protein-specific immunoglobulin-binding

Peanut-specific immunoglobulin-binding was examined using immunoblotting methodology with both individual and pooled sera of peanut-allergic (PA), allergic but not peanut-allergic (ANPA) or non-allergic (NA) subjects. Figure 1 shows the binding of IgA, IgE, IgM, IgG and IgG-subclasses to CPE on immunoblots after incubation with pooled plasma of the 3 groups.

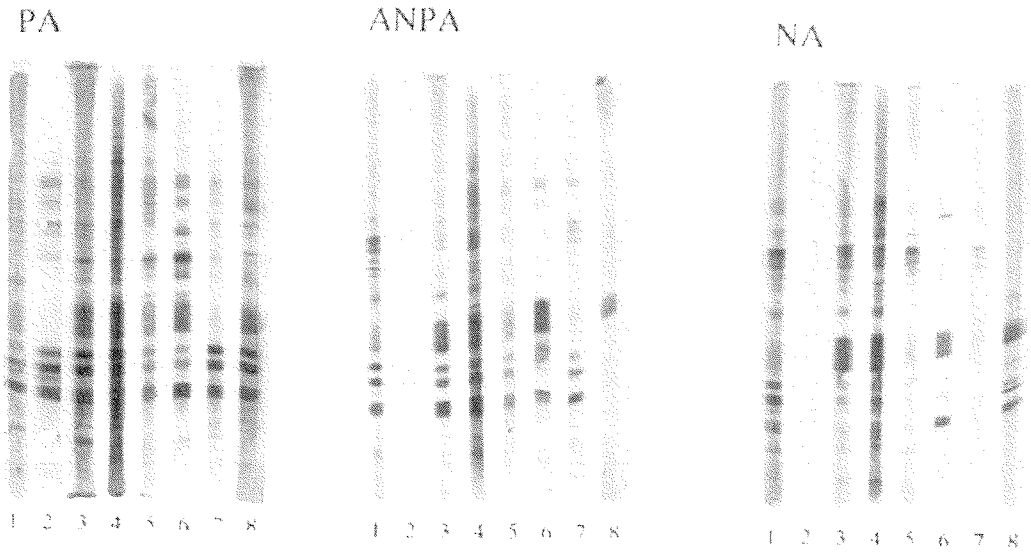


Figure 1: Recognition of CPE-specific antibodies in pooled plasma from the PA, ANPA or NA group.
Lane 1: IgM; lane 2: IgE; lane 3: IgG; lane 4: IgG1; lane 5: IgG2; lane 6: IgG3; lane 7: IgG4 and lane 8: IgA.
Western blots were incubated overnight with pooled plasma (1:25 v/v). Subsequently, CPE-specific antibodies were detected with Antibodies against IgA, IgE, IgM, IgG and IgG subclasses.

As expected, this figure shows that CPE contains multiple binding sites f all examined antibodies of the IgA, IgM, IgG classes and IgG-subclasse However, CPE-specific IgE could exclusively be detected in the sera of P subjects.

Using the immunoblots incubated with pooled plasma, the diversity of I binding proteins of the 3 groups were determined by counting the numb of recognized proteins using the Diversity One computer programm Except for IgM, the most diverse binding was found in the PA group as shown in Figure 2. The ANPA and NA group showed a comparable diversi in recognition of CPE by all Ig and IgG-subclasses (except IgE). Wi respect to IgM-binding, a more diverse binding to the various pean proteins was observed in the ANPA and NA groups compared to the P group. In the binding pattern of IgG1 no significant differences we observed between the groups.

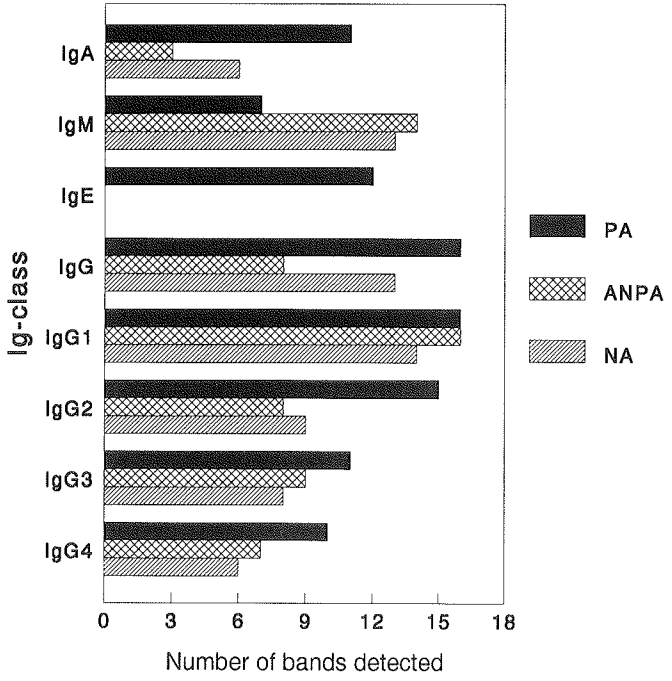


Figure 2: Number of Ig-binding proteins detected using pooled plasma sample of the PA, ANPA or NA group as estimated using the Diversity On computer programme.

Coomassie brilliant blue staining of the proteins after SDS-PAGE demonstrated 14 bands in CPE with molecular weights ranging from about 10 to 70 kD (Figure 3). In general, immunoblotting demonstrated a similar protein pattern as Coomassie brilliant blue gel staining, although some additional protein bands could be discovered. As is shown by incubating the immunoblot with an individual plasma (Lane 3) and pooled plasma of the PA group (Lane 4), not all proteins present in the CPE were recognized. IgE-specific binding to the 14 peanut proteins visible on the SDS-PAGE gel was investigated by immunoblotting analysis using the individual plasma samples of all 14 PA patients.

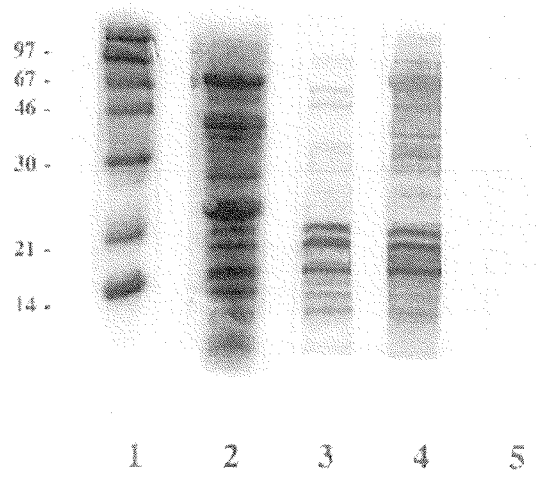


Figure 3: Detection of CPE-specific IgE in plasma of the PA subjects.
Lane 1: Coomassie brilliant blue staining of molecular weight markers.
Lane 2: Coomassie brilliant blue staining of CPE (10 µg of a 5 mg/ml solution).
Lane 3: Detection of CPE-specific IgE on immunoblot of CPE incubated overnight with individual plasma (1:10 v/v diluted) of donor R.
Lane 4: Detection of CPE-specific IgE on immunoblot incubated overnight with pooled plasma of the PA group (1: 10 v/v diluted).
Lane 5: Controle immunoblot which was not incubated with plasma, otherwise following the same procedure.

In Figure 4 the percentage of plasma samples of the PA individuals containing IgE to the detected proteins is presented based on the analysis of

the various immunoblots by the Diversity One computer programme. Ig binding occurred to almost all peanut proteins, although the highe percentage of IgE-binding was found for 6 peanut protein bands that we designated protein band number 3, 4, 6, 10, 11 and 12. The estimated mo ecular weights of these proteins are 44, 40, 33, 21, 20 and 18 kD. The last proteins were recognized by specific IgE in more than 70% of the inve tigated plasma samples. Remarkably, one higher located protein band approximately 23 kD, which is clearly present on SDS-PAGE (Figure 3), d not bind any IgE.

As the electrophoretic analysis followed by immunoblotting were carrie out under reduced conditions, it is not possible to indicate if these protei are actual individual proteins or subunits derived from higher molecul weight proteins. The identity of these protein bands was not further inves gated.

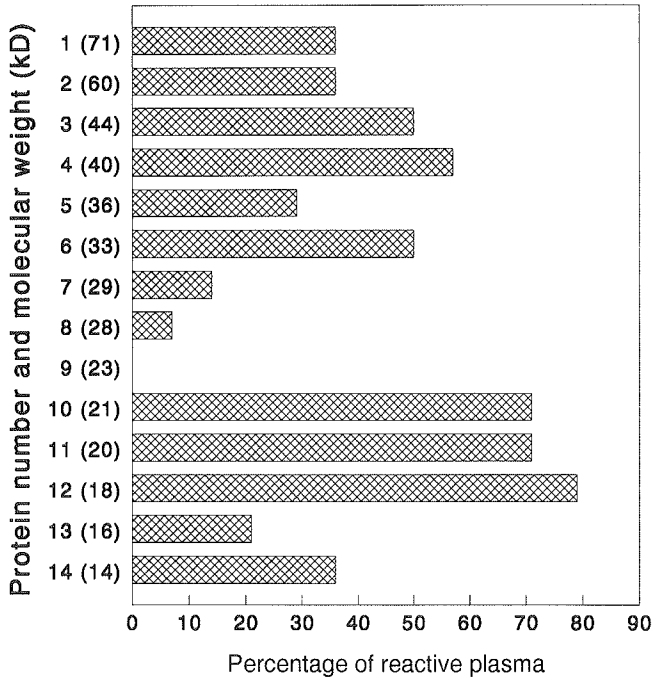


Figure 4: Percentage of plasma samples of the PA individuals showing specif IgE binding to the different proteins of CPE.

The CPE-specific binding of non-IgE immunoglobulins to the 3 main alle

gens was determined using individual plasma of all subjects (Figure 5). No significant differences could be observed between the percentages of positive sera with IgA and IgM. Approximately 90% of the plasma samples of the PA and ANPA subjects contained IgG which recognized these 3 main allergens, and only 60% of the NA subjects. This higher percentage in the PA and ANPA group is due to the increased recognition of these allergens by IgG2 and IgG4 antibodies compared to the NA group.

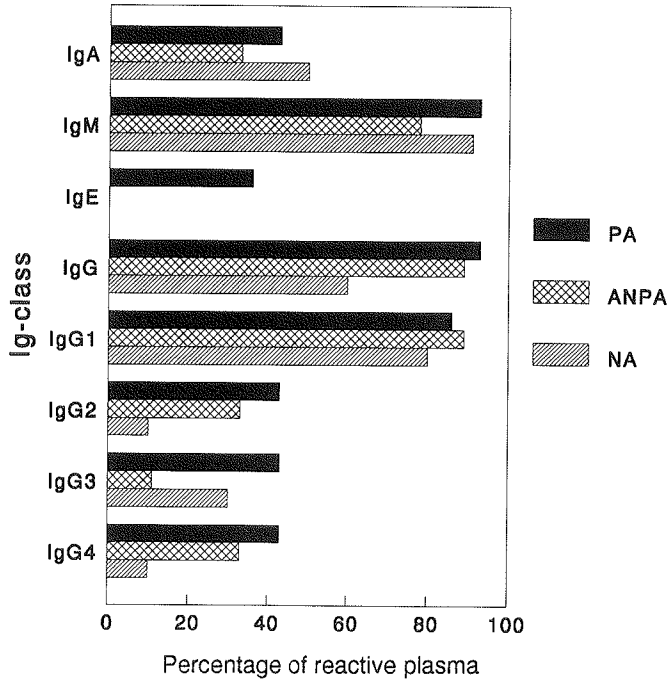


Figure 5: Percentage of plasma samples of the PA, ANPA and NA subjects containing Igs specific for the 3 major allergens.

Analysis of peanut protein-specific antibodies by ELISA methodology

To appreciate the above described immunoblotting analysis in a more quantitative manner, ELISA's were developed for the measurement of CPE-specific immunoglobulins in the plasma of all subjects and expressed as arbitrary units (Table 2). Statistical analysis showed no significant differences between the 3 groups. IgG appears to be elevated in the PA group, which is mostly due to the elevated levels of IgG1 and IgG4, but a large variation

in plasma-CPE-specific IgG between the different subjects was measure
Especially the levels of CPE-specific IgG4 varied strongly in all 3 groups.
some plasma samples IgG4 was present at a concentration little above th
detection limit while other plasma samples contained approximately 10,0
units of CPE-specific IgG4.

Table 2: Mean levels (± standard deviation) of CPE-specific immunoglobulins
plasma of patients and controls expressed as arbitrary units.

	PA	ANPA	NA
IgA	1243 (1313)	1497 (1338)	1632 (1069)
IgM	1633 (772)	1645 (833)	1340 (896)
IgG	8763 (10162)	3850 (1408)	5395 (4282)
IgG1	2787 (1470)	1923 (801)	4642 (3825)
IgG2	999 (1270)	999 (682)	2486 (5141)
IgG3	1127 (607)	1849 (1206)	1043 (434)
IgG4	3450 (10418)	844 (1900)	1634 (3485)

Discussion

The present study shows that the various proteins present in peanut conta
multiple allergens, which are recognized by more than 50% of PA subject
Moreover, 3 protein bands with a molecular weight of approximately 18-2
kD were recognized by plasma-IgE of more than 70% of our PA subject
Considering the molecular weights of these proteins of 18-21 kD, one
these protein bands, probably the 18 kD protein, might be similar to th
previously designated *Ara h* II with a molecular weight of 17 kD
described by Burks (21). As the N-terminal amino acid sequence of *Ara h*
is not known, no attempts were made to further identify our 18-21 k
proteins. The other peanut-allergen described by Burks et al (20), *Ara h*
was not found to be a major allergen for our population of patients.
A recent study by Hefle at al. (22) demonstrated not only IgE-binding to s

proteins between 15 and 25 kD, which is comparable to the molecular weights of the here described major allergens, but also skin test reactivity in 12 peanut-allergic patients. This could indicate that these proteins play a major role in the allergenicity of peanuts. But, as this study shows, also other proteins in peanuts do contain IgE binding sites. The multiplicity of peanut allergens, as shown by Hefle et al. (22), is confirmed by several studies on peanut allergens (17-22). But, as this study shows, also other proteins in peanuts contain IgE binding sites.

Differences in the recognition pattern of peanut allergens observed in a number of studies, can be explained in different ways. Firstly, since neither the peanuts were obtained from the same variety nor their extracts prepared with a standardized method, differences in protein composition of the extracts will have occurred (30). Secondly, diverse food habits between populations, for instance a high peanut consumption in the USA compared to other countries (30, 23), and consumption of different peanut varieties could lead to differences in recognitions patterns of the different peanut proteins by the various immunoglobulins investigated. Thirdly, the genetic background of peanut-allergic individuals from various parts of the world, could also play a role in the development of specific antibodies to an antigen. Most studies on peanut allergy have either been performed in the USA (17, 20-22) or in Australia (18, 19) whereas the present study was conducted in The Netherlands, this is another factor contributing to some differences.

In the plasma of ANPA and NA subjects, IgA, IgG and IgM against the 3 main allergens and to other protein bands in CPE could also be detected. Analyzing the number of immuno-reactive peanut proteins in pooled serum of each group, the most diverse recognition pattern of the protein bands in all immunoglobulin-classes was found in the PA group, although no differences were found in the quantitative analysis of CPE-specific antibodies in the plasma of the 3 groups. For several respiratory allergens a higher level of IgG, especially of the IgG4 subclass, has been detected in the sera of allergic individuals compared to non-allergic individuals (32, 33). However, this has not been found for cat allergen and it has been suggested that the presence of specific IgG4 in sera of non-allergic individuals is a result of high exposure (34). Several studies investigated the role of food-specific IgG and IgG subclass antibodies in food-allergic patients both in children (35-37) and adults (39-41). Both in children as in adults a higher level of

food-specific IgG4 was found (36-41) although this has been contradicte by others (35). Moreover, in children who became tolerant to cow's mi the casein-specific IgG1 and IgG4 antibodies diminished (37). In our stuc we did not find a significant higher concentration of peanut-specific IgG IgG4 in plasma of PA allergic individuals. Although the mean concentrati of both IgG and IgG4 was increased, there was a wide variation in th concentration of the immunoglobulins between patients, resulting in a hig standard deviation. In some of the sera of all groups, we could detect or very low concentrations of peanut-specific IgG4.

No significant differences were observed in peanut protein-specific Ig levels in the PA group compared to the ANPA and NA groups. It has bee suggested that IgA possesses a protective entity for the development food allergy and therefore, would be enhanced in allergic individuc Specific IgA antibodies bind to the specific antigen in the gut lume thereby preventing absorption of the antigen and making the antige available for digestion by intraluminal proteolytic enzymes. During the ea days of life, the human intestines are unable to produce IgA and the chi has to depend on mother's milk as sole IgA-source for the protecti against all kinds of viral and bacterial infections (41, 42). Savilahti et al. (4 showed that mothers of breast-fed children who developed cow's m allergy had a lower level of colostral IgA compared to mothers of no allergic children indicating an important role for colostral IgA. Since in se of the children no differences were observed, an organ-specific role for Ig may be involved. However, this has been contradicted by Falth-Magnu son (44) who did not find a difference in food-specific IgA or IgG in th colostrum of mothers of atopic and non-atopic children. Our study in adu also did not show any difference in specific IgA concentrations in sera allergic patients.

From the results of this study it can be concluded that peanut protei consist of various protein bands which express multiple allergens. A tot number of 6 protein bands showed binding to plasma-IgE in more than 50 of our PA patients, whereas to 3 proteins of 18-21 kD, plasma IgE-bindir was observed for more than 70% of the PA patients. The recognition peanut proteins by plasma Ig and IgG subclasses was very diverse plasma samples of PA individuals and more restricted in plasma samples ANPA and NA individuals. This more diverse recognition pattern in P patients did not lead to increased levels of peanut protein-specific imm

noglobulins of the various investigated classes, although IgE was only found in plasma samples of the PA patients.

Aknowledgments

The authors would like to thank Riek Vlooswijk and Henriette Bleeker for technical assistence and Dr. Martin Hessing for critical reading of the manuscript (TNO Nutrition and Food Research Institute).

References

1 **Belanti JA.** (1991) Developmental aspects of food allergy in infancy and childhood. *Immunol Allergy Clinics North America* **11**:885-891.

2 **Schreiber RA and Walker WA.** (1989) Food allergy: facts and fiction. *Mayo Clinics Proc* **64**:1381-1391.

3 **Bock SA.** (1982) The natural history of food sensitivity. *J Allergy Clin Immunol* **69**:173-177.

4 **Metcalfe DD.** Food allergy. *Curr Opin Immunol* **3**:881-886.

5 **Sampson HA.** (1983) Role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* **71**:473-480.

6 **Burks AW, Williams LW, Mallory SB, Shirell MA and Williams C.** (1989) Peanut protein as a major cause of adverse food reactions in patients with atopic dermatitis. *Allergy Proc* **10**:265-269.

7 **Yunginger JW, Squillace DL, Jones RT and Helm RM.** (1989) Fatal anaphylactic reactions induced by peanuts. *Allergy Proc* **10**:249-253.

8 **Elsayed S and Apold J.** (1983) The immunochemical analysis of cod fish allergen M: Location of the immunoglobulin binding sites as demonstrated on the native and synthetic peptides. *Allergy* **38**:449-459.

9 **Hoffman DR, Day ED and Miller JS.** (1981) The major heat stable allergen of shrimps. *Ann Allergy* **47**:17-22.

10 **Lehrer SB, Ibanez MD, McCants ML, Daul CB and Morgan JE.** (1990) Characterization of water soluble shrimp allergens released during boiling. *J Allergy Clin Immunol* **85**:1005-1013.

11 **Daul CB, Slattery M, Morgan JE and Lehrer SB.** (1991) Isolation and characterization of an important 36 kD shrimp allergen. *J Allergy Clin Immunol* **87**:192.

12 **Hoffman DR.** (1983) Immunochemical identification of the allergens in hen's egg white. *J Allergy CLin Immunol* **71**:481-486.

13 **Langeland T.** (1983) A clonical and immunological study of allergy to hen's e white. IV. specific IgE antibodies to individual allergens in hen's egg white related clinical and immunological parameters in egg-allergic patients. *Allergy* **38**:493-500.
14 **Ball G, Shelton MJ, Walsh BJ, Hill DJ, Hosking CD and Howden ME.** (1994) A mc continous allergenic epitope of bovine beta-lactoglobulin recognized by human I binding. *Clin Exp Allergy* **24**:758-764.
15 **Kohno Y, Honma K, Saito K, Tsunoo H, Kaminogawa S and Niimi H.** (1994) Preferen recognition of primary protein structures of alpha-casein by IgG and IgE antibod of patients with milk allergy. *Ann Allergy* **73**:419-422.
16 **Ogawa T, Bando N, Tsuji H, Okajima H, Nishikawa K and Sasaoka K.** (1991) Inve gation of the IgE-binding proteins in soybeans by immunoblotting with sera soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitaminol* **37**:555-565.
17 **Sachs MI, Jones RT and Yunginger JW.** (1981) Isolation and partial characterization a major peanut allergen. *J Allergy Clin Immunol* **67**:27-34.
18 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts *Allergy Clin Immunol* **72**:61-68.
19 **Barnett D and Howden MEH.** (1986) Partial characterization of an allergenic glyc protein from peanut (*Arachis hypogaea* L.). *Biochim Biophys Acta* **882**:97-105.
20 **Burks WA, Williams LW, Helm RM, Connaughton C, Cockrell G and Helm RM.** (19 Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermat and positive peanut challenges. *J Allergy Clin Immunol* **88**:172-179.
21 **Burks WA, Williams LW, Connaughton C, Cockrell G, O'Brien T and Helm RM.** (19 Identification and characterization of a second major peanut allergen, *Ara h* I, W use of sera of patients with atopic dermatitis and positive peanut challenges *Allergy Clin Immunol* **88**:172-179.
22 **Hefle SL, Folgert JP, Bush RK and Sun Chu F.** (1994) Monoclonal antibodies agai selected peanut allergens: production and use as affinity agents. *Food Agric Immunol* **6**:197-208.
23 **Fries JH.** (1982) Peanuts: allergic and other untoward reactions. *Annals of Aller* **48**:220-226.
24 **Ahmed EH and Young CT.** (1982) Composition, nutrition, and flavor of peanuts. Peanut Science and Technology. Eds: Pattel HE and Young CT. American Pear Research and Education Society, Inc. 655-688.
25 **Chapman MD.** (1989) Purification of allergens. *Curr Opin Immunol* **1**:647-53.
26 **de Jong EC, Spanhaak S, Pellegrom H, Wierenga EA and Penninks AH.** (1995) Diver protein specificity of peanut-specific Th2 clones from a peanut-allergic patie *Submitted for publication.*
27 **de Jong EC, Spanhaak S, Martens BPM, Kapsenberg ML, Penninks AH and Wieren EA.** (1995) Food allergen (peanut)-specific Th2 clones generated from the periphe blood of a peanut-allergic patient. *J Allergy Clin Immunol* In press.

28 **Laemmli UK.** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

29 **Towbin HJ, Staehelin T and Gordon J.** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc Natl Acd Sci USA* **76**:4350-4354.

30 **Basha SM.** (1979) Identification of cultivar differences in seed polypeptide composition of peanuts (*Arachis hypogae* L.) by two dimensional polyacrylamide gel electrophoresis. *Plant Physiol* **63**:301-306.

31 **Kistenmaker C, Aarnink EJM and Hulshof KFAM.** (1992) De consumptie van afzonderlijke producten door Nederlandse bevolkingsgroepen. Voedselconsumptiepeiling 1992. *TNO rapport,* V93.418.

32 **Gwynn CM, Smith JM and Leon GL.** (1987) Role of IgG4 subclass in childhood allergy. *Lancet* **1**:910-911.

33 **Chapman MD, Rowntree S, Mitchell EB, di Frisco de Fuenmajor MC and Platts-Mills TAE.** (1983) Quantitative assesments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitus. *J Allergy Clin Immunol* **72**:27-33

34 **Homburger HA, Mauer K, Sachs MI, O'Connel EJ, Jacob GL and Caron JC.** (1986) Serum IgG4 concentrations and allergen-specific IgG4 antibodies compared in adults and children with asthma and non-allergic individuals. *J Allergy Clin Immunol* **19**:427-434.

35 **Host A, Husby S, Gjesing B, Larsen JN and Lowenstein H.** (1992) Prospective estimation of IgG, IgG subclasses and IgE antibodies to dietary proteins in infants with cow milk allergy. Levels of antibodies to challenge and clinical course of cow milk allergy. *Allergy* **47**:218-229.

36 **Roger A, Pena M, Botey J, Esseverri JL and Marin A.** (1994) The valus of specific IgG4 determination in childhood allergy to egg in relation to specific IgE and the provocation test. *J Investig Allergol Clin Immunol* **4**:87-90.

37 **James JM and Sampson HA.** (1992) Immunologic changes associated with the development of tolerance in children with cow milk allergy. *J Pediatr* **121**:371-377.

38 **Stoger P and Wuthrich B.** (1993) Type I allergy to cow milk proteins in adults. A retrospective study of 34 adult milk- and cheese-allergic patients. *Int Arch Allergy Immunol* **102**:399-407.

39 **Husby S, Schultz-Larsen F and Svehag SE.** (1989) IgG subclass antibodies to dietary antigens in atopic dermatitis. *Acta Derm Venereol Suppl Stockh* **144**:88-92.

40 **El-Rafei A, Peters SM, Harris N and Bellanti JA.** (1989) Diagnostic value of IgG4 measurements in patients with food allergy. *Ann Allergy* **62**:94-99.

41 **Hanson LA, Ahlstedt S, Andersson B, Carlsson B, Fällström SP and Mellander L.** (1985) Protective factors in milk and the development of the immune system. *Pediatrics* **75**:172-176.

42 **Mazanec MB, Nedrud JG, Kaezal CS and Lamm ME.** (1993) A three-tiered vieuw of the role of IgA in the mucosal defence. *Immunol Today* **14**:430-435.

43 **Savilahti E, Taino VM, Salmenperä L, Arjomaa M, Kallio J, Perheentupa J and Siim MA.** (1991) Low colostral IgA associated with cow's milk allergy. *Acta Paedi Scand* **80**:1207-1213.

44 **Falth-Magnusson K.** (1989) Breast milk antibodies to foods in relation to matern diet, maternal atopy and the development of atopic disease in the baby. *Int Ar Allergy Appl Immunol* **90**:297-300.

Chapter 4

Food allergen (peanut)-specific Th2 clones generated from the peripheral blood of a peanut-allergic patient

Esther C. de Jong[1,2], Steven Spanhaak[1], Ben P.M. Martens[3], Martien L. Kapsenberg[4], André H. Penninks[1] and Eddy A. Wierenga[4]

[1]TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[2]Utrecht Toxicology Center, Utrecht, The Netherlands
[3]Allergy Center Utrecht, Utrecht, The Netherlands
[4]Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, The Netherlands

J Allergy Clin Immunol, *In press*

Abstract

Increasing evidence indicates a prominent role of allergen-specific Th cells with high IL-4 and IL-5 production and low IFN-γ production, in th regulation of IgE and eosinophil production in allergic disorders. Howeve most of these studies have concentrated on T cells reactive with inhalatio allergens, whereas little is still known about the properties of food-allerge reactive T cells.

In the present study, we therefore characterized peanut-specific T cell cloned from a severe peanut allergic patient.

Peripheral blood mononuclear cells (PBMC) from peanut-allergic and nor allergic individuals were stimulated with crude peanut extract (CPE) t compare the proliferative responses and to select a suitable patient for th cloning of CPE-specific T cells. The resultant panel of CPE-reactive T lym phocyte clones was serologically phenotyped by flowcytometry and ana lyzed for cytokine secretion by ELISA.

The patients' PBMC showed a dose-dependent proliferative response t CPE which was significantly higher ($p<0.05$) than in PBMC of non-allergi donors. The CPE-specific TLC generated from the selected patient were c CD4+/ CD8- T helper cells with a Th2 cytokine profile, secreting hig amounts of IL-4 and IL-5, but little or no IFN-γ.

This study demonstrates that peanut-specific T cells do occur in the perip heral blood of peanut-allergic patients and suggests an increased frequen cy of these T cells in patients, as compared to non-allergic control individu als. The CD4+ phenotype and the Th2 cytokine profile of the CPE-specifi TLC suggest a functional role of allergen-specific Th2 cells in the pathophy siology of food allergy, similar to the function of inhalation allergen-specifi Th2 cells.

Introduction

Food allergies occur in 1-10% of the Western population (1, 2), especially in young children (3). Most common food allergens are proteins in cow's milk, hen's egg, peanut and soy bean (4) which may induce a type I food allergic reaction in sensitized individuals, characteristically associated with high serum levels of specific immunoglobulin E (IgE) (5) and eosinophilia (6, 7). So far, little is known about the mechanism underlying the development of a type I food allergy.

For allergy to inhalation allergens, like house dust mites (HDM), various pollen and domestic animals, several studies revealed that allergen-specific T cells generated from peripheral blood (8-11) or skin (12, 13) of atopic donors are Th2 cells producing high levels of interleukin-4 (IL-4) and IL-5 but little or no interferon-γ (IFN-γ). IL-4 plays an important role in the regulation of production of IgE antibodies, both *in-vitro* (14-18) and *in-vivo* (19), by inducing an isotype switch to IgE in B cells (14, 17, 18). This IL-4-induced isotype switch is inhibited by IFN-γ (20-22). Consequently allergen-specific Th2 cells from atopic donors are efficient helper cells for IgE synthesis (9, 23-25). IL-5 has been shown to induce eosinophil proliferation and activation (26, 27). Eosinophils play a role in the late phase allergic reaction causing tissue damage by toxic mediators released by degranulation (28, 29).

Some clinical symptoms of food allergy such as the oral allergy syndrome (OAS) (30) and the gastro-intestinal problems (31) appear to be related to allergen uptake in the digestive tract and therefore to be confined to this type of allergy. Other clinical manifestations, however, are very similar to those seen in inhalation allergy, i.e. atopic dermatitis, asthma, anaphylaxis and eosinophilia. Because of the key role of allergen-specific T cells in the pathophysiology of these disorders, we investigated whether food allergen-specific T cells have properties similar to those of the above described inhalation allergen-specific T cells. To address this question we generated and characterized peanut protein-specific T cell clones from a peanut-allergic patient. We focussed on peanut allergy because this is a common food allergy in adults which is very persistent and can cause severe symptoms like anaphylactic shock followed by death (32-34). For this study peanut proteins were extracted from raw, unshelled peanuts. The obtained

crude peanut extract (CPE) was used to compare peanut-specific T ce reactivity in PBMC of non-allergic (NA) and peanut-allergic (PA) individua The proliferative response to peanut agglutinin (PNA), the naturally occ ring lectin in peanuts, was studied as a parallel control because this lect may induce mitogenic stimulation as demonstrated in bovine PBMC (35 Based on this comparative study one peanut-allergic patient was selecte for the generation and characterization of CPE-specific T cell clones.

Materials and methods

Subjects

The non-allergic control group (NA) consisted of 10 individuals (2 males, females; mean age 31.9 ±10.0 years) with no history of allergy and nega ve radio-allergo-sorbent-test (RAST) and skin-prick-test (SPT) scores f peanut allergen and 7 common other allergens (grass, cat, dog, alternari birch, bird and house dust mite). The mean total serum IgE titer was 1((±148) IU/ml. The peanut-allergic group (PA) consisted of 7 patients male, 6 females; mean age 27.1 ±6.7 years) with a peanut allergy as i dicated by clinical symptoms and positive peanut-specific RAST (>3+) an SPT scores. Approval for this study was obtained from an independe Medical-Ethical Commitee. An informed consent was obtained from subjects. Peanut specific T cell clones were generated from a severe P subject (NvD), giving strong proliferative responses to CPE in PBMC. This w a 15-year-old girl with a severe peanut and coriander allergy causi symptoms like the oral allergy syndrome, asthma and anaphylactic shoc At the time of blood collection, 4 weeks after a severe anaphylactic shoc the total serum IgE level was 708 IU/ml with high levels of peanut specif IgE (5+) as determined by RAST.

Peanut protein extraction

Peanut proteins were extracted from raw, unshelled peanuts of the Runn Jumbo 38/42 variety (van Zijl, Hilversum, The Netherlands) using a metho modified from Barnett et al. (36). Briefly, peanuts were ground and fat w extracted by Soxhlet using petroleum ether at 40-60^{0}C. The defatted flo was ground again and suspended in 0.1 M ammoniumbicarbonate. Th

suspension was stirred for 4 h and centrifugated at 10,000g for 30 min at 4^0C to remove insoluble compounds. The supernatant was dialysed over night against distilled water using a 3.5 kD cut-off membrane (Spectrum Medical Industries, Inc., Houston, TX). The dialysed extract, referred to as crude peanut extract (CPE), was lyophilized and stored at -20^0 C. The obtained CPE consisted of approximately 90% protein as determined with the Bio-Rad protein assay (Bio-Rad, München, Germany). On SDS-PAGE with silver staining, CPE consisted of several bands between 70 and 10 kD. The PNA concentration in CPE was estimated by intensity of the bands at approximately 1%.

Culture media

The cloning procedure and proliferation assays were performed in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 10% pooled complement inactivated normal human serum (HS; Central Laboratory Blood Transfusion Service (CLB), Amsterdam, The Netherlands) and gentamycine (50 μg/ml; Flow Laboratories, Irvine, UK).
Epstein Barr-virus (EBV) transformed B cells were cultured in IMDM supplemented with 10% Fetal Calf Serum (Hyclone, Logan, Utah) and gentamycine (50 μg/ml).

Proliferation assays with PBMC

PBMC were isolated from heparinized venous blood using density gradient separation on Ficoll-Plaque (Pharmacia LKB) essentially as described by Böyum (37). Recovered cells were cultured (2×10^5/200 μl) in triplicate at 37^0C and 5% CO_2 for 7 days in IMDM supplemented with HS in the absence or presence of increasing concentrations of CPE (1, 10, 50, 100, 150, 200 and 250 μg/ml) or peanut agglutinin (PNA; lectin from *Arachis Hypogaea*; 1, 10, 50 and 100 μg/ml; Sigma, St. Louis, MO) in 96-well flat-bottom culture plates (NUNC, Roskilde, Denmark). Proliferation was measured using ^{3}H-thymidine (^{3}H)-TdR) incorporation. After 7 days, (^{3}H)-TdR (0.4 μCi/well; Amersham, Aylesbury, UK) was added and the cells were incubated for another 18 h and then harvested (Harvester 96, Tomtec, Orange, USA). (^{3}H)-TdR incorporation was measured using a 1450 Microbeta-counter (Wallac, Turku, Finnland).

T cell cloning procedure

CPE-specific T cell clones were generated from heparinized venous bloo as described previously (25). In parallel to the above described prolifer tion experiments, identical triplicate cultures were started in seperat culture plates. If cultures in the first plate indicated PNA- or CPE-induced cell proliferation on day 7, cultures in the parallel plate were used for th generation of specific T cell clones. In order to further promote the expans on of CPE- or PNA-reactive, recombinant interleukin-2 (rIL-2; Eurocetu Amsterdam, The Netherlands) was added to each well on day 7 at a fin concentration of 20 IU/ml. After another 7 days of culture, cells wer cloned from the thus formed short-tern CPE-specific T cell lines by limitin dilution at 1 or 0.3 cells/100 µl/well in 96-well flat-bottom culture plates an mitogenic stimulation with PHA (final concentration 5 µg/ml; Difco, Detroi MI). To all wells 100 µl of a feedermix was added consisting of irradiate (3000 rad) PBMC of two unrelated donors (each 10^6 cells/ml) irradiate (3000 rad) cells of the Epstein-Barr virus transformed B cell line JY (2x1 cells/ml) and rIL-2 (20 IU/ml) as growth factor. Approximately 3 weeks late expanded clones were tested for CPE-reactivity in an antigen-specifi proliferation assay performed in IMDM supplemented with HS in duplicat in 96-well flat-bottom culture plates. To 100 µl T-cell suspension (10^5 cells) 5 µl irradiated (3000 rad) freshly isolated autologous PBMC ($2x10^5$) as source of antigen presenting cells (APC), and 50 µl CPE (200 µg/ml) wer added. After 48 hours proliferation was measured by (^{3}H)-TdR incorporatio as described before. Criteria for antigen specific proliferation were minimum of 1000 cpm and a stimulation index (SI) of 5 or more. SI was th measured cpm in the presence of antigen divided by the measured cpr in the absence of antigen. CPE-specific T cell clones were maintained i culture by bi-weekly restimulation with PHA in the presence of the feede mix as described above. All experiments were performed on day 10 afte restimulation.

Surface marker analysis of T cell clones

For analysis of surface marker expression by the CPE-specific T cell clone the following fluorescein (FITC-) or phycoerythrin (PE)-conjugated monoclo nal antibodies were used: CD3-PE (T3); CD4-FITC (T4); CD8-PE (T8); CD45RA PE (2H4); CD29-PE (4B4) and HLA-DR-FITC (I3) all purchased from Coulte (Hialeah, FL). Before labeling, the T cell clones were seperated from th

remaining feedermix cells using Ficoll-Paque as described before for the isolation of PBMC. Per experiment, 10^5 cells were suspended in 100 µl PBS (Coulter) containing 1% BSA (Organon Teknika, Boxtel, The Netherlands) and labelled with 10 µl undiluted solution of the appropriate antibody. After 30 min of incubation at 4^0C the cells were washed twice by centrifugation for 5 min at 250g at 4^0C and resuspending the pellet in 2 ml PBS containing 1% BSA. The final pellet was resuspended in 300 µl PBS containing 1% BSA. Analysis was performed using an Epics Elite flow cytometer (Coulter). Results were expressed as percentage of positive cells.

Epstein Barr-virus transoformation of autologous B cells

Antigen-specific proliferation assays with T cell clones to obtain culture supernatant for the determination of cytokine production, were performed using Epstein-Barr virus (EBV) transformed autologous B cells (EBV-B) as antigen presenting cells (APC). For EBV transformation 10^6 PBMC in 500 µl IMDM supplemented with 10% FCS and 4 µg/ml cyclosporin-A were added to 500 µl supernatant of a 10-day-culture of the Marmoset monkey B95-8 cell line (38) containing EBV, and cultured overnight at 37^0C in a 24-well culture plate (NUNC). Cells were washed 3 times and resuspended in 200 µl IMDM/FCS and cultured in a 96-well flat-bottom culture plate at 37^0 C and 5% CO_2 until proliferating cells were observed by light microscopy. EBV-B cells were maintained at a concentration of approximately 10^6 cells/ml in IMDM with 10% FCS.

Cytokine assays

Cytokine secretion profiles of the CPE-specific T cell clones were determined after both mitogeneic stimulation with monoclonal antibodies against CD3 and CD28 as well as after specific stimulation with CPE in the presence of APC. For mitogeneic stimulation, triplicate cultures of 10^5 cells/well were stimulated in a 96 well flat bottom culture plate with anti-CD3 monoclonal antibody (CLB-1xE; CLB) and anti-CD28 monoclonal antibody (CLB-28/1; CLB) both at a final dilution of 1:1000 in a volume of 200 µl/well in IMDM with 10% HS. For antigen-specific stimulation 10^5 cells/well in triplicate cultures were stimulated in 200 µl IMDM/10% HS with CPE (final concentration 50 µg/ml) in the presence of $2x10^4$ EBV-B/well as APC. After 36 h the culture plates were centrifugated at 300g for 10 min at room temperature. Supernatants from the triplicate cultures were collected, pooled and stored

at -20^0 C until determination of the cytokine concentration.
Both IFN-γ and IL-4 secretion were measured using ELISA kits from Gibc and CLB, respectively and IL-5 using an ELISA as described before (39).

Statistical analysis
Statistical analysis was performed by creating a orthogenale polynom with a linear, square and cubic component. These polynomes wer created for the proliferative response to ioncreasing amounts of CPE every subject. The mean polynomes of the PA and NA group were teste for statistical significant differences using a Students' *t*-test taking $p<0.05$ level of significance.

Results

Peanut specific proliferation in PBMC of peanut allergic and non-allergi donors.
A clear dose-dependent proliferative response was measured upo exposure of PBMC of peanut-allergic patients (PA; n=7) to CPE of 991 (±6834) cpm in the presence of 250 μg/ml CPE (915 ±816 without CPE whereas PBMC of non-allergic control individuals (NA; n=10) showe onlyvery weak responses to CPE of 393 (±329) cpm in the presence of 25 μg/ml CPE (163 ±183 without CPE). In Figure 1 these data are expressed stimulation index (SI) which represents the measured cpm in the presenc of CPE divided by the measured cpm in the absence of CPE.
Responses in PBMC of the PA group were significantly higher ($p<0.05$) tha in PBMC of the NA group from CPE concentration of 50 μg/ml. PNA, th naturally occuring lectin in peanuts, induced only very weak responses PBMC of both groups. The responses were not significantly different be tween the two groups (Figure 1).
After the demonstration of CPE-specific T cells reactivity in PBMC of th peanut-allergic patients, CPE-specific T cell clones were generated fro one of the peanut-allergic patients, NvD, selected for showing a relative strong PBMC response reaching a plateau level of 5317 cpm in the preser ce of 150 μg/ml CPE (444 cpm without CPE). Also in PBMC of th patient stimulation with purified PNA induced only a weak proliferativ

response.

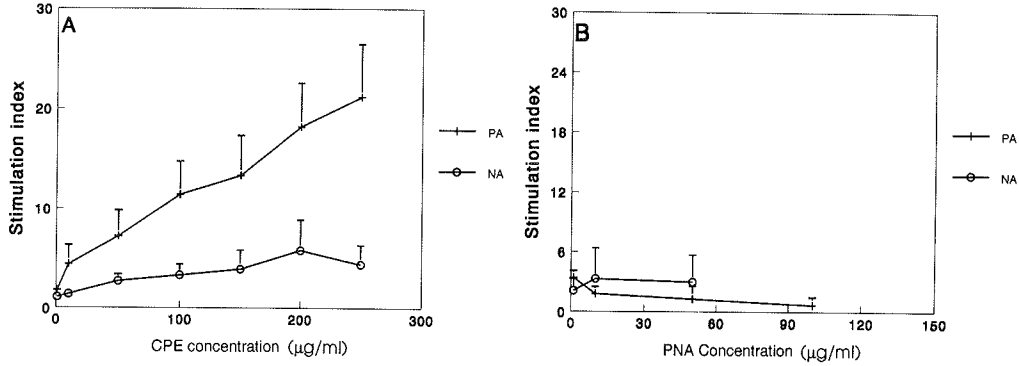


Figure 1: Proliferative response of PBMC from peanut-allergic (PA; n=7) and non-allergic donors (NA; n=10), A) to peanut proteins (CPE) and B) to PNA.
Proliferation of PBMC ($2x10^5$ cells/well) was determined by measuring (^{3}H)-TdR incorporation after 7 days of incubation in the absence or presence of increasing amounts of CPE or PNA.
Results are expressed as mean stimulation index (SI) ± SEM.

CPE-specific T cell clones

From a CPE-reactive T cell line obtained by stimulation of PBMC of this patient with 150 µg/ml CPE for 14 days, 49 T cell clones were generated. Testing this panel of T cell clones for CPE-specificity, 10 clones proved to be reactive, which were designated GB101, GB102, GB105, GB106, GB107, GB108, GB109, GB110, GB111 and GB114 (Figure 2). These 10 clones were further studied for their expression of certain surface markers by flow cytometry and for their cytokine secretion profile by ELISA.

Surface marker expression by CPE-specific T cell clones.

The surface marker phenotype of the 10 CPE specific T clones, was determined by flow cytometric analysis 10 days after restimulation with feeder-mix. All T cell clones expressed a typical phenotype of activated memory Th cells: $CD3^+$, $CD4^+$, $CD8^-$, $CD45RA^-$, $CD29^+$, $HLA\text{-}DR^+$. The percentage of

activated cells, expressing HLA-DR varied strongly (10 to 100%) betwee
the different clones.

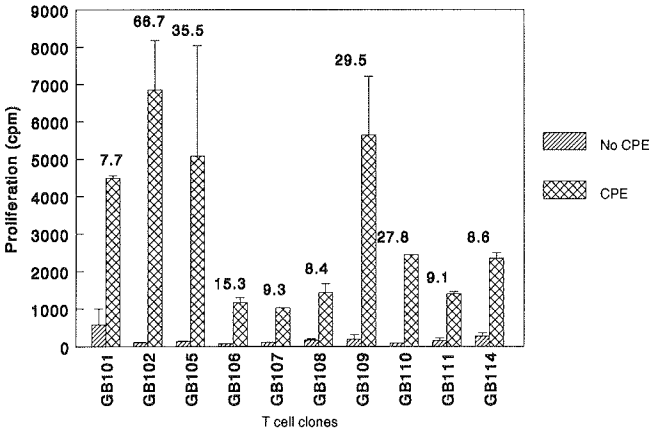


Figure 2: CPE-specific proliferative responses of T cell clones from peanu allergic patient NvD. Cloned cells (10^5 cells/well) were cultured w 10^5 3000 rad irridiated autologous PBMC as APC in the absence presence of 50 µg/ml. Proliferation was measured after 48 h of inc bation using (^{3}H)-TdR incorporation.
The bars express mean values ± SEM of triplicate cultures, whereas th stimulation index is given on top of the bars.

Th2 cytokine secretion profile of CPE-specific T cell clones.

Cytokine secretion of the CPE-specific T cell clones was measured in 36 supernatants after stimulation of 10^5 cells with either a combination of an CD3 and anti-CD28 or CPE in the presence of APC. Reactivity of the clon to these different modes of stimulation was confirmed by (^{3}H)-TdR incorp ration in identical parallel cultures. As is shown in Figure 3, the CPE-reactiv T cell clones secreted high levels of IL-4 and IL-5 and little no IFN-γ after strong mitogenic stimulation indicating a Th2 pr file. Stimulation with CPE showed a similar profile only low concentrations of the secreted cytokines were measured. IL-4 w produced at concentration of 200 to 500 pg/ml, IL-5 was produce at a non-detectable concentration (200 pg/ml) to 600 pg/ml an IFN-γ was produced less then 20 to 450 pg/ml. The cytokine produ

tion was compared to the prevoiusly described, well charcterized HDM-specific T cell clones with a Th1 (MBE.AA40 and MBE.AA42) or Th2 (MBB.AA60) phenotype measured under identical experimental condition (40).

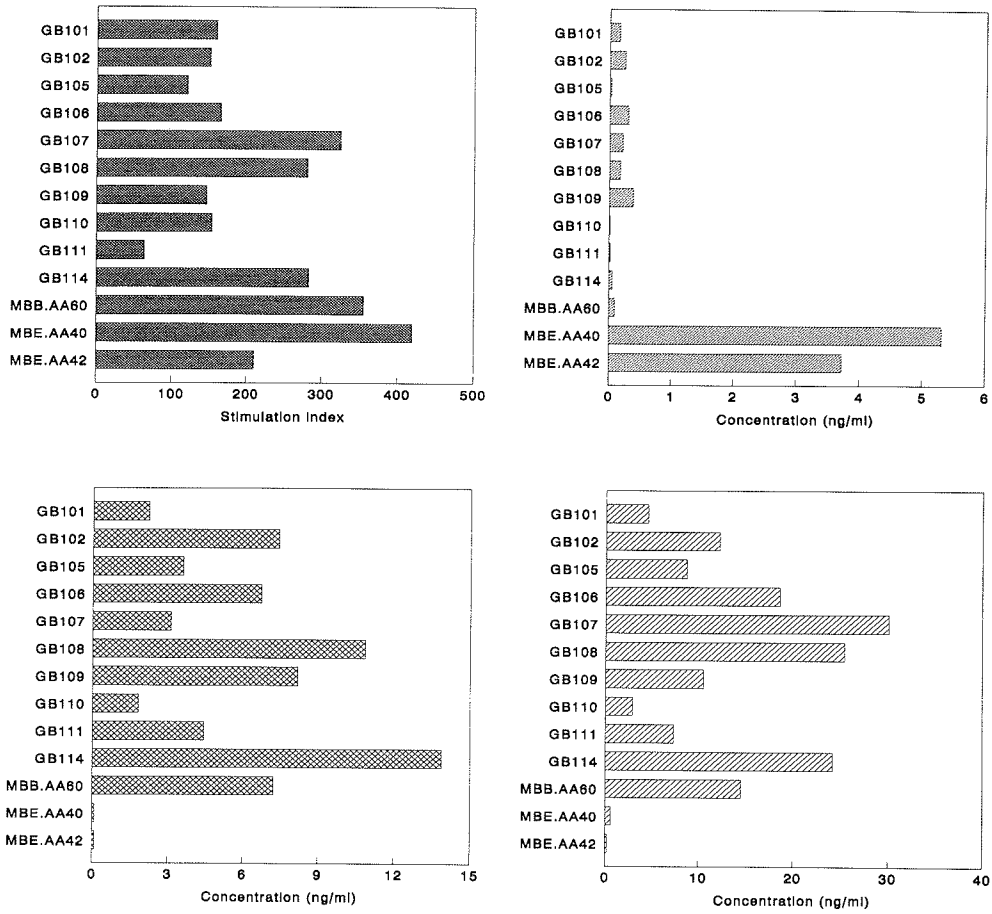


Figure 3: Cytokine secretion by CPE-specific T cell clones. Clone cells were stimulated (10^5 cells/well) for 36 h with anti-CD3 plus anti-CD28 (both 1:1000 final dilution) after which the cytokine concentrations in the culture supernatants were measured by ELISA. Secretion levels were compared to those of well-characterized aeroallergen-specific Th1 (MBA.AA40 and MBE.AA42) and Th2 (MBB.AA60) clones tested identically.

Detection levels: IFN-γ: 20 pg/ml, IL-4: 2.4 pg/ml, IL-5: 2.5 ng/ml.

Discussion

The present study shows that PBMC of peanut-allergic patients harbour pe
anut protein-responsive T cells, which upon cloning appeared to be Th
cells producing high levels of IL-4 and IL-5 but little or no IFN-γ.
The proliferative responses of PBMC of peanut-allergic patien
to CPE were significantly higher than those of non-allergi
individuals. This phenomenon has been described previously fo
inhalation (25, 41, 42) and food-induced intolerances (43-45) an
this could indicate a higher frequency of responsive T cells. Th
has been contradicted by others (46, 47). Recently Dorion et a
(47) showed no difference between peanut allergic, asthmatic an
non-allergic patients in response to *Ara h* II, a major peanut allerge
which has been identified by immunoblotting techniques using patient
sera (48). This difference in response could be due to the amount o
consumption of peanuts which is much higher in the American populatio
compared to the Dutch population. Another explanation could be th
fact that the lymphocyte stimulation studies of Dorion et al. were perfo
med in medium without serum while we used medium supplemented wi
10% pooled HS. This serum could contain certain factors which could inhib
or favour the outgrowth of certain cells. Also Higgins et al. (49) studied th
proliferative response to peanut proteins of peanut allergic and no
allergic individuals. They found a good response in non-allergic individuo
as well although this response tended to be lower than the respons
observer in peanut-allergic individuals and higher concentration of peanu
extract (up to 400 μg/ml) were needed. It could very well be that if w
had used higher concentrations of our extract we would have found
response.
It should be mentioned, however, that some peanut-allergic patien
whose PBMC showed no significant proliferative response to CPE we
excluded from this comparative study. The non-responsiveness of this sm
subgroup of patients could be a result of their peanut-free diet resulting
a very low frequency of reactive T cells in peripheral blood (50). Anoth
possible explanation is that these patients selectively respond to no
soluble peanut compounds or to allergens smaller than 3.5 kD, which bo
are not present in our CPE preparation.

Although PNA, the naturally occurring lectin in peanuts, can induce strong proliferative responses in bovine PBMC by means of agglutination (35), it induced a slight increase in (^{3}H)-TdR incorporation in human PBMC, both of peanut-allergic and non-allergic control individuals. This weak PNA-induced proliferative response can not account for the specific CPE-induced response in the patients' PBMC, because CPE used in this study contained only about 1% of PNA (data not shown). Indeed, compared to the proliferation induced by 1 or even 10 μg/ml of PNA, 150 μg/ml of CPE induced a significantly higher proliferation ($p < 0.05$). Moreover, if the proliferative response induced by CPE was caused by the mitogenic activity of PNA, a similarly high proliferative response should also have been observed in PBMC of non-allergic individuals. However, it can not be excluded that agglutination of the PBMC by PNA facilitated the CPE-specific T cell response to some extend.

The 10 CPE-specific T cell clones generated from patient NvD, with a strong PBMC response to CPE, all expressed the characteristic phenotype of activated T helper cells, i.e. $CD3^+$, $CD4^+$, $CD8^-$, $CD29^+$, $CD45RA^-$. No CPE-specific CD8+ T cells were cloned although approximately 10% of the obtained non-CPE-specific T cell clones were $CD8^+$. The strong differences in HLA-DR expression tested for all clones in parallel, at the same time point in the restimulation cycle, suggest that the panel comprises of different CPE-specific T cell clones, although future experiments on their protein-specificity and HLA-restriction of antigen recognition will provide more definite data on this matter.

Upon strong mitogenic stimulation with anti-CD3 plus anti-28 monoclonal antibodies, all CPE-specific T cell clones produced considerable to high levels of IL-4 and IL-5 but little or no IFN-γ, indicating a Th2 cytokine profile (24) as has been observed by Higgins et al. (49) as well. Antigen specific stimulations with 50 μg/ml CPE and autologous Epstein Barr virus transformed B cells as APC, resulted in the same cytokine profile but in lower absolute concentrations. Some of the CPE-specific T cell clones produced detectable amounts of IFN-γ (20-500 pg/ml), but compared to IFN-γ production by the well-characterized HDM-reactive Th1 clones (MBE.AA40 and MBE.AA42) the concentrations are very low. Compared to a HDM-specific Th2 clone, MBB.AA60, tested under identical experimental conditions, and to observations of others with several inhalation allergen-specific T cell clones (8-13, 25), the CPE-specific T cell clones produced IL-4

within a similar concentration range. IL-5 was not detectable in the supe natant of 2 clones after stimulation with CPE although these clones ar capable of producing high concentrations of IL-5 after strong mitogenei stimulation (Figure 3). This could be due to the high detection level of th used method or to the small amount of cells used to obtain the supern tant.

So far the precise protein-specificity of these clones has not been determ ned. Little is known about peanut protein constitution and the allergens may contain, but recently Dorion et al. (47) showed that PBMC of peanut allergic patients proliferate in response to *Ara h* II. Current isolation o proteins from CPE in our laboratory may enable us to determine th specificity and the deversity of the proteins that are recognized by ou panel of CPE-specific T cell clones.

The observation that all T cell clones reactive with the total peanut protei extract, are IL-4 and IL-5 producing Th2 cells and the commonly increase production of IgE and eosinophilia in food allergic patients suggest a important role of food allergen-specific Th2 cells in the pathophysiology o food allergy, similar to the role of Th2 cells in inhalation allergy.

Acknowledgements

The authors would like to thank Dr. Catharien Hilkens (Department of Ce Biology and Histology, University of Amsterdam) for the measurements of IL 5, Hillie Pellegrom (TNO Nutrition and Food Research) for technical assistan ce and Dr. Geert Houben (TNO Nutrition and Food Research) for critica reading of the manuscript.

References

1 **Belanti JA.** (1991) Developmental aspects of food allergy in infancy and childhood *Immunol Allergy Clin* **11**:885-891.

2 **Schreiber RA and Walker WA.** (1989) Food allergy: facts and fiction. *Mayo Clin Proc* **64**:1381-1391.

3 **Kajosaari M.** (1982) Food allergy in Finnish children aged 1 to 6 years. *Acta Paediat Scan* **71**:815-819.

4 **Burks AW, Williams LW, Mallory SB, Shirrell MA and Williams C.** (1989) Peanut protein as a major cause of adverse reactions in patients with atopic dermatitis. *Allergy Proc* **10**:265-269.

5 **Sampson HA and Metcalf DD.** (1992) Food allergies. *J Am Med Assoc* **20**:2840-2844.

6 **Businco L, Meglio P and Ferrara M.** (1993) The role of food allergy and eosinophils in atopic dermatitis. *Pediatr Allergy Immunol* **4(4 suppl)**:33-37.

7 **Jaffe JS and Metcalf DD.** (1993) Cytokines and their role in the pathogenesis of severe food hypersensitivity reactions. *Ann Allergy* **71**:362-364.

8 **Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM and Kapsenberg ML.** (1990) Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes in atopic patients. *J Immnunol* **144**:4651-4656.

9 **Del Prete G.** (1992) Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* **47**:450-455.

10 **Ebner E, Széphalusi Z, Ferreira F, Jilek A, Valenta R, Parronchi P, Maggi E, Romagnani A and Kraft D.** (1993) Identification of multiple T cell epitopes on *Bet v* I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* **150**:1047-1054.

11 **van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RC and Kapsenberg ML.** (1994) Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol* **152**:4203-4210.

12 **van der Heijden FL, Wierenga EA, Bos JD and Kapsenberg ML.** (1991) High frequency of IL-4 producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesional skin. *J Invest Dermatol* **97**:389-394.

13 **Ramb-Lindhauer Ch, Feldmann A, Rotte M and Neumann Ch.** (1991) Characterization of grass pollen reactive T cell lines derived from lesional atopic skin. *Arch Dermatol Res* **183**:71-76.

14 **Snapper CM, Finkelman FD and Paul WE.** (1988) Differential regulation of IgG1 and IgE synthesis by interleukin-4. *J Exp Med* **167**:183-196.

15 **Romagnani S, Del Prete G and Maggi E.** (1989) Role of interleukins in induction and regulation of human IgE synthesis. *Clin Immunol Immunopathol* **50**:S13-S23.

16 **Schultz CL and Coffman RL.** (1991) Control of isotype switching by T cells and cytokines. *Curr Opin Immunol* **3**:350-354.

17 **Vercelli D and Geha RS.** (1991) Regulation of IgE synthesis in humans: a tale of two signals. *J Allergy Clin Immunol* **88**:285-285.

18 **Heusser ChH, Bews J, Brinkmann V, Delespesse G, Kilchherr E, Ledermann F, Le Gros G and Wagner K.** (1991) New concepts in IgE regulation. *Int Arch Allergy Appl Immunol* **94**:87-90.

19 **Lebedin YS, Raudla LA, Chuchalin AG.** (1991) Serum levels of interleukin 4, interleukin 6 and interferon-gamma following in vivo isotype-specific activation of IgE synthesis in humans. *Int Arch Allergy Appl Immunol* **96**:92-94.

20 **Coffman RL and Carty J.** (1986) T cell activity that enhances polyclonal IgE production and it's inhibition by interferon-γ. *J Immunol* **136**:949-954.

21 **Snapper CM and Paul WE.** (1987) Interferon-γ and B cell stimulatory factor-1 reciproc ally regulated Ig isotype production. **Science 236**:944-947.

22 **Pene J Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, Yokota T, Arai N, Arai I Bancherau J and de Vries J.** (1988) IgE production by normal human lymphocytes induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E *Proc Natl Acad Sci USA* **85**:6880-6884.

23 **Del Prete G, Maggi E, Parronchi P, Chrétien I, tiri A, Macchia D, Ricci M, Bancherau de Vries J and Romagnani S.** (1988) IL-4 is an essential factor for IgE synthes induced *in vitro* by human T cell clones. *J Immunol* **140**:4193-4198.

24 **Mosmann TR and Coffman RL.** (1989) Th1 and Th2 cells: different patterns of lym phokine secretion lead to different functional properties. *Annu Rev Immunol* **7**:145 173.

25 **Wierenga EA, Snoek M, Bos JD, Jansen HM and Kapsenberg ML.** (1990) Compariso of diversity and function of house dust mite-specific T lymphocyte clones fron atopic and non-atopic donors. *Eur J Immunol* **20**:1519-1526.

26 **Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG and Vadas MA.** (1988 Recombinant human interleukin 5 is a selective activator of human eosinoph function. *J Exp Med* **167**:219-223.

27 **Clutterbuck EJ, Hirst EMA and Sanderson CJ.** (1989) Human interleukin-5 (IL-5) regulc tes the production of eosinophils in human bone marrow cultures: Comparison an interaction with IL-1, IL-3, IL-6 and GM-CSF. *Blood* **73**:1504-1512.

28 **Gleich GJ.** (1990) The eosinophil and bronchial asthma: current understanding. *Allergy Clin Immunol* **85**:422-436.

29 **Weller PF.** (1991) The immunobiology of eosinophils. *N Engl J Med* **324**:1110-1118.

30 **Enberg RN.** (1991) Food induced oropharyngeal symptoms: the oral allergy syndro me. *Immunol Allergy Clin* **11**:767-772.

31 **Metcalfe DD.** (1991) Food allergy. *Curr Opin Immunol* **3**:881-886.

32 **Bock SA and Atkins FM.** (1989) The natural history of peanut allergy. *J Allergy Cli Immunol* **83**:900-904.

33 **Bush RK, Taylor SL and Nordlee JA.** (1989) Peanut sensitivity. *Allergy Proc* **10**:261-264.

34 **Yunginger JW, Squillace DL, Jones RT and Helm RM.** (1989) Fatal anaphylacti reactions induced by peanuts. *Allergy Proc* **10**:249-253.

35 **Nagi AM and Babiuk LA.** (1989) Peanut agglutinin (PNA): binding and stimulation c bovine intestinal and peripheral blood leukocytes. *Vet Immunol Immunopatho* **22**:67-79.

36 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts. *Allergy Clin Immunol* **72**:61-68.

37 **Böyum A.** (1968) Isolation of leucocytes from human blood. *Scand J Clin Lab Inves* **21**:9-14.

38 **Miller G and Lipman M.** (1973) Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc Nat Acad Sci USA* **70**:190-194

39 **McNamee LA, Fattah DI, Baker TJ, Bains SK and Hissey PH.** (1991) Production, characterisation and use of monoclonal antibodies to human interleukin-5 in an enzyme-linked immunosorbent assay. *J Immunol Methods* **141**:81-88.

40 **Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RAW and Kapsenberg ML.** (1991) Human atopen-specific type 1 and 2 T helper cell clones. *J Immunol* **147**:2942-2949.

41 **Rawle FC, Mitchell EB and Platts-Mill TAE.** (1984) T cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* **133**:195-201.

42 **Burastero SE, Fenoglio D, Crimi E, Brusasco V and Rossi GA.** (1993) Frequency of allergen-specific T lymphocytes in blood and bronchial response to allergen in asthma. *J Allergy Clin Immunol* **91**:1075-1081.

43 **Scheinmann P, Gendrel D, Charlas J and Paupe J.** (1976) Value of lymphoblast transformation test in cow's milk protein intestinal intolerance. *Clin Allergy* **6**:515-521.

44 **Van Sickle GJ, Keating Powell G, McDonald PJ and Goldblum RM.** (1985) Milk- and soy protein-induced enterocolitis: Evidence for lymphocyte sensitization to specific food proteins. *Gastroenterology* **88**:1915-1921.

45 **Kondo N, Agata H, Fukutomi O, Motoyoshi F and Orii T.** (1990) Lymphocyte responses to food antigens in patients with atopic dermatitis who are sensitive to foods. *J Allergy Clin Immunol* **86**:253-260.

46 **May CD and Alberto R.** (1972) *In vitro* responses of leukocytes to food proteins in allergic and normal children: lymphocyte stimulation histamine release. Clin Allergy **2**:335-340.

47 **Dorion BD, Burks AW, Harbeck R, Williams LW, Trumble A, Helm RM and Leung DY.** (1994) The production of interferon-γ in response to a major peanut allergy, *Ara h* II, correlates with serum levels of IgE anti-*Ara h* II. *J Allergy Clin Immunol* **93**:93-99.

48 **Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ and Helm RM.** (1992) Identification and characterization of a second major peanut allergen, *Ara h* II, with use of sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* **90**:962-969.

49 **Higgins JA, Lamb JR, Lake RA and O'Hehir RE.** (1995) Polyclonal and clonal analysis of human $CD4^+$ T-lymphocytes responses to nut extracts. *Immunology* **84**:91-97.

50 **Shinoda S, Kondo N, Fukutomi O, Agata H, Suzuki Y, Shimozama N, Tomatsu S, Yamada Y, Takemura M, Noma A and Orii T.** (1993) Suppressive effects of elimination diets on T cell responses to ovalbumin in hen's egg-sensitive atopic dermatitis patients. *Clin Exp Allergy* **23**:689-695.

Chapter 5

Diverse protein specificity of peanut-specific Th2 cell clones from a peanut-allergic patient

Esther C. de Jong[1,2], Steven Spanhaak[1], Hillie Pellegrom[1], Eddy A. Wierenga[3], André H. Penninks[1]

[1]TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[2]Utrecht Toxicology Center, Utrecht, The Netherlands
[3]Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, The Netherlands

Submitted for publication

Abstract

Allergen-specific T cells play an important role in the pathogenesis allergic disease. Identification of the proteins recognized by these T ce could lead to more insight into the pathophysiology of the disease an possibly, to an improvement of specific immunotherapy. Characterizatio of several major allergens in allergenic entities such as house dust mit grass pollen and cat dander indicated that most allergens contain multip T cell epitopes complicating the design of immunotherapy protocols. So f little is known about the specificities of food-allergen specific T cells. In th present study, the protein specificity of peanut specific T cell clone generated from a severe peanut-allergic patient, was analyzed.

From raw, unshelled peanuts a crude peanut extract (CPE) was prepare The 2 main protein fractions, arachin and conarachin, were isolated fro CPE by ion exchange chromatography. CPE was further separated base on hydrophobicity using HPLC and based on size using gelelectrophores Using a panel of CPE-reactive T cell clones, the obtained fractions we analyzed for their ability to induce proliferation.

This panel of CPE-specific T cell clones recognized either arachin, conar chin or peanut agglutinin (PNA), the naturally occurring lectin in peanu Upon fractionation by HPLC, only highly hydrophobic fractions we recognized. The separation by size showed that the arachin specific clon recognized a protein band of approximately 30-35 kD, while 2 conarachi reactive clones responded to a protein fraction of 25-40 kD, and anoth clone to a fraction of 18-25 kD.

This study shows that CPE-specific T cell clones generated from a seve peanut-allergic patient recognize various peanut proteins.

Introduction

Allergen-specific T cells play an important role in the pathophysiology of allergic disease (1). Several studies have shown that allergen-specific T cells generated from patients suffering a respiratory allergy to house dust mite (HDM), grass pollen or cat dander, are Th2 cells producing high levels of IL-4 and IL-5 but little or no IFN-γ (2-5). IL-4 induces isotype switching in B cells to IgE or IgG4 which can be inhibited by IFN-γ (6-9) while IL-5 is a potent inducer of eosinophil production and activation (10, 11). Both specific IgE production and eosinophilia are characteristic features of atopic allergy. Recently it has been shown that peanut-specific T cell clones generated from a peanut-allergic patient were also of a Th2 phenotype (12, 13) suggesting a similar mechanism in food allergy.
Peanut, *Arachis hypogaea*, is a common cause of food allergy (14). Along with cow's milk and hen's egg, peanut account for approximately 80% of the adverse reactions to food products in patients with atopic dermatitis (15). Fatal or near-fatal anaphylactic reactions due to the consumption of small amounts of peanuts are not uncommon among peanut-allergic patients (16, 17). Once sensitized to peanuts, the allergy is very persistent (18).
Peanuts consist for 53% (w/w) of fat and 28% of protein (19). The protein fraction can be separated in albumins, and the two major storage proteins arachin and conarachin (20), which account for 87% of the protein content of peanuts (21). Although several peanut allergens have been identified by immuno-blotting procedures using specific IgE-containing sera from peanut allergic individuals (22-26) little is known about the proteins recognized by peanut-specific T cells. Identification of such proteins could lead to more insight into the mechanism of peanut allergy. Also for the improvement of specific immunotherapy the identification of allergens and their T cell epitopes could be of importance. So far, whole protein mixtures have been used for immunotherapy risking severe side effects, such as anaphylactic shock, via cross-linking of specific IgE on mast cells and basophils. Immunotherapy with purified peptides containing T cell epitopes could minimize this risk because epitopes recognized by IgE antibodies are distinctly different from the epitopes recognized by T cells (28). T cell epitopes could therefore be used in higher concentrations to more effec-

tively induce tolerance.
In this study we analyzed the protein specificity within a previous described panel of crude peanut extract (CPE)-reactive Th2 clones gene ated from a severe peanut-allergic patient. Initially the proliferativ response of these CPE-specific T cell clones was tested in reaction wi arachin and conarachin, the 2 main fractions, and soy as a negativ control. Furthermore, CPE was fractionated based on hydrophobicity usin HPLC. CPE, arachin and conarachin were also separated based o molecular size using Western blotting techniques. The proliferative respons to all obtained fractions was measured.

Materials and methods

Peanut protein purification

Crude peanut extract (CPE) was prepared from raw, unshelled peanuts a described before (24). In short, peanuts were ground and fat wa extracted by Soxhlet using petroleum ether at 40-60°C. The defatted flou was ground again and suspended in ammoniumbicarbonate (0.1 M). Th suspension was stirred for 4 h and insoluble particles were removed b centrifugation for 30 min at 10,000g at 4°C. The supernatant was dialyse overnight against distilled water using a 3.5 kD cut-off membrane (Spec trum Medical Industries, Inc, Houston, Tx), lyophilized and stored at -20°C.
Arachin and conarachin were isolated from CPE using a diethylaminoeth (DEAE) Sephacel (Pharmacia LKB, Uppsala, Sweden) column of 1.5 x 5 cm. CPE was eluted with 0.1 M sodium phosphate buffer, pH 8.0 and sodium chloride gradient from 0 M to 0.5 M. Protein contents of the frac tions was measured at 280 nm. Fractions forming one peak were poole As has been described before (24), conarachin emerged first at approx mately 0.25 M NaCl followed by arachin at 0.35 M NaCl. These fractior were dialysed against distilled water, lyophilized and stored at -20°C.
For gelelectrophoresis, protein samples were reduced by incubation for 1 min at 100°C with sample buffer consisting of 1% DTT (Sigma Chemica Co., St. Louis, MO), 63 mM-Tris-HCl, 2% (w/v) SDS, 0.01% (w/v) bromophen blue, 20% (v/v) glycerol, pH 6.8. SDS-PAGE was performed essential according to Laemmli (29) using precasted 15% Tris HCl polyacrylamid

gels (Bio-Rad Laboratories, Hercules, CA). To visualize the protein bands, the gels were stained with Coomassie brilliant blue R-250. For the determination of the molecular weights of the protein bands, pre-stained molecular weight markers (Bio-Rad) with molecular weights of 200, 97.4, 69, 46, 30, 21.5 and 14.3 kD were used.

High performance liquid chromatography (HPLC) fractionation of CPE

For separation of CPE using HPLC a reversed phase Ultrapore™ C3 column with a pore width of 300 Å (Beckman Instruments, San Diego, CA) of 10 x 250 mm was used. As elution buffer A 0.1% (v/v) trifluor acetic acid (TFA) in water was used and buffer B consisted of 0.1% (v/v) TFA in 70% (v/v) acetonitril in water. The gradient was 0%-20% buffer B in 5 min, 20%-50% buffer B in 22 min and 50%-100% buffer B in 13 min. The eluate was collected in 40 fractions (1 fraction per minute).

Of each fraction, 400 µl was lyophilized and resuspended in 30 µl sample buffer and loaded on precast gradient (10-15%) gels (PhastGel, Pharmacia) with molecular weight markers (Pharmacia) of 94, 68, 43, 30, 20 and 14 kD.

Protein assay

The protein concentration of the different samples was measured using a Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) following the manufacturers instructions.

Preparation of immunoblots and nitrocellulose particle suspension

To study the proliferative response of CPE-specific T cell clones to proteins, CPE, arachin and conarachin were separated based on size by SDS-PAGE as described above and transferred to nitrocellulose membranes (0.2 µM, Bio-Rad) as described by Towbin (30). These membranes were used to obtain nitrocellulose particle suspensions which contained antigen.

From the membranes both left and right sides were cut off and stained with 0.2% Ponseau S (Sigma) in 3% trichloride acetic acid/3% sulfosylial acid to visualize the protein bands. The rest was used to prepare suspensions of nitrocellulose particles containing the separated proteins. To this aim, horizontal strips covering an area of molecular weight were cut and, as previously described by Abou-Zeid (31), dissolved in 2 ml DMSO and rotated at room temperature (RT) for 2 h. To precipitate the nitrocellulose-

protein complexes, an equal volume of 0.05 M carbonate/bicarbona buffer, pH 9.6 was added and after vortexing the tubes were centrifuge at 10,000g at RT for 10 min. The pellet was resuspended in 5 ml Iscove Modified Dulbecco's Medium (IMDM; Gibco, Paisley, UK) and washe once more to remove the remaining DMSO. The recovered pellet w resuspended in 1 ml IMDM.

T cell clones and proliferation assays

For all experiments CPE-specific T cell clones were used generated from severe peanut allergic patient as described before (13) all showing a T cytokine secretion profile. To maintain the T cell clones in culture, they we restimulated every 2 weeks with PHA (20 μg/ml PHA (Difco, Detroit, MI) ar a feedermix consisting of peripheral blood mononuclear cells (PBMC) of unrelated donors (each 10^6cells/ml, 3000 rad irradiated), $2x10^5$ cells/ (3000 rad irradiated) of the Epstein Barr virus transformed B cell line JY ar 20 IU/ml recombinant IL-2 (Eurocetus, Amsterdam, The Netherlands) as growth factor. Proliferation assays were always performed 10 days aft restimulation.

Experiments were performed in triplicate cultures in 96-well flat-botto culture plates (NUNC, Roskilde, Denmark) in IMDM supplemented with 1 pooled complement inactivated normal human AB serum (BioWhitthake Walkersville, ML), gentamycin (50 μg/ml; Gibco) and fungizone (2.5 μg/ Gibco).

To measure proliferation, 10^5 T cells were cultured in 200 μl comple medium in the presence of $5x10^4$ 3000 rad irradiated Epstein Barr-vir transformed autologous B cells (EBV-B) as antigen presenting cells (AP and in the absence or presence of the protein samples. CPE, arachi conarachin and soy protein (unheated soy flour, USF; ILOB-TNO, Wageni gen, The Netherlands) were used at a final concentration of 50 μg/ml, PN (Sigma) at 25 μg/ml, HPLC fractions at 10 μg/ml and the nitrocellulo particle suspension at 5 μg/ml. After 48 h, proliferation was measured b adding 0.4 μCi/well of ^{3}H-Thymidine (^{3}H)-TdR) (Amersham, Aylesbur UK) after which the cells were incubated another 18 h and harvested wi a cell harvester (Tomtec, Orange, US). Beta-emission was counted by liqu scintillation spectroscopy with a 1450 Microbeta (Wallac, Turku, Finland). A increase in cpm in the presence of antigen of at least 3 times the cpm the absence of antigen was seen as a specific response.

Results

Separation of crude peanut extract

Proteins were extracted from raw, unshelled peanuts and after fat extraction, a water soluble CPE was obtained consisting for 90% of protein. By SDS-PAGE (Figure 1) CPE was separated into several proteins with molecular weights varying from 10 kD to 70 kD. Peanut agglutinin (PNA), the naturally occurring lectin in peanuts, is present in CPE at a concentration of approximately 1% as determined by measuring the intensity of the protein bands on SDS-PAGE.

Using DEAE column chromatography, 2 fractions were collected which represent the 2 main storage proteins, arachin and conarachin.

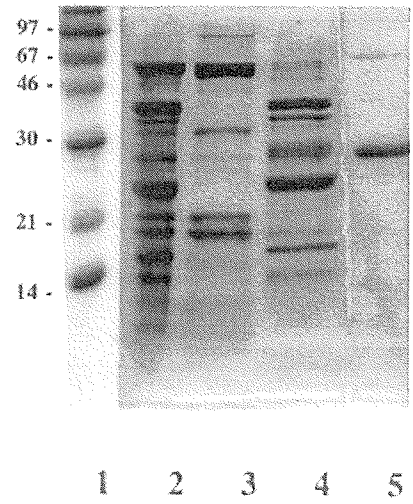


Figure 1: SDS-PAGE of CPE (lane 2), conarachin (lane 3), arachin (lane 4), PNA (lane 5) and molecular weight markers (lane 1). In each lane 5 µg protein was loaded. The gel was stained with Coomassie brilliant Blue to visualize the protein bands.

The SDS-PAGE profile of arachin showed several bands with molecular weights between approximately 10 and 50 kD with prominent bands of 25, 42 and 43 kD. Conarachin consists of several bands with molecular weights

between 10 and 73 kD with prominent bands of approximately 63 and 7 kD and 20 and 22 kD (Figure 1). The ratio arachin to conarachin is approx mately 2:1 as estimated from the area under the curve.

In order to further fractionate CPE, HPLC was applied to separate th extract on the basis of hydrophobicity. The SDS-PAGE profile of the 4 fractions is shown in Figure 2.

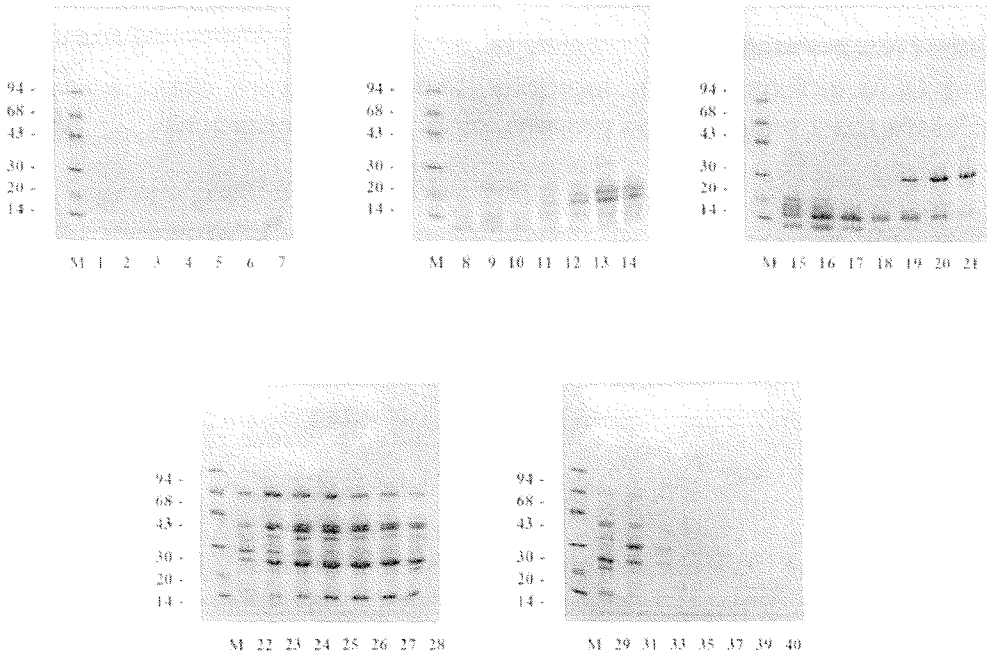


Figure 2: SDS-PAGE of the 40 collected HPLC fractions from CPE. In each fir lane of the gels, molecular weight markers were loaded. Gels wer stained with Coomassie brilliant Blue to visualize the protein bands.

Proliferative response of CPE-specific T cell clones to different proteins an fractions

In Table 1 the proliferative responses of the CPE-specific T cell clones t arachin, conarachin, and soy protein are shown. Of this panel of clone

from patient G (GB-series clones) only one clone recognized arachin while 3 other clones recognized conarachin. Clone GB110 did not respond to either arachin or conarachin but showed to be responsive to PNA (1542 cpm without PNA, 49350 cpm in the presence of PNA).

Table 1: Protein specificity of peanut-specific T cell clones.

	Proliferation (cpm)				
Clone	Control	CPE	Ara	Conar	Soy
GB102	1604	**14278**	**16113**	1687	1536
GB109	1595	**32655**	2504	**20238**	1424
GB110	1542	**27345**	1522	1816	1397
GB111	1516	**31891**	2439	**21967**	1508
GB114	1477	**14580**	1354	**13911**	1472

T cells (10^5) were cultured with APC ($5x10^4$) in the absence or presence of CPE (50 µg/ml), arachin (50 µg/ml), conarachin (50 µg/ml) and unheated soy flour (50 µg/ml). Proliferation was measured after 48 h of incubation using (^{3}H)TdR incorporation and is expressed as counts per minute (cpm).

The results of the proliferative response of 4 clones to the 40 HPLC fractions are presented in Figure 3. Four different response patterns were observed. Clone GB102, arachin-specific, recognized only fractions 29 and 30 while clone GB109 responded to the fractions 29, 30, and 32 to 35 and GB111 recognized fractions 30 and 32 to 36. Clone GB110 did not respond to any of the HPLC fractions.

To determine which of the proteins observed on SDS-PAGE (Figure 1) is responsible for the proliferative response of the CPE-specific T cell clones, the proteins were transferred to nitrocellulose and suspensions were made from horizontal molecular weight range strips as indicated in Figure 4 which shows the proliferative responses to the different suspensions. The proteins recognized by the arachin-specific T cell clone has a molecular weight of approximately 30-35 kD as is shown by the combination of the CPE- and arachin-suspension results. Combining the CPE results with the conarachin results, the protein recognized in conarachin has a molecular weight of ap-

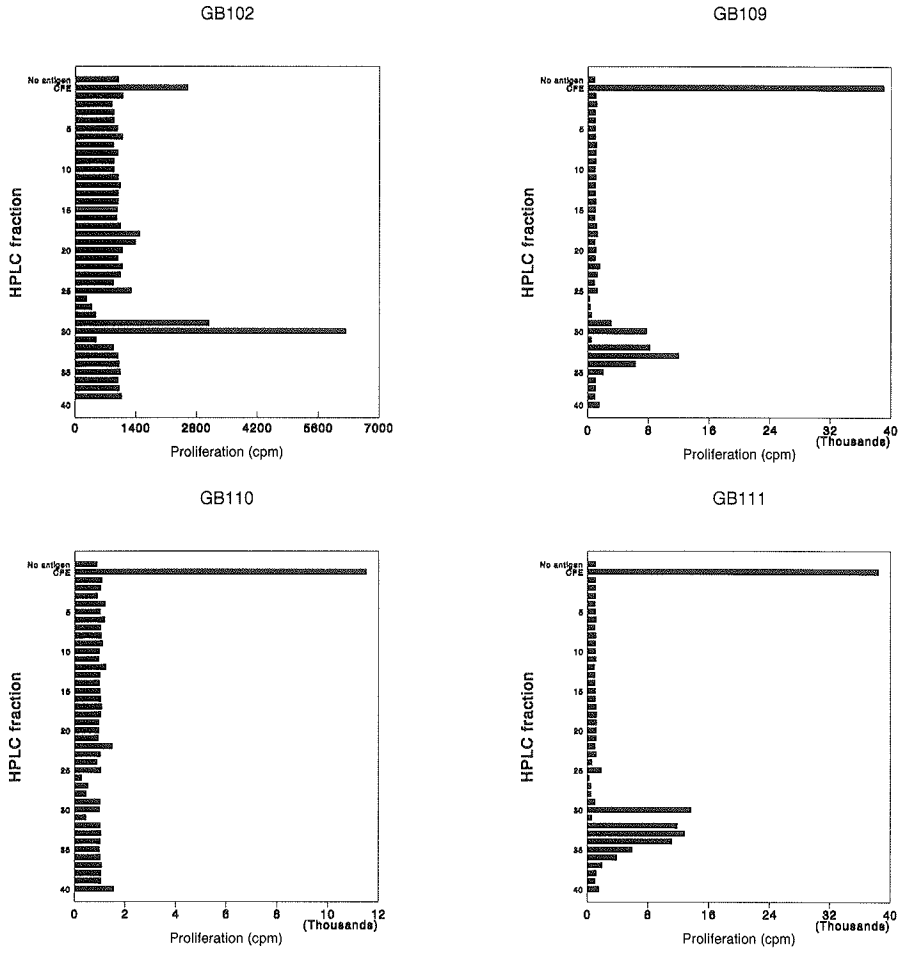


Figure 3: Proliferative response of the T cell clones encoded GB102, GB10 GB110 and GB111 to the 40 protein fractions separated by HPL from CPE.
Cloned cells (10^5 cells/well) were cultured with APC ($2x10^4$ cells/we in the absence or presence of CPE (50 µg/ml) or HPLC fraction 1 t 40 at a concentration of approximately 10 µg/ml. Proliferation wc measured after 48 h of incubation using (^{3}H)TdR incorporation. Pro feration is expressed as counts per minute (cpm).

proximately 30-35 kD (GB109 and GB111). GB114 responded to 2 CPI suspensions (26-28 and 14-18 kD) as well as to 2 conarachin suspensior

(17-30 and <17 kD) indicating that the recognized protein band must have a molecular weight of approximately 14-28 kD. Clone GB110, which did not respond to arachin or conarachin neither to any of the HPLC fractions, showed to be responsive to PNA, the naturally occurring lectin in peanuts.

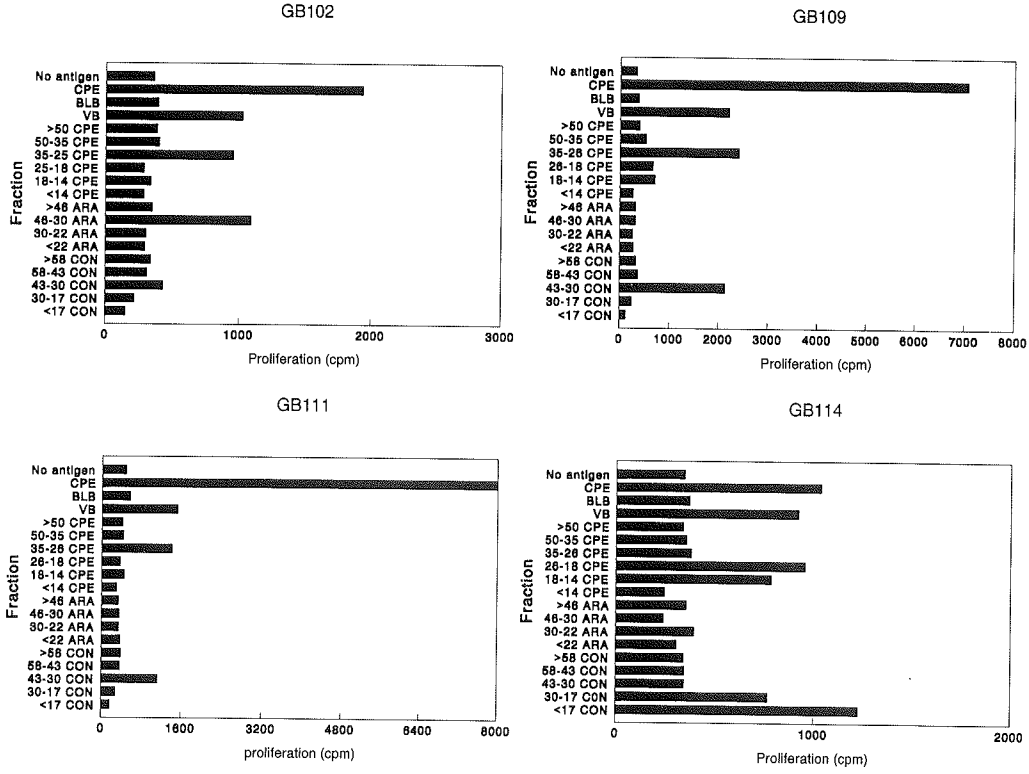


Figure 4: Proliferative response of the T cell clones encoded GB102, GB109, GB111 and GB114 to protein fractions of CPE separated on SDS-PAGE and bound to nitrocellulose.
Cloned cells (10^5 cells/well) were culture with APC ($1x10^5$ cells/well) in the absence or presence of CPE (50 µg/ml), control blot (BLB), vertical blot stripe (VB) and the different blot stripes of CPE (CPE), arachin (ARA) and conarachin (CON). Nitrocellulose blot suspensions were added at a concentration of approximately 10 µg/ml. Proliferation was measured after 48 h of incubation using (^{3}H)TdR incorporation. Proliferation is expressed as counts per minute (cpm).

Discussion

From this study we can conclude that the two main storage prote complexes in peanuts, arachin and conarachin, play a major role in the cell responses to peanut proteins. The recognition of proteins by th described peanut-specific T cell clones is very diverse. At least 5 differe proteins are recognized and probably even more T cell epitopes. This is accordance with studies on specificity of HDM-, grass pollen- and c dander-specific T cell clones which revealed that T cells of differe patients recognized different peptides and moreover, the different T c clones from one patient responded to distinct epitopes.

The CPE-reactive T cells clones recognized either arachin or conarachi One clone, GB110, responded only to PNA. Although it has been shov that PNA has a mitogenic capacity in mononuclear cells of other speci (32), in our study the observed response is not considered to be mitogeni Apart from the observation that not all clones showed a proliferati response to PNA, and in the absence of APC no response was observe PNA was also not able to induce a Ca^{2+}-influx in these T cell clones (da not shown). The strongest argument against a possible mitogenic effect PNA is the observation that anti-HLA class II and anti-HLA-DP monoclor antibodies blocked the proliferative response to PNA (data not shown). F these reasons, as well as the described binding of IgE from peanut-allerg patients to PNA (24, 33), we suggest that the responses of peanut specific cell clones to PNA is immunogenic and not mitogenic.

Although soy beans and peanuts are closely related phytogenetically ar a serological cross-reactivity is thought to exist (34), no proliferati response was observed of the CPE-specific T cell clones in the presence soy proteins.

HPLC separation of CPE showed that the CPE-specific T cell clones we stimulated by different protein fractions or not stimulated at all. The arach specific T cell clone (GB102) demonstrated a proliferative response to or 2 fractions, 29 and 30 while the conarachin-specific T cell clone GB1 recognized fractions 29 and 30 as well as 32 to 35 and clone GB114 w activated by fractions 30 and 32 to 36. The PNA-responsive T cell clor (GB110) did not react to any of the HPLC fractions, which may be due to too low concentration of PNA to stimulate the T cell clone, as PNA being

approximately 1% of CPE, will be divided into several fractions.
The proteins recognized by the T cell clones in this study are highly hydrophobic since upon HPLC separation they were collected in the final elution fractions. As hydrophobic proteins are more easily transported through membranes (35) this implicates that these hydrophobic peanut proteins might be taken up in the gastro-intestinal tract more easily. Most food allergens are also found to be heat- and acid-stable (36), and Burks et al. (37) even showed that treatment of peanut proteins with human digestive enzymes did not reduce IgE-or IgG-specific binding activity. Taken together, these observations indicate that apart from more individual conditions of the patient, susceptibility for food allergens may also depend on special properties of the proteins involved. Hydrophobicity and stability upon heat-, acid- and enzymatic-treatment may all increase the bioavailability of intact epitopes of food allergens to the lymphoid tissue of the gastro-intestinal tract and more easily lead to sensibilisation in susceptible individuals.
Using the nitrocellulose bound proteins as antigen source, 3 different fractions were recognized: The arachin-specific clone, GB102 responded to a fraction that contained proteins in the molecular weight range of 30-35 kD in the arachin fraction. GB109 and GB111 both recognized a protein of approximately 26-30 kD while GB114 responded to 2 fractions in both CPE as well as conarachin with a molecular weight between 29 and 14 kD. This could either be due to break-down of the protein or due to a shared T cell epitope in both fractions.
Combining the molecular weights of the proteins bound to nitrocellulose recognized by the T cell clones with the proteins recognized in the HPLC fractions, these data suggest that at least 5 different CPE-specific T cell clones were isolated which all recognized different proteins.
Several peanut allergens have been described previously by immunoblotting procedures using specific IgE containing sera from peanut-allergic patients (22-26). Sachs et al. (22) described an acidic glycoprotein, Peanut-1, with 2 subunits between 20 and 30 kD. Another allergen, the so called concanavaline A-reactive protein, with a molecular weight of approximately 65 kD has been identified by Barnett et al. (23) and, more recently, Burks et al. (24, 25) described two major allergens *Ara h* I and *Ara h* II of respectively 63.5 and 17 kD. Whether the described allergens belong to the arachin or conarachin complex of proteins is unknown. Based on the

molecular weights of the described allergens, *Ara h* I probably belongs conarachin, which shows in our hands two major bands of approximate 73 and 65 kD, whereas arachin does not contain proteins with a molecul weight of more then 50 kD. *Ara h* II could belong to both arachin and co arachin, although our SDS-PAGE profile of arachin shows a distinct band 19 kD. Probably *Ara h* I will not account for the observed proliferati response of our peanut specific T cell clones based on comparison of th molecular weights. However, clone GB114 recognizes a protein band approximately 14 to 30 kD which could be *Ara h* II. Recently Dorion et (38) showed that PBMC of peanut-allergic patients proliferate in respon to *Ara h* II. Although the majority of our T cell clones would probably n react to this protein, it is very well possible that T cells from other patien would recognize this protein, since in our study already 5 different protei were recognized by T cell clones of one patient.

This study shows that in peanut allergy, as has previously been shown f respiratory allergy to HDM (39, 40), pollen (41) and cat-dander (5), a wid variety of proteins is recognized by peanut specific T cell clones. This cou suggest that also in peanut proteins multiple T cell epitopes are prese and a wide variety between persons will exist. This complicates the desig for immunotherapy and would implicate that specific immunotherap should consist of a mixture of all relevant peptides.

Acknowledgements

The authors would like to thank Dr. H.M. van Noort for the performance the HPLC fractionation and gelelectrophoresis of the obtained fraction Maaike van Zijverden for technical assistance and Dr. G.F. Houben f critical reading of the manuscript.

References

1 **Del Prete G.** (1992) Human Th1 and Th2 lymphocytes: their role in the pathophysi ogy of atopy. *Allergy* **47**:450-455.

2 **Wierenga EA, Snoek M, de Groot CJ, Chrétien I, Bos JD, Jansen HM and Kapsenberg ML.** (1990) Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes in atopic patients. *J Immunol* **144**:4651- .

3 **O'Hehir RE, Garman RD, Greenstein JL and Lamb JR.** (1989) The specificity and function of T cell responsiveness to allergens. *Annu Rev Immunol* **9**:67.

4 **Parronchi P, Macchia D, Piccinni MP, Biswas P, Simonelli C, Maggi E, Ricci M, Ansari AA and Romagnani S.** (1991) Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. *Proc Natl Acad Sci USA* **88**:4538-4542.

5 **van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RCand Kapsenberg ML.** (1994) Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol* **152**:4203-4210.

6 **Coffman RL and Carty J.** (1986) T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J Immunol* **136**:949-954.

7 **Snapper CM and Paul WE.** (1987) Interferon-γ and B cell stimulatory factor-1 reciprocally regulated Ig isotype production. *Science* **236**:944-947.

8 **Schultz CL and Coffman RL.** (1991) Control of isotype switching by T cells and cytokines. *Curr Opin Immunol* **3**:350-354.

9 **Vercelli D and Geha RS.** (1991) Regulation of IgE synthesis in humans: a tale of two signals. *J Allergy Clin Immunol* **88** 285.

10 **Tagari P, Pecheur EI, Scheid M, BrownP, Ford-Hutchinsons AW and Nicholson D.** (1993) Activation of human eosinophils and differential HL-60 cells by IL-5. *Int Arch Allergy Immunol* **101**:227-233.

11 **Hamid Q, Boguniewics M and Leung DY.** (1994) Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* **94**:870- .

12 **Higgins JA, Lamb JR, Lake RA and O'Hehir RE.** (1995) Polyclonal and clonal analysis of human $CD4^+$ T-lymphocytes responses to nut extracts. *Immunology* **84**:91-97.

13 **de Jong EC, Spanhaak S, Martens BPM, Kapsenberg ML, Penninks AH and Wierenga EA.** (1995) Allergen-specific Th2 clones generated from the peripheral blood of a peanut-allergic patient. *J Allergy Clin Immunol* In press.

14 **Kalliel JN, Klein DE and Settipane GA.** (1989) Anaphylaxis to peanuts: clinica; correlation to skin tests. *Allergy Proc* **10**:259-.

15 **Burks AW, Williams LW, Mallory SB, Shirell MA and Williams C.** (1989) Peanut protein as a major cause of adverse food reactions in patients with atopic dermatitis. *Allergy Proc* **10**:265-269.

16 **Yunginger JW, Squillance DL, Jones RT and Helm RM.** (1989) Fatal anaphylactic reactions induced by peanuts. *Allergy Proc* **10**:249-253.

17 **Assem ESK, Gelder CM, Spiro SG, Baderman H and Armstrong RF.** (1990) Anaphylaxis induced by peanuts. *Br Med J* **300**:1377-1378.

18 **Bock SA and Atkins FM.** (1989) The natural history of peanut allergy. *J Allergy Clin Immunol* **93**:900-904.

19 **Pancholy SK, Deshpande AS and Krall S.** (1987) Amino acids, oil and protein content of some selected peanut cultivars. *Proc Amer Peanut Res Educ Assoc* **10**:30-37.

20 **Johns CO and Jones DB.** (1916) The proteins of the peanut, *Arachis hypogaea*. I. Tl globulins arachin and conarachin. *J Biol Chem* **28**:77-87.

21 **Basha SMM and Pancholy SK.** (1981) Polypeptide composition of arachin and no arachin proteins from early bunch peanut (*Arachis hypogaea* L.) seed. *Pear Science* **8**:82-88.

22 **Sachs MI, Jones RT and Yunginger JW.** (1981) Isolation and partial characterizati of a major peanut allergen. *J Allergy Clin Immunol* **67**:27-34.

23 **Barnett D and Howden MEH.** (1986) Partial characterization of an allergenic glyc protein from peanut (*Arachis hypogaea* L.). *Biochim Biophys Acta* **882**:97-105.

24 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts. *Allergy Clin Immunol* **72**:61-68.

25 **Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G and O'Brien T.** (199 Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermat and positive peanut challenges. *J Allergy Clin Immunol* **88**:172-179.

26 **Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien T and Helm RM.** (199 Identification and characterization of a second major peanut allergen, *Ara h* II, wi the use of sera of patients with atopic dermatitis and positive peanut challenge. *Allergy Clin Immunol* **90**:962-969.

27 **Heymann PW, Chapman MD, Aalberse RC, Fox JW and Platts-Mills TEA.** (198 Antigenic and structural analysis of group II allergens (*Der f* II and *Der p* II) fro house mites (*Dermatophagoides* spp). *J Allergy Clin Immunol* **83** 1055- .

28 **O'Hehir RE, Hoyne GF, Thomas WR and Lamb JR.** (1993) House dust mite allergy: fro T-cell epitopes to immunotherapy. *Eur J Clin Invest* **23**:763- .

29 **Laemmli Uk.** (1970) Cleavage of structural proteins during the assembly of the hea of bacteriophage T4. *Nature* **227**:680-685.

30 **Towbin HJ, Staehelin TH and Gordon J.** (1979) Electrophoretic transfer of protein fro polyacrylamide gels to nitrocellulose sheets. *Proc Natl Acad Sci USA* **76**:4350-4354.

31 **Abou-Zeid c, Filley E, Steele J and Rook GAW.** (1987) A simple new method for usir antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocyt in vitro after converting bands cut from Western blots into antigen-bearing particle *J Immunol Methods* **98**:5-.

32 **Nagi AM and Babiuk LA.** (1989) Peanut agglutinin (PNA): binding and stimulation bovine intestinal and peripheral blood leukocytes. *Veterinary Immunol Immun pathol* **22**:67-76.

33 **Burks AW, Cockrell G, Connaughton C, Guin J, Allen W. and Helm RM.** (1994) Ide tification of peanut agglutinin and soybean trypsin inhibitor as minor legum allergens. *Int Arch Allergy Immunol* **105**:143-149.

34 **Barnett D, Howden D, Bonham B and Howden MEH.** (1987) Allergenicity crossrea tions among legume foods-an in vitro study. *J Allergy Clin Immunol* **79**:433- .

35 **Klaassen CD.** (1986) Distribution, excretion and absorption of toxicants. IN: Casare and Doull's toxicology. The basis of science of poisons. Eds: Klaassen CD, Amdur M and Doull J. Macmillan Publishing Company USA

36 **Taylor SL, Lemansky RF, Bush RK and Busse WM.** (1987) Food allergens: structure and immunologic properties. *Annals Allergy* **59**:93-99.

37 **Burks WA, Williams LW, Thresher W, Connaughton C, Cockrell G and Helm RM.** (1992) Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenge. *J Allergy Clin Immunol* **90**:889-897.

38 **Dorion BJ, Burks AW, Harbeck R, Williams LW, Trumble A, Helm RM and Leung DY.** (1994) The production of interferon-γ in response to a major peanut allergen, *Ara h* II, correlates with serum levels of IgE anti-*Ara h* II. *J Allergy Clin Immunol* **93**:93-99.

39 **IJssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R and de Vries JE.** (1992) T cell activation-inducing epitopes of house dust mite allergen *Der p* I. *J Immunol* **148**:738-745.

40 **van Neerven RJJ, van 't Hof W, Ringrose JH, Jansen HM, Aalberse RC, Wierenga EA and Kapsenberg ML.** (1993) T cell epitopes of house dust mite major allergen *Der p* II. *J Immunol* **150**:2326-2335.

41 **Ebner E, Széphalusi Z, Ferreira F, Jilek A, Valenta R, Parronchi P, Maggi E, Romagnani A and Kraft D.** (1993) Identification of multiple T cell epitopes on *Bet v* I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* **150**:1047-1054.

Chapter 6

Enhanced proliferative response of peripheral blood T cells of allergic donors to a non-relevant allergen, peanut protein

Esther C. de Jong[1,2], Steven Spanhaak[1], Hillie Pellegrom[1], Joost P. Bruyntjes[1], Andre H. Penninks[1] and Martien L. Kapsenberg[3]

[1]TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[2]Utrecht Toxicology Center, Utrecht, The Netherlands
[3]Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Stimulation of peripheral blood mononuclear cells (PBMC) from allerg donors with a specific allergen induces a strong proliferative response *vitro*. PBMC of non-allergic donors do proliferate in response to allerge but weakly compared to allergic donors. However, the proliferati responses of PBMC of allergic donors to allergens that are not relevant f these donors, are less well studied. In the framework of studies on th characteristics of the allergic response to peanut proteins, we measure the response of PBMC of allergic but not peanut-allergic (ANPA) donc and non-allergic (NA) donors to peanut protein and compared thes responses to the proliferation of PBMC from peanut-allergic (PA) donors. A enhanced response to peanut proteins was observed in the ANPA grou which was comparable to the response measured in PA donors and statis cally significantly enhanced compared to the response measured in N donors. This enhanced proliferation was not found in response to oth food allergens (soy and ovalbumin) to which none of the donors we allergic, or to the inhalant cat dander allergen, to which a subgroup of th ANPA donors was allergic. This suggests that the observed enhance response in allergic donors to this non-relevant allergen, is specific f peanut allergen.

As it has been demonstrated that $CD8^+$ T cells can inhibit the IgE respons the proliferation of PBMC depleted of $CD8^+$ T cells of all 3 groups to pean proteins was studied. The CD8-depleted PBMC of the PA group showed higher response to peanut proteins compared to undepleted PBM suggesting that $CD8^+$ T cells play a role in peanut allergy. In contrast, n evidence was obtained for a role of $CD8^+$ T cells in the peanut response the ANPA group, since the response observed in the $CD8^+$ T cell deplete PBMC of ANPA and NA donors was not altered.

Because IgE production is regulated by the reciprocal Th1 cytokine IFN and Th2 cytokine IL-4, the secretion of these cytokines was measured in th supernatants of PBMC of all 3 groups after both specific stimulation wi peanut allergens and mitogenic stimulation with PHA and PMA, but n differences were observed.

From this study it is concluded that PBMC from ANPA donors stimulated with several food allergens to which non of the patients was allergic, only showed an enhanced proliferative response to peanut proteins. The lack of correlation between peanut-specific T cell proliferation and peanut-specific IgE could not be explained by differences in quality of the T cell response.

Introduction

Stimulation of peripheral blood mononuclear cells (PBMC) from individuals allergic to a specific allergen induces a strong proliferative response *in vitro* to this allergen. This response is weak in PBMC of non-allergic donors as has been demonstrated for both inhalant allergens (1, 2) and food allergens (3, 4). Moreover, the frequency of specific T cells in target organs is elevated in allergic donors compared to non-allergic donors (2). The proliferative response of PBMC of allergic donors to allergen to which these donors do not have IgE, is less well studied.

In the framework of studies on the characteristics of peanut allergy, the proliferative response of PBMC isolated from non-allergic (NA) and allergic but not peanut allergic donors (ANPA) to the strong but non-relevant peanut proteins as allergens was investigated and compared to the response of PBMC from peanut-allergic (PA) donors. The observed differences in response were studied in more detail paying special attention to the role of $CD8^+$ T cells and cytokine production.

It has been demonstrated that $CD8^+$ T cells inhibit IgE production by the production of the Th1-associated cytokine IFN-γ which may inhibit the outgrowth of Th2 cells or act directly on the B cells (5, 6). $CD8^+$ T cells could play a role in the differential response of PBMC of NA, ANPA and PA donors. To investigate the role of $CD8^+$ T cells, proliferation experiments were carried out with both PBMC and CD8-depleted PBMC.

The cytokine production of the proliferating cells was studied because IgE production is regulated by the countermanding signals of interleukin-4 (IL-4) and interferon-γ (IFN-γ). IL-4 induces an immunoglobulin switch in B cells to IgE and IgG4 while IFN-γ suppresses the production of IgE (7-10).

Materials and methods

Donors

Nine allergic, 10 non-allergic donors and 5 peanut-allergic patients partic pated in this study after a medical examination, and informed consent wc obtained. Except for the PA patients, the medical examination an selection consisted of a general physical examination, a Radio-Allerge Sorbent Test (RAST) for 8 allergens (grass, cat, dog, alternaria, birch, bir house dust mite and peanut) and total IgE and a Skin-Prick-Test (SPT) as shown in Table 1. Non-allergic individuals were selected on the absence positive reaction to the SPT of the tested allergens and no clinical symp toms of any allergy. Allergic patients were selected on a positive skin-pric test reaction to one or more allergens, no reaction to peanuts and n clinical symptoms after consumption of peanuts. The mean total IgE wc 462 ± 528 IU/ml in the ANPA group and 102 ± 148 IU/ml in the NA group. A donors were on a peanut free diet for two weeks before blood collection. The peanut-allergic patients (n=5), all with a proven peanut allergy b means of a food challenge, SPT and RAST, had a mean age 26 (± 6) yea and a mean IgE of 2139 (±2287) IU/ml.

This study was approved by an independent Medical-Ethical committee.

Table 1: Patients' characteristics.

	NA	ANPA	PA
n	10	9	5
Mean age (years)	32.3	29	26
Sex (M/F)	2/8	2/7	1/4
Total IgE (IU/ml)	102 ±148	462 ±528	2139 ±2287
SPT[1]	negative	positive	positive
RAST[1]	negative	positive	positive
SPT[2]	negative	negative	positive
RAST[2]	negative	negative	positive

[1] : SPT/RAST total; [2] : SPT/RAST peanut-spefific.

Preparation of cell suspensions

Heparinized peripheral blood was collected by venapunction and PBMC were isolated using density gradient separation on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) essentially as described by Böyum (11). Recovered PBMC were resuspended at a concentration of $2x10^6$ cells/ml in Iscove's Dulbecco's Modified Medium (IMDM; Gibco, Paisly, UK) supplemented with 10% complement inactivated pooled normal human serum (HS) (Central Laboratory Blood Transfusion Service, CLB, Amsterdam, The Netherlands).
To deplete the $CD8^+$ T cells from PBMC, magnetic beads with anti-CD8 antibodies on the surface (Dynal, Oslo, Norway) were used. PBMC (2 ml, $17.5x10^6$ cells/ml) in PBS (Gibco)/2% FCS (Hyclone, Logan, Utah) were incubated at 4^0C with 500 µl washed beads. After 30 min, 8 ml PBS/2%FCS was added and the tube was placed in a magnet. The supernatant, containing the CD8-depleted PBMC was collected, washed and resuspended in IMDM at a concentration of $2x10^6$ cells/ml.

Flow cytometric analysis

For immunophenotyping the subpopulation of cells, the following combinations of fluorescein (FITC) or phycoerythrine (PE) conjugated monoclonal antibodies were used: CD4 FITC (T4)/ CD8 PE (T8); CD19 FITC (B4)/ CD2 PE (T11); HLA-Dr FITC (I3)/ CD3 PE (T3); CD3 FITC (T3)/ CD16+CD56 PE (Leu 11+Leu 19); CD4 FITC (T4)/ CD29 PE (4B4); CD4 FITC (T4)/ CD45-RA PE (2H4) and CD45 FITC (KC56)/ CD14 PE (Mo2). These antibodies were obtained from Coulter (Hialeah, FL) except the CD3/CD16+56 antibodies which were obtained from Becton & Dickinson (Mountain View, CA).
Per experiment, 10^5 PBMC were labelled in 100 µl PBS containing 1% BSA (Organon Teknika, Boxtel, The Netherlands). To each tube 10 µl undiluted Coulter antibody or 20 µl undiluted Becton & Dickinson antibody was added. After 30 min of incubation at 4^0C the cells were washed twice in 2 ml PBS+1% BSA by centrifugation for 5 min at 400g at 4^0C. The final pellet was resuspended in 300 µl PBS+1% BSA. Analysis was performed using an Epics Elite flow cytometer (Coulter).

Proliferation assay

PBMC or CD8 depleted PBMC ($2x10^5$ cells/well in 200 µl) were cultured in the absence or presence of PHA (Difco, Detroit, MI) or several antigens in 96-well flat-bottom culture plates (NUNC, Roskilde, Denmark). The following

antigens were used: crude peanut extract (CPE; final concentrations 1, 1
50, 100, 150, 200 and 250 μg/ml), Peanut agglutinin (PNA, Lectin from *Ara*
his Hypogaea; Sigma, St. Louis, MO; final concentrations 1, 10 and
μg/ml), soy (TNO-ILOB, Wageningen The Netherlands; final concentratio
200, 300 and 400 μg/ml), ovalbumin (Serva, Heidelberg, Germany; fin
concentrations 100, 150, 200 μg/ml) and cat-allergen (ALK-Benelux, Houte
The Netherlands; final concentrations 100, 150, 200 μg/ml).
After 7 days of incubation in the absence or presence of the appropria
allergen, proliferation was measured using ^{3}H-thymidine incorporation (^{3}
TdR). After addition of ^{3}H-thymidine (0.4 μCi/well) (Amersham, Aylesbur
UK) the plates were incubated for 18 h before harvesting (Harvester 9
Tomtec, Orange). Beta-emission was counted with a 1450 Microbe
(Wallac, Turku, Finland). Proliferation is expressed as stimulation index (S
which is the mean cpm of triplicate cultures in the absence of antige
divided by the mean cpm of triplicate cultures in the presence of antigen

Preparation of supernatant

Cytokine production was measured in culture supernatant at several tim
points using different stimuli. To obtain these supernatants, $2x10^{5}$ PBMC
200 μl/well were cultured in 96-well flat-bottom culture plates (NUNC)
triplicate in the presence of both phytohaemagglutinin (PHA, 10 μg/m
Difco) and phorbol myristate acetate (PMA, 1 ng/ml; Sigma) or CPE (2(
μg/ml). After 36 h of incubation at 37^{0}C and 5% CO_2 humidified atmo
phere, the supernatants of the PHA/PMA stimulation were collected aft
centrifugation of the plates at 400g at RT, triplicates were pooled ar
stored at -20^{0}C until cytokine determination. The supernatants of th
cultures stimulated with CPE, were collected after 4 and 10 days
described above.

IL-4 and IFN-γ assays.

IL-4 secretion in supernatant was determined using an ELISA kit (CL
following the manufacturers instructions. IFN-γ was measure
using an ELISA kindly provided by Dr. P. van der Meide (TNO Preve
tion and Health, Rijswijk, The Netherlands). Briefly, monoclon
antibody (MD-2) directed against human IFN-γ, was used to coat 9
well ELISA plates (Maxisorp, NUNC) before incubation with th
collected supernatants. To detect the bound IFN-γ a second an

human IFN-γ biotinylated monoclonal antibody, MD-1 (22-3A) was used. Biotin was detected using peroxidase conjugated streptavidin. Colour was developed using 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate and the reaction was stopped with 2 N H_2SO_4. Absorption was measured at 450 nm. Recombinant human IFN-γ was used as a reference.

Statistical analysis

Statistical analysis was performed by Students' *t*-test taking $p<0.05$ as level of significance.

Results

Proliferation assays with PBMC from PA, ANPA and NA donors

In a first series of experiments the proliferative response to crude peanut extract (CPE) of PBMC from peanut allergic donors (PA), allergic but not peanut allergic (ANPA) and non-allergic donors (NA) was compared. The basic finding of these studies was that PBMC from not only the PA but also from the ANPA group showed an enhanced proliferative response to CPE (Figure 1). This enhanced proliferative response of PBMC from the ANPA group has been observed in 3 separate experiments performed over a period of approximately 1.5 year and was statistically significant ($p<0.05$) at CPE concentrations of a 100 μg/ml and higher (Figure 1) compared to the response of the NA donors. The enhanced proliferation of PBMC from the ANPA group can not be explained by a preferential susceptibility to the mitogenic effect of the naturally occurring lectin in peanuts, since purified PNA, which constitutes approximately 1% of CPE, did not induce proliferation in PBMC of any of the groups tested (Figure 1).

To study whether the enhanced proliferative response of the ANPA group to CPE was specific for peanut allergens or could also be observed for other common food and inhalant allergens, the proliferative responses of PBMC from the ANPA and NA group were measured to soy, ovalbumin and cat-allergen. None of the donors was allergic to soy or ovalbumin, whereas a subgroup of the ANPA group was not allergic to cat-allergen. As is demonstrated in Figure 2, no statistically significantly differences between PBMC from ANPA and NA donors were observed in the responses

to soy or ovalbumin. As expected, the PBMC of the cat-allergic don (CA) did demonstrate an increased proliferation to cat-allergens co pared to the PBMC from the NA group. However, an enhanced prolife tive response to cat-allergens was not observed in the PBMC of allergic b not cat allergic (ANCA) donors. These results suggest that the enhanc response to CPE in PBMC of the ANPA group is a specific effect to pea allergens.

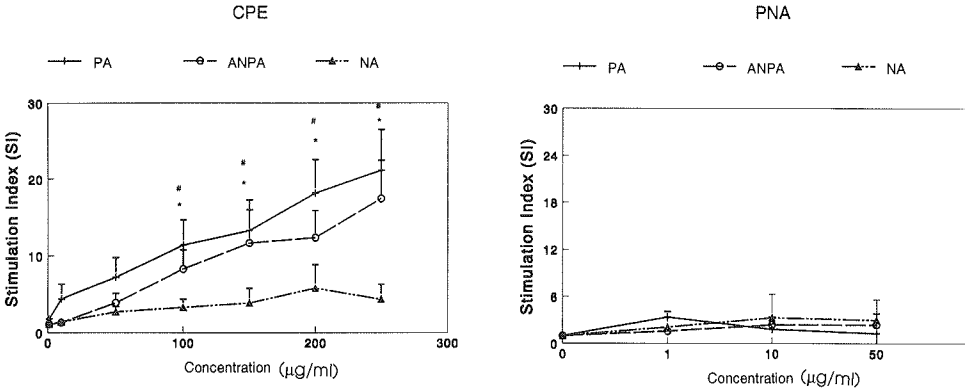


Figure 1: Proliferative response of PBMC from peanut-allergic (PA), allergic b not peanut-allergic (ANPA) and non-allergic donors (NA), to pea proteins (CPE, upper panel) and to peanut agglutinin (PNA, low panel).

Proliferation of PBMC ($2x10^5$ cells/well) was determined by measuri (^{3}H-TdR) incorporation after 7 days of incubation in the absence presence of increasing amounts of CPE or PNA.

Results are expressed as mean stimulation index (SI) ± SEM.

*: The response of the ANPA group is statitically sign cantly ($p<0.05$) increased compared to the response of t NA group.

#: The response of the PA group is statitically significa ly ($p<0.05$) increased compared to the response of the N group.

Proliferation assays with CD8$^+$-depleted PBMC from PA, ANPA and N donors

The next series of experiments were focused on the mechanism that m

underlie the observed enhanced response to CPE in the PA and ANPA groups. The enhanced proliferative response of the ANPA group could notbe related to a different subset composition of PBMC or to an enhanced activation state of the T cells, because no significantly different frequencies of $CD3^+$ T cells or $CD3^+$ T cells expressing HLA-DR, which is an activation marker, could be found after flow cytometric analysis (data not shown).

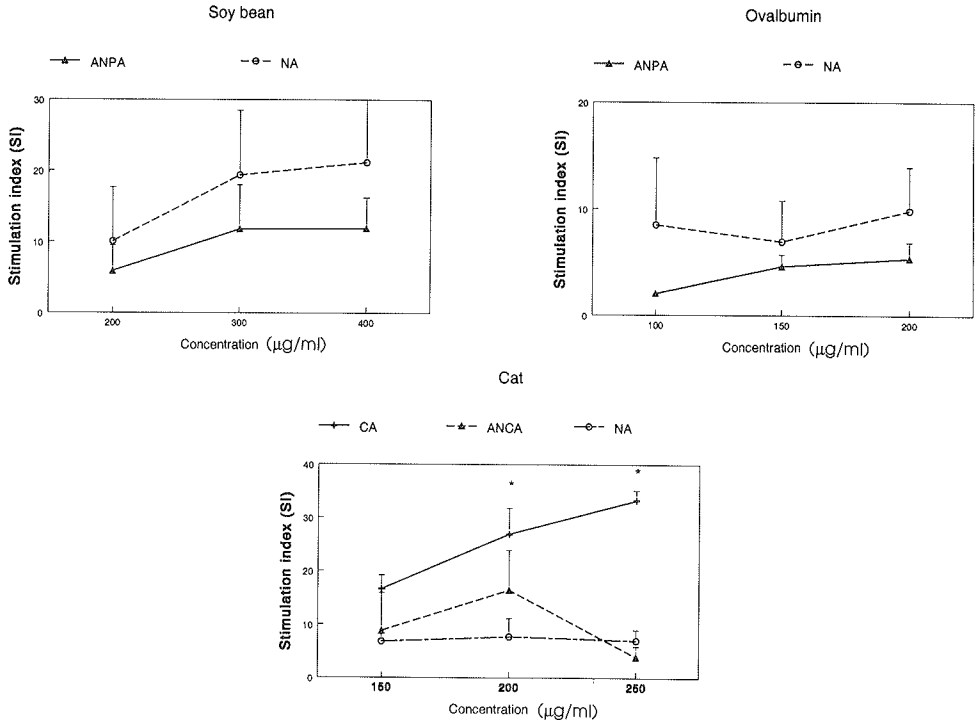


Figure 2: Proliferative response of PBMC of ANPA and NA subjects to soy bean (upper panel, left), ovalbumin (upper panel, right) and cat extract (lower panel). The ANPA-group has been divided in cat-allergic (CA), allergic but not cat allergic (ANCA) for the response to cat extract.
Proliferation of PBMC ($2x10^5$ cells/well) was determined by measuring(^{3}H-TdR) incorporation after 7 days of incubation in the absence or presence of the appropriate allergen.
Results are expressed as mean SI ± SEM.
*: The response of the CA group is statistically significantly ($p<0.05$) increased compared to the response of the NA group.

The role of CD8$^+$ T cells in the response of PBMC of all 3 groups to CPE w
studied by depleting CD8$^+$ T cells from the PBMC of 5 donors of the P
group and 4 donors of each of the ANPA and NA groups. The proliferati
of the CD8$^+$ depleted PBMC upon incubation with CPE is shown in Figure

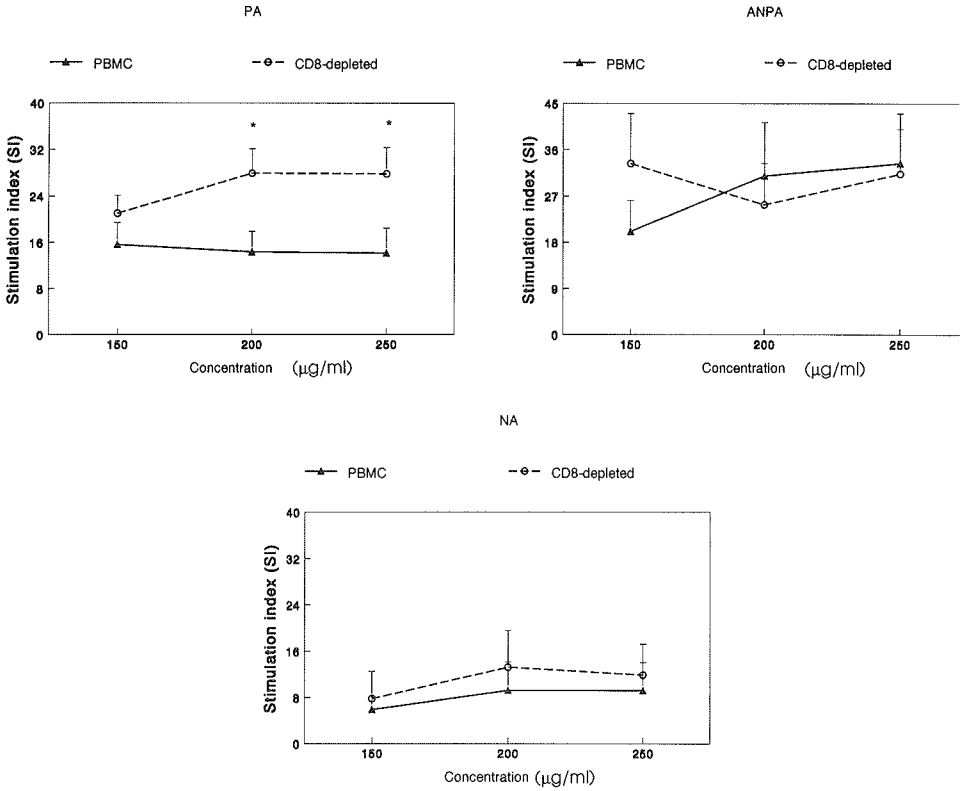


Figure 3: Proliferative response of PBMC and CD8-depleted PBMC (CD8) of P
(upper panel, left), ANPA (middle panel, right) and NA (lower pan
individuals.
Proliferation of PBMC ($2x10^5$ cells/well) was determined by measuri
(^{3}H-TdR) incorporation after 7 days of incubation in the absence
presence of increasing amounts of CPE.
Results are expressed as mean SI ± SEM.
*: The response of CD8$^+$ T cell-depleted PBMC is statitically significan
($p<0.05$) increased compared to the PBMC response.

Depletion of CD8$^+$ T cells resulted in the PA group in an increased c

proliferation in 4 out of 5 donors and was statistically significantly different ($p<0.05$) from the unseparated PBMC at a CPE concentration of 200 and 250 μg/ml. It should be noted that this increase was higher than could be expected due to the increase of the frequency of potentially CPE-reactive $CD4^+$ T cells of approximately 20%. This finding suggests that $CD8^+$ T cells play a suppressive role in the proliferative response of PBMC from the PA group to peanut proteins. In the PBMC of the ANPA group, however, no such difference was observed, although 3 of the 4 donors did show a slightly, but not statistically significant increased response. Depletion of $CD8^+$ T cells from PBMC of the NA group did not show any change of the low proliferative responses.

Cytokine production

To study whether the response of PBMC from the PA and ANPA group show qualitative differences rather than quantitative differences in the production of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4, these cytokines were measured in supernatant of PBMC stimulated with both CPE and a combination of the mitogens PHA and PMA. After 36 h of stimulation with PHA and PMA, no differences were observed in the levels of IL-4 and IFN-γ (Figure 4, left panel). In another experiment IL-4 and IFN-γ secretion was determined in supernatant of PBMC of the PA and ANPA group after incubation with CPE for 4 and 10 days. After 4 days of incubation, IFN-γ was present in the supernatant, although in low concentrations between 3 to 20 U/ml, whereas IL-4 could not be detected. Although the IFN-γ level in the PA group was higher compared to the ANPA group, this was statistically not significant ($p=0.07$) (Figure 4, right panel). At day 10 IL-4 could be detected at level of the detection limit of the assay without significant differences for both groups. PBMC from the NA group, which did show a very low proliferative response, produced approximately 200 U/ml IFN-γ in this assay (data not shown). The PBMC of the PA and the ANPA groups produced approximately 300 U/ml and 500 U/ml respectively, but these differences proved not to be statistically significant (Figure 4, right panel).

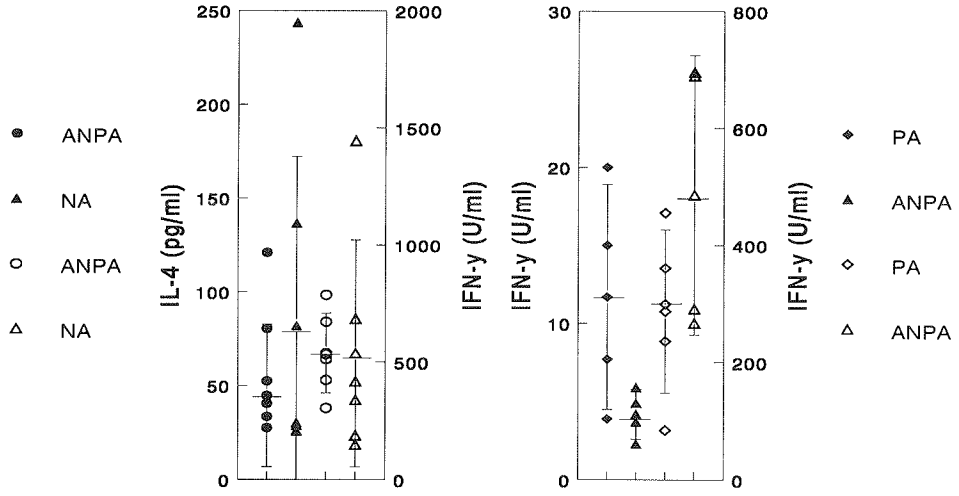


Figure 4: Cytokine production in supernatant of PBMC of PA, ANPA and NA i dividuals.
Left panel: IL-4 and IFN-γ production after mitogenic stim lation with 10 µg/ml PHA and 1 ng/ml PMA. Supernatants we collected after 36 h of incubations. Closed symbols represe the IL-4 production in pg/ml and the open symbols the IFN-γ pr duction in U/ml.
Right panel: IFN-γ production of PBMC of PA and ANPA indivi uals, stimulated with CPE (200 µg/ml). Closed symbols repr sent concentration in U/ml measured after 4 days of stimul tion whereas open symbols represent the measured concentr tions at day 10.

Discussion

In this study we show an enhanced proliferative response of PBMC f peanut proteins of donors allergic to one or more allergens but not f peanuts in a comparative study with non-allergic donors. The observe response of PBMC of the ANPA group was comparable to the response PBMC of peanut allergic donors (PA). This enhanced response is peanu

specific and has been observed repeatedly. Recently it has been demonstrated that human serum in culture medium may play a role in the higher proliferative responses to several allergens observed in PBMC of allergic individuals compared to PBMC from non-allergic donors (12). This indicates that T cells from non-allergic donors are inhibited *in vitro* by a serum-component. In our study, the PBMC of ANPA donors respond in the same fashion as PBMC of PA donors to peanut proteins, suggesting that the peanut-specific T cells from the ANPA group were also insensitive to such an inhibitory factor.

The enhanced proliferative response of PBMC of the ANPA compared to non-allergic donors could not be due to a susceptibility to an aspecific effect of PNA, the naturally occurring lectin in peanuts, because PNA did not induce a proliferative response in neither of the groups. Moreover, if PNA would induce a proliferative response by means of agglutination as has been described for bovine PBMC (13), this should also have been observed in the non-allergic group.

The proliferative response of these allergic donors to other common food or inhalant cat dander allergens was not enhanced. This implies that the enhanced response to peanut proteins observed in PBMC of ANPA donors, must be specific for peanut proteins and is not be due to a higher activation state of T cells in the peripheral blood of allergic donors. This was confirmed by flow cytometric analysis as the percentage of activated T cells in the PBMC of the ANPA group and the NA group were comparable.

The enhanced response to peanut proteins of ANPA donors could also be due to a different frequency of peanut-specific T cells, to a different subset of peanut-specific T cells or to a difference in cytokine production.

Several studies suggested an altered subset composition in the peripheral blood of allergic individuals (14-17). It has been demonstrated that allergic donors possess a decreased total $CD3^+$ population which is caused by a decreased $CD8^+$ T cell subset and consequently a higher ratio of $CD4^+$/-$CD8^+$ cells (15, 16). It has been postulated that this reflects a decreased number of $CD8^+$ T cells with suppressor activity which facilitates allergic reactions. However, a decreased frequency of $CD8^+$ T cells has been contradicted (17) and also in the present study no difference in subset composition was observed.

To study the possible role of $CD8^+$ T cells in the differential responses to peanut proteins, the PBMC were depleted of $CD8^+$ T cells. In PA individuals

the proliferative response was enhanced in the $CD8^+$-depleted populatio compared to PBMC, whereas there was no significant effect in the othe groups after depletion. The slight proliferative response observed in bot PBMC and $CD8^+$ T cell-depleted PBMC of the NA donors may be due to low frequency of both $CD4^+$ and $CD8^+$ peanut-specific T cells in th peripheral blood of NA donors or to the possible inhibitory factor in serur (12). The responses of the $CD8^+$ T cell depleted PBMC from the PA an ANPA donors, however, were in contrast to our expectation, of a unchanged and enhanced response, respectively. It has been postulate that $CD8^+$ T cells have a suppressive effect on IgE production by th production of IFN-γ which inhibits either the outgrowth of $CD4^+$ Th cells or directly the IgE switch in B cells (5, 6). Therefore, peanut-specifi $CD8^+$ T cells were not expected to be responsible for an increase of th proliferative response observed in PBMC from PA donors, whereas th response of ANPA donors who do not have peanut-specific IgE, migh have been explained by the proliferation of active $CD8^+$ T cells wit suppressor activity. The increased response after $CD8^+$ depletion ma indicate that in PA donors $CD8^+$ T cells possess a suppressor activity tha decreases the proliferative response. Alternatively, it has recently bee shown that in the presence of IL-4, activated $CD8^+$ T cells can switch t produce Th2 associated cytokines (18, 19) and may enhance the IgE mediated response. However, the response to peanut proteins of PA donors was increased after depletion of $CD8^+$ T cells from PBMC. Thi suggests that type 2 $CD8^+$ T cells do not play a role in peanut allergy.

As the response of $CD8^+$ T cells-depleted PBMC from ANPA donors wa unchanged suggesting that $CD8^+$ T cells are not responsible for the enhanced proliferative response observed in PBMC, the enhanced prolife ration to peanut proteins of ANPA donors may be explained by the prolife ration of peanut allergen-specific $CD4^+$ Th1 cells, which do not support IgI production. Therefore, the production of Th1 and Th2 associated cytokine was measured of PBMC from all groups after mitogenic and specific (CPE stimulation but no differences were observed. The secretion of IL-4 and IFN γ by PBMC both after specific and mitogenic stimulation of allergic com pared to non-allergic individuals have been studied by several groups (21 26). IL-4 was found to be increased (20-22), whereas IFN-γ secretion wa found to be diminished (20, 23, 24) or not different (21, 22). Tang (25 showed an diminished IFN-γ production by PBMC only during acute exacer

bation of atopic dermatitis. This suggests that IFN-γ levels are more important than IL-4 levels in atopic dermatitis.
The enhanced response of PBMC of the ANPA group to peanut proteins has consequences for the use of a lymphocyte stimulation test (LST) which has been suggested to be a possible diagnostic assay to detect allergy to inhalation allergens (26, 27) and food allergens (4, 28, 29). With a suspected peanut allergy in combination with other allergies, a proliferative response or positive LST would not be a reliable indicator for a peanut allergy. To diagnose IgE mediated food allergy one still has to rely on SPT and RAST in combination with the "Golden Standard", the double-blind, placebo-controlled food challenge (30).
This study demonstrates that the peanut-specific proliferative response in ANPA donors is enhanced. The underlying mechanism is not clear, since no indications were found that this can be explained by qualitative differences of the T cell response. To elucidate the mechanism responsible for the enhanced proliferative response, more detailed studies are needed.

Acknowledgements

The authors would like to thank Dr. P. van der Meide (TNO Prevention and Health, Rijswijk, The Netherlands) for the kind gift of the monoclonal antibodies and standard for the IFN-γ-ELISA.

References

1 **Rawle FC, Mitchell EB and Platts-Mill TAE** (1985) T cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* **133**:195-201.

2 **Burastero SE, Fenoglio D, Crimi E, Brusasco V and Rossi GA.** (1993) Frequency of allergen-specific T lymphocytes in blood and bronchial response to allergen in asthma. *J Allergy Clin Immunol* **91**:1075-1081.

3 **May CD and Alberto R.** (1972) *In vitro* responses of leucocytes to food proteins in allergic and normal children: lymphocyte stimulation histamine release. *Clin Allergy* **2**:335-340.

4 **Kondo N, Agata H, Fukutomi O, Motoyoshi F and Orii T.** (1990) Lymphocyte repons to food antigens in patients with atopic dermatitis who are sensitive to foods. *Allergy Clin Immunol* **86**:986-260.

5 **McMenamin C and Holt PG.** (1993) The natural immune response to inhaled solub protein antigens involves major histocompatibility complex (MHC) class I-restricte CD8+ T cell-mediated but MHC class II-restricted $CD4^+$ T cell-dependent immur deviation resulting in selective suppression of immunoglobulin E production. *J E Med* **178**: 889-899.

6 **Kemeny DM and Diaz-Sanchez D.** (1993) The role of $CD8^+$ T cells in the regulation IgE. *Clin Exp Allergy* **23**:466-470.

7 **Coffman RL and Cartly J.** (1986) T cell activity that enhances polyclonal IgE produ tion and inhibition by interferon-γ. *J Immunol* **136**:949-954.

8 **Cartly J and Paul WE** (1987) Interferon-γ and B cell stimulatory factor-1 reciproca regulate Ig isotype production. *Science* **236**:944-947.

9 **Schultz CL and Coffman RL.** (1991) Control of isotype switching by T cells and cytoki es. *Curr Opin Immunol* **3**:350-354.

10 **Vercelli D and Geha RS.** (1991) Regulation of IgE synthesis in humans: a tale of tw signals. *J Allergy Clin Immunol* **88**:285-295.

11 **Böyum A.** (1968) Isolation of leucocytes from human blood. *Scand J Clin Lab Inve* **21**:9-14.

12 **Upham JW, Holt BJ, Baron-Hay MJ, Yabuhara A, Hales BJ, Thomas WR, Loh RK O'Keefe PT, Palmer L, Le Souef WR, Sly PD, Burton PR, Robinson BWS and Holt P** (1995) Inhalant allergen-specific T-cell reactivity is detectable in close to 100% atopic and normal individuals: covert responses are unmasked by serum-fre medium. *Clin Exp All* **25**:634-642.

13 **Nagi AM and Babiuk LA.** (1989) Peanut agglutinin (PNA): binding and stimulation bovine intestinal and peripheral blood leucocytes. *Veterinary Immunol Immunopa hol* **22**:67-79.

14 **Kagi MK, Wuthrich B, Montana E, Barandun J, Blaser K and Walker C.** (1994) Differer tial cytokine profiles in peripheral blood lymphocytes supernatants and skin biopsie from patients with different forms of atopic dermatitis, psoriasis and normal indivi uals. *Int Arch Allergy Immunol* **103**:332-340.

15 **Strannegard O and Strannegard IL.** (1979) In Vitro differences between th lymphocytes of normal subjects and atopics. *Clin Allergy* **9**:637-643.

16 **Valdes Sanchez AF, Gómez Echevarría AH and Lastra Alfonso G.** (1991) Atopi dermatitis: serum immunoglobulins and T-lymphocyte subpopulation. *J Invest Allerg Clin Immunol* **1**:154-158.

17 **Hsieh KH.** (1984) Changes of lymphoproliferative responses of T cell subsets t allergen and mitogen after hyposensitization in asthmatic children. *J Allergy CL Immunol* **74**:34-40.

18 **Croft M, Carter L, Swain SL and Dutton RW.** (1994) Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* **180**:1802-1205.

19 **Seder RA and Le Gros GG.** (1995) The functional role of $CD8^+$ T helper type 2 cells. *J Exp Med* **181**:5-7.

20 **Jujo K, Renz H, Abe J, Gelfand EW and Leung DYM.** (1992) Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. *J Allergy Clin Immunol* **90**:323-331.

21 **Jaffe JS, James SP, Mullins GE, Braun-Elwert L, Lubensky I and Metcalfe DD.** (1994) Evidence for an abnormal profile of interleukin-4 (IL-4), IL-5, and γ-interferon (γ-IFN) in peripheral blood T cells from patients with allergic eosinophilic gastroenteritis. *J Clin Immunol* **14**:299-309.

22 **Byron KA, O'Brien RM, Varigos GA and Wootton AM.** (1994) *Dermatophagoides pteronyssinus* Il-induced interleukin-4 and interferon-γ expression by freshly isolated lymphocytes of atopic individuals. *Clin Exp Allergy* **24**:878-883.

23 **Hill DJ, Ball G, Hosking CS and Wood PR.** (1993) Gamma-interferon production in cow milk allergy. *Allergy* **48**:75-80.

24 **Suomalainen H, Soppi E, Laine S and Isclauri E.** (1993) Immunologic disturbances in cow's milk allergy, 2: evidence for defective interferon-gamma generation. *Pediatr Allergy* **4**:203-207.

25 **Tang M, Kemp A and Varogos G.** (1993) IL-4 and interferon-gamma production in children with atopic disease. *Clin Exp Immunol* **92**:120-124.

26 **O'Brien RM, Thomas WR and Wootton AM.** (1992) T cell responses to purified major allergens from house dust mite *Dermatophagoides pteronyssinus. J Allergy Clin Immunol* **89**:1021-1031.

27 **van Bever HP, Bridts CH, Moens MM, de Rijck TE, Mertens AV, de Clerck LS and Stevens WJ.** (1993) Lymphocyte transformation test with house dust mite (*Dermatophagoides pteronyssinus*) in normal children, asthmatic children and asthmatic children receiving hyposensitization. *Clin Exp Allergy* **23**:661-668.

28 **Albani S, Avanzani MA and Plebani A.** (1989) Diagnostic value of lymphocyte stimulation test in cow milk protein intolerance. *Ann Allergy* **63**:489

29 **Ownby DR.** (1991) In vitro assays for the evaluation of immunologic reactions to foods. *Immunol Allergy Clin North America* **11**:851-862.

30 **Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sacks M, Bush RK and Metcalfe DD.** (1988) Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: A manual. *J Allergy Clin Immunol* **82**:986-997.

Chapter 7

General discussion

Introduction

The aim of the research described in this thesis was to obtain more insight in some of the mechanisms of food allergy. Food allergy is a complex disease that can cause symptoms not only at the place of entry of the allergen, the gastro-intestinal tract, but throughout the whole body. The symptoms can be very severe, especially in peanut allergy in which case death due to an anaphylactic shock is not uncommon. In this thesis several aspects of peanut allergy have been studied. The second chapter of this thesis describes the difficulties in diagnosing peanut allergic patients, due to the differences in responses, such as differences in display and time of onset of symptoms, differences in severity of the response as well as the ill-characterized peanut-extracts used for diagnostic tests. Identification of peanut proteins recognized either by IgE, or by T cells may be important for the development of both diagnostic tools and specific immunotherapy. The diversity of proteins recognized by B and T cells is described in chapters 3 and 5. In chapter 4 it is shown that peanut-specific T cell clones generated from a peanut-allergic patient have a Th2 phenotype, producing high levels of IL-4 and IL-5 but little or no IFN-γ. Moreover the responses to peanut proteins of peripheral blood mononuclear cells (PBMC) from peanut-allergic (PA), allergic but not to peanuts (ANPA) and non-allergic (NA) subjects were investigated as described in chapter 6.

Recognition of peanut proteins by B and T lymphocytes

Using the plasma of a panel of peanut-allergic adults (n=14), we hav demonstrated that peanut proteins contain multiple allergens recognize by IgE as described in chapter 3. Three of these proteins or protein banc were recognized by more than 70% of the individuals and had molecul weights of approximately 18, 20 and 21 kD. In addition, 3 other protei were recognized by more than 50% of the patients with molecular weigh of 33, 40 and 44 kD.

The recognition of peanut proteins by peanut-specific T cell clones gene ated from the peripheral blood of a severe peanut allergic patient described in chapter 5. This recognition proved to be extremely diverse f the various T cell clones as has also been described previously for inhala allergens found in HDM, grass pollen and cat dander (1-6). These studi implicate an even more diverse recognition of peanut proteins by T cells. has been demonstrated that the proteins recognized by these T cell clon have molecular weights of approximately 30 to 35 kD or 14 to 26 kD. Bo arachin and conarachin, the 2 major proteins on a weight basis of pe nuts, are recognized by the described peanut-specific T cell clones.

Comparing the proteins recognized by IgE with those recognized by the cell clones, it may be concluded that B and T cell epitopes are present the same protein bands. However, the protein bands to which 70% of th patients showed IgE binding, were only recognized by 1 of the 5 T c clones.

Several peanut allergens have been described previously which we detected using IgE-containing sera of peanut-allergic patients (7-11). Th well-characterized *Ara h* I and *Ara h* II (9, 10) have molecular weights approximately 63 and 17 kD, respectively. Comparison of the molecul weight of *Ara h* I with the molecular weights of the proteins recognized b either our peanut-specific T cell clones or the peanut-specific IgE-co taining plasma samples obtained from Dutch peanut-allergic patient suggests that *Ara h* I is not the most important allergen for the Dutch popu ation. Based on the molecular weights of the recognized proteins by th IgE-containing plasma samples of the Dutch peanut-allergic patients, can be concluded that *Ara h* II probably is one of the major allerge because it is most likely one of the three proteins that are recognized b

more than 70% of the IgE containing plasma samples used in our study and is most likely also recognized by one of the T cell clones. Dorion et al. (12) also demonstrated responses of PBMC to this allergen. Whether other described allergens, such as Peanut-1 and the Concanavaline-a reactive protein (7, 8) are recognized by IgE-containing plasma samples of our population or are recognized by the described T cell clones has not been investigated.

Implications for immunotherapy

Since immunotherapy was first described in 1911 (13), it has been widely used to reduce or overcome the allergic state of the patient. Immunotherapy is either based on the induction of cells with suppressor activity, on inducing tolerant or anergic CD4+ T cells or on inducing a switch from a Th2 towards a Th1 response. In several studies it has been shown that treatment of allergic patients with increasing doses of the relevant allergen resulted in a lower proliferative response of PBMC to the allergen and a reduced IL-4 and IL-5 production whereas IFN-γ production increased (14-16). Induction of T cells with antigen-specific suppressor activity has also been demonstrated in immunotherapy (17). These therapies are based on T cell recognition of allergens. So far, whole protein mixtures have been used for desensitization with the risk of severe side effects, especially in peanut allergy (18-20). Therefore, it would be an improvement for immunotherapies to be able to use only peptides which do contain the T cell epitopes but little or no B cell epitopes recognized by IgE which may be responsible for the severe side effects. However, the results described in chapter 5 clearly indicate that also in case of peanuts, the recognition of allergens by T cells is very diverse which may be a complicating factor for the design of specific immunotherapy. This diverse recognition is in agreement with the results obtained for several inhalant allergens found in house dust mite, various pollen and cat dander (3-6). This indicates that the T cell epitopes would have to be established for every patient individually which is hardly feasible in practice. However, peptide cocktails expressing various T cell epitopes may be beneficial. Immunotherapy conducted with a pepsin-digested short ragweed extract and complete protein mixtures

showed comparable results (21). However, one should be cautious with th type of therapy because digested extracts could still contain IgE-bindir sites which would increase the risk of side effects, especially in the case peanut-allergy. Recently it has been shown that the use of immun dominant T cell peptides can induce a non-responsiveness to the who allergen: Allergen primed mice were treated with *Der p* I (purified fro house dust mite allergens) or *Fel d* I (purified from cat dander allergens) ir muno-dominant peptides which resulted in a reduced T cell responsivene and reduced production of IL-2, IL-4 and IFN-γ (22, 23).
These results indicate that there may still be possibilities for immunotherap This may be very important for peanut-allergic patients because not or the symptoms can be very severe (24, 25) but also because peanut is ve difficult to eliminate from the diet. Peanut flour is used in many produc such as cookies, soups and sauces. The only study that has been publishe using immunotherapy for the treatment of peanut allergy was terminate early because one of the patients died from an anaphylactic shock whic however, was due to a switch in syringes with placebo and peanut-extra (19). The use of immunotherapy with immuno-dominant peptides which c not contain IgE-binding sites may therefore be a very useful and sa therapy for such a severe allergy.

What makes the peanut more hazerdous than many other foo allergens?

Peanut allergy is one of the few allergies in which relatively frequent th patient may suffer from an anaphylactic shock followed by death (24, 25 It is thought to occur more often due to peanut than to bee venom. F example, in 1993, 4 people died due to an peanut-induced anaphylact shock in the United Kingdom (26). Although the mechanism of this stron allergic reaction is unknown, some properties of peanuts, such as the hig fat contents, the lipophilicity of the proteins and the stability for acid, hea and enzymatic treatment may play a role. However, this kind of propertie are not specific for peanut but may also be found in other food allergen But combined these features may contribute to the allergenicity of peanu Another important factor that may be involved in the severity of th

response could be the high allergen dose in case of peanut allergy compared to inhalant allergies. If a peanut-allergic patient eats peanut proteins, for instance hidden in a meal, at least several micrograms will be consumed. Compared to the inhaled picograms of HDM-allergen, this is a relatively high exposition. This could lead to massive mast cell degranulation and mediator release. Although this high allergen dose may also apply for all food allergies, cow's milk allergy, for instance, does not as often results in an anaphylactic shock compared to peanut. Possibly the combination of high allergen load and the specific properties of peanuts may be of importance.

Of these specific properties especially the high fat content of 55% in peanut (27) together with the lipophilic character of the peanut proteins may play an important role in the more severe reactions observed. Due to this high fat content, at least part of the protein could be surrounded by fat resulting in the creation of micelles, which have also been described for other plant proteins (28). It may be hypothesized that these protein containing micelles may be differently absorbed in the gastro-intestinal tract than soluble proteins. This may not only play an important role in the induction but also in the effector phase of peanut allergy.

Role of peanut properties in sensitization

In general, soluble dietary antigens are absorbed partly intact through the epithelial cells towards the lamina propria (LP) (29). The intestinal epithelial cells (IEC) process the antigens and present the resulting peptides in context of MHC class I to the $CD8^+$ T cells which may induce tolerance (30, 31). Under certain circumstances, as for instance during inflammation when the expression of MHC class II molecules on epithelial cells is increased, they are aslo capable of activating $CD4^+$ T cells (32).

In contrast, protein containing micelles may be absorbed and presented via a different route. This may be via absorbtion by M cells, the specialized epithelial cells covering the intestines at the sites of the Peyer's Patches (PP), and presentation to immuno-competent cells of the PP (29). This may lead to sensitization rather than oral tolerance as has been demonstrated with ovalbumin which in general may induces tolerance but when expressed in *E. coli* and absorbed via M cells towards the PP, induces a strong immune response (33).

Another possibility could be that the peanut proteins are delivered to the

cells of the reticuloendothelial system (RES) in area's of the gut wall. Th RES contains large numbers of macrophages which will produce IL-1 an IL-6 after activation. These cytokines are necessary for antigen presentatio and especially Th2 cells seem to depend on the production of IL-1 to b activated by APC (34, 35). If food proteins are presented to the RES in th presence of bacteria, the RES can be activated by the bacteria whic may result in a Th2 response to the food protein rather than tolerance via T suppressor response. Several agents have been shown to activate th RES and induce an immune response to food proteins (36, 37).

Although it is generally thought that sensitization to food proteins take place in the gastro-intestinal tract, there is no evidence to support this. Als in the case of peanut proteins, the gastro-intestinal tract may not be th place of sensitization. This is supported by the observation that in th allergy clinic, young children are presented with a clinically manife peanut allergy, although they were assumed not to have eaten an peanuts yet. The obvious explanation may be that the child is breast-fe and that the mother has eaten peanuts. But this is not always the answe Some of these children are bottle-fed or the breast-feeding mother ha followed an elimination diet. An elimination diet may be followed by mother who is expecting a child with a high risk of developing a foo allergy. Such an diet may eliminate several allergenic food products tha cause the majority of the allergies such as cow's milk, henn's egg, peanu and soy, or food products to which the mother, father or siblings of th expected child are allergic. An elimination diet is thought to lower or delc the onset of allergic diseases in the child (38). The observation of childre with a clinically manifest peanut allergy who are though to have had n contact with peanut proteins, suggests that sensitization has already take place in utero or that the child has come into contact with peanu through another route, for instance through the skin or the respiratory trac Still, it can not be excluded completely that the child may have had a oral exposure to peanut proteins via licking of antigens from the skin of th hands.

Role of peanut properties in effector phase

The above decribed mechanism of induction of an immune response ma play an important role in the induction phase of a peanut allergy but doe not explain the strong anaphylactic responses upon repeated exposure.

fast, relatively high allergen concentration in the circulation may be responsible for the severe reactions as often seen with peanut. Several already mentioned properties of peanuts and peanut proteins may indeed result in a fast transport of peanut allergens across the gastro-intestinal tract. First, the high fat content of peanuts, possible micelle formation, and the mainly hydrophobic character of peanut proteins as demonstrated in chapter 5, may all facilitate a rapid uptake by the gastro-intestinal tract and into the circulation. Because of the rapid uptake of the allergens, binding of specific IgA is prevented which normally makes the allergen more prone to digestion by intraluminal proteolytic enzymes and ridding the body of locally formed immunecomplexes containing the antigen which can cause damage and induce an immune response (39-41). Taken into account that peanut proteins, do not loose their allergenicity by treatment with heat (roasting), acid or digestive enzymes (42, 43), high levels of peptides with intact IgE-binding sites will cross the gastro-intestinal tract. These properties, however, may not be specific for peanuts. Also other fatty substances as for example nuts, could be delivered to the immune system in a same fashion and most food allergens are relatively stable for heat, acid and digestive enzymes. However, these combined features may result in a high concentration of allergen in the circulation. Moreover, in chapter 3 it has been demonstrated that almost all protein bands present in CPE were recognized by more than 20% of IgE-containing plasma samples used in this study and 6 by more than 50%. Although it should be recognized that multiple binding of IgE to peanut proteins may partially be due to break-down of allergenic proteins, the broad recognition may play a role in the severity of responses to minute amounts of peanut protein.

Role of PNA

Another property of peanuts that may play a role in the allergenicity is the presence of peanut agglutinin (PNA). PNA is a lectin that can bind to sugars with a Gal(β1→3)GalNac specificity (44). Some lectins, such as concanavaline A and phytohaemaglutinin are known inducers of proliferation in PBMC. Moreover, several lectins have IgE-binding capacities and histamine-release activities but PNA does not induce release histamine (45). Although it has been demonstrated that PNA can induce proliferation in bovine PBMC (46), in our studies (chapter 4 and 6) human PBMC from both

peanut-allergic and non-allergic individuals were not found to proliferate in response to PNA. However, PNA-specific IgE has been found in sera of peanut-allergic patients and PNA has been indicated as a minor allergen (11). The sugar-binding properties of PNA may play a role in the induction of IgE-mediated responses based on the observations that PNA binds to a IgE-suppressive (IgE-SF) form of soluble CD23 (sCD23) derived from T cells in the rat and human (47, 48). CD23 is the low-affinity receptor of IgE (FcεRII) which may be present both membrane-bound (mCD23) and in a soluble form and plays a regulatory role in IgE production (49). Membrane-bound CD23 facilitates the antigen uptake by B-cells and induces a far more efficient antigen presentation than classical endocytotic antigen uptake (50). Soluble CD23 is a cleavage product from mCD23 and excists in 2 forms, an IgE-potentiating factor (IgE-PF) and an IgE-SF (51, 52). IL-4 is an inducer of both mCD23 and sCD23 (53, 54). As PNA binds to IgE-SF, the balance between IgE-PF and IgE-SF could be disturbed and upregulation or facilitation of IgE production may occur in a microenvironment where peanut proteins or other allergens are present. If this mechanism indeed plays a role *in vivo*, it would be of relevance for not-peanut allergic, and thus probably peanut consuming, individuals. However, no epidemiological data are availlable to support a relationship between the consumption of peanuts and the general occurence of allergic disorders. Moreover, although PNA-specific IgE has been detected in sera of peanut-allergic patients, absorption of intact PNA through the gastro-intestinal tract has not been proven. Nevertheless, this may be an interesting subject for future studies.

Although knowledge about mechanisms of allergic reactions has increased strongly during the last decade, still little is known about sensitization to food proteins. More knowledge on local conditions at the site of sensitization, whether this is the gastro-intestinal tract or other tissues, that may favour the development of a sensitization will in the future offer more possibilties for prophylactic interference in food allergy. For studying the specific initial immune responses in the gastro-intestinal tract, an animal model would be very helpful. Recent studies at our laboratory are focused on the induction of food allergy in rats both studying the responses in local as well as peripheral lymphoid tissues. Hopefully these studies will lead to more insight into the mechanism of sensitization to food proteins.

References

1 **Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RAW and Kapsenberg ML.** (1991) Human atopen-specific types 1 and 2 T helper clones. *J Immunol* **147**:2942-2949.

2 **Del Prete G.** (1992) Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* **47**:450-455.

3 **Ebner C, Széphalusi Z, Ferreira F Jilek A, Valenta R, Parronchi P, Maggi E, Romagnani S and Kraft D.** (1993) Identification of multiple T cell epitopes on *Bet v* I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol;* **150**: 1047-1054.

4 **van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RC and Kapsenberg ML.** (1994) Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol;* **152**: 4203-4210.

5 **IJssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R and de Vries JE.** (1992) T cell activation-inducing epitopes of house dust mite allergen Der p 1. Proliferation and lymphokine production patterns by Der p 1-specific CD4+ T cell clones. *J Immunol* **148**:738-745.

6 **van Neerven RJJ, van t Hoff W, Ringrose J, Jansen HM, Aalberse RC, Wierenga EA and Kapsenberg ML.** (1993) T cell epitopes of housedust mite major allergen Der p 2. *J Immunol;* **151**: 2326-2335.

7 **Sachs MI, Jones RT and Yunginger JW.** (1981) Isolation and partial characterization of a major peanut allergen. *J Allergy Clin Immunol* **67**:27-34.

8 **Barnett D and Howden MEH.** (1986) Partial characterization of an allergenic glycoprotein from peanut (*Arachis hypogaea* L.). *Biochim Biophys Acta* **882**:97-105.

9 **Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G and O'Brien T.** (1991) Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* **88**:172-179.

10 **Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien T and Helm RM.** (1992) Identification and characterization of a second major peanut allergen, *Ara h* II, with the use of sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* **90**:962-969.

11 **Burks AW, Cockrell, Connaughton C, Guin J, Allen W. and Helm RM.** (1994) Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. *Int Arch Allergy Immunol* **105**:143-149.

12 **Dorion BJ, Burks AW, Harbeck R, Williams LW, Trumble A, Helm RM and Leung DY.** (1994) The production of interferon-γ in response to a major peanut allergen, *Ara h* II, correlates with serum levels of IgE anti-*Ara h* II. *J Allergy Clin Immunol* **93** 93-99.

13 **Noon L and Cantab BL.** (1911) Prophylactic inoculation against hay fever. *Lancet* **1**:1572-1574.

14 **Secrist H, Chelen CJ, Wen Y, Marshall JD and Umetsu DT.** (1993) Allergen immunothe rapy decreases interleukin production in CD4+ T cells from allergic individuals. *J Ex Med* **178**:2123-2130.

15 **Varney VA, Hamid QA, Gaga M, Ying S, Jacobson M, Frew AJ, Kay AB and Durhar SR.** (1993) Influence of grass pollen immunotherapy on cellular infiltrations an cytokine mRNA expression during allergen induce late phase cutaneous responses. *Clin Invest* **92**:644-651.

16 **Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C and Müller UR.** (1995) Be venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- secretion in specific allergen-stimulated T cell cultures. *J Immunol* **154**:4187-4184.

17 **Rocklin RE, Sheffner AL, Greineder DK and Melmon KL.** (1980) Generation of antigen specific suppressor cells during allergy desensitization. *N Engl J Med* **302**:1213-1219.

18 **Hejjaoui A, Ferrando R, Dhiver H, Michel FB and Bousquet.** (1992) Systemic reaction occurring during immunotherapy with standardized pollen extracts. *J Allergy Cli Immunol* **89**:935-933.

19 **Oppenheimer JJ, Nelson HS, Bock SA, Christensen F and Leung DYM.** (1992) Treat ment of peanut allergy with rush immunotherapy. *J Allergy Clin Immunol* **90**:256-262.

20 **Stewart II GE and Lockey RF.** (1992) Systemic reactions from allergens Im munotherapy. *J Allergy Clin Immunol* **90**:567-578.

21 **Litwin A, Pesce AJ, Fisher T, Michael M and Michael JG.** (1991) Regulation of the human immune response to ragweed pollen by immuno-therapy. A controlled tria comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. *Clin Exp Allergy* **21**:457-465.

22 **Briner TJ, Kuo MC, Keating KM, Rogers BL and Greenstein JL.** (1993) Peripheral T cel tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen *Fel d* I. Proc Natl Acad Sci USA **90**:7608-7612.

23 **Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR and Lamb JR.** (1993) Inhibition of T cel and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* **178**:1783-1788.

24 **Bock SA and Atkins FM.** (1989) The natural history of peanut allergy. *J Allergy Clin Immunol* **93**:900-904.

25 **Yunginger JW, Squillance DL, Jones RT and Helm RM.** (1989) Fatal anaphylactic reactions induced by peanuts. *Allergy Proc* **10**:249-253.

26 **Frankland B.** (1994) Peanut and nut allergy. *Leatherhead Peanut Allergy Seminar Conference Programme*, 3-4.

27 **Pancholy SK, Deshpande AS and Krall S.** (1987) Amino acids, oil and protein content of some selected peanut cultivars. *Proc Amer Peanut Res Educ Assoc* **10**:30-37.

28 **Ismond MAH, Arntfield SD and Murray ED.** (1991) Formation and interactions of plant protein micelles in food systems. *Symp Series Am Chem Soc* **454**: 91-103.

29 **Walker WA and Isselbacher KJ.** (1974) Uptake and transport of macromolecules by the intestines. Possible role for clinical disorders. *Gastroenterology* **67**:531-537.

30 **Mayer L and Shlien R.** (1987) Evidence for function of Ia molecules on gut epithelials in man. *J Exp Med* **166**:1471-1483.

31 **Holmes R and Lobley RW.** (1989) Intestinal brush border revisited. *Gut* **30**:1667-1678.

32 **Mason SD and Perdue MH.** (1990) Changes in distribution of Ia on epithelium of the jejunum and ileum in rats infected with *Nippostrongylus brasiliensis. Clin Immunol Immunopathol* **57**:83-95.

33 **Dahlgren UIH, Wold AE, Hanson LA and Midtvedt T.** (1991) Expression of a dietary protein in *E. coli* renders strongly antigenic to gut lymphoid tissue. *Immunology* **73**:394-397.

34 **McKenzie D.** (1988) Alloantigen presentation by B cells. Requirements for IL-1 and IL-6. *J Immunol* **141**:2907-2911.

35 **Kurt-Jones EA, Hamberg S, Ohara J, Paul WE and Abbas AK.** (1987) Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J Exp Med* **166**:1774-1787.

36 **Mowat AMcI and Parrot DMV.** (1983) Immunological responses to fed protein antigens in mice. IV. Effect of stimulating the reticuloendothelial system on oral tolerance and intestinal immunity to ovalbumin. *Immunology* **50**:547-554.

37 **Strobel S and Fergusson A.** (1986) Modulation of intestinal and systemic immune responses to a fed protein antigen, in mice. *Gut* **27**:829-837.

38 **Zieger RS, Heller S, Mellon M, Forsythe AB, O'Conner R, Hamburger RN and Schatz M.** (1989) Effect of combined maternal and infant food-allergen avoidance on the development of atopy in early infancy: A randomized study. *J Allergy Clin Immunol* **84**:72-89.

39 **Husby S.** (1988) Dietary antigens: uptake and humoral immunity in man. *Acta Pathol Microbiol et Immunologica Scand* **96**:1-40.

40 **Sanderson I and Walker WA.** (1993) Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology* **104**:622-639

41 **Mazanec MB, Nedrud JG, Kaetzel CS and Lamm ME.** (1993) A three-tiered view of the role of IgA in the mucosal defense. *Immunol Today* **14**:430-435.

42 **Burks WA, Williams LW, Thresher W, Connaughton C, Cockrell G and Helm RM.** (1992) Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenge. *J Allergy Clin Immunol* **90**:889-897.

43 **Barnett D, Baldo BA and Howden MEH.** (1983) Multiplicity of allergens in peanuts. *J Allergy Clin Immunol* **72**:61-68.

44 **Torres-Pinedo R.** (1993) Lectins and the intestines. *J Pediatr Gastroenterol Nutrition* **2**:588-594.

45 **Shibasaki M, Sumazaki R, Isoyama S and Takita H.** (1992) Interactions of lectins with human IgE: IgE-binding property and histamine-releasing activity of twelve plant lectins. *Int Arch Allergy Immunol* **98**:18-25.

46 **Nagi AM and Babiuk LA.** (1989) Peanut agglutinin (PNA): binding and stimulation bovine intestinal and peripheral blood leucocytes. *Veterinary Immunol Immunopathol* **22**:67-79.

47 **Yodoi J, Hirashima M and Ishizaka K.** (1982) Regulatory role of IgE-binding facto from rat T lymphocytes. V. The carbohydrate maieties in IgE-potentiating factors ar IgE-suppressive factors. *J Immunol* **128**:289-295.

48 **Huff TF, Jardieu P and Ishizaka K.** (1986) regulatory effects of human IgE-bindir factors on the IgE responses of rat lymphocytes. *J Immunol* **136**:955-962.

49 **Flores-Romo L, Shields J, Humbert Y, Graber P, Aubrey JP, Gauchat JF, Auala G, All B, Chavez M and Bazin H.** (1993) Inhibition of an in vivo antigen-specific IgE respons by antibodies t CD23. *Science* **261**:1038-1041.

50 **van der Heijden FL, van Neerven RJJ, van Katwijk M, Bos JD and Kapsenberg M** (1993) Serum IgE-facilitated antigen presentation in atopic disease. *J Immun* **150**:3643-3650.

51 **Seumura M, Yodoi J, Hirashima M and Ishizaka K.** (1980) Regulatory role of Ig binding factors from rat T lymphocytes. I. Mechanism of enhancement of Ig response by IgE-potentiating factor. *J Immunol* **125**:148-154.

52 **Hirashima M, Yodoi J, and Ishizaka K.** (1980) Regulatory role of IgE-binding facto from rat T lymphocytes. III. IgE-specific suppressivefactor with IgE-binding activity. *Immunol* **125**:142-148.

53 **Keegan AD, Snapper CM, van Dusen R, Paul WE and Conrad DH.** (1989) Superindu tion of the murine B cell FcεRII by T helper cell clones. Role of IL-4. *J Immun* **142**:3868-3874.

54 **Vercelli D, Leung DYM, Jabara HH and Geha RS.** (1989) Interleukin 4 depender induction of IgE synthesis and CD23 expression by supernatants of human helper cell clones. *Int Arch Allergy Appl Immunol* **88**:119-121.

Samenvatting voor niet-ingewijden

Ongewenste effecten van voedingsmiddelbestanddelen komen regelmatig voor en kunnen worden onderverdeeld in toxische reacties, niet-toxische reacties of te wel overgevoeligheidsreacties en aversies. Toxische reacties worden veroorzaakt door een giftige component in het voedingsmiddel. Dit kan een natuurlijk voorkomend gif zijn, danwel een giftige stof ontstaan bij het koken of verwerken van het product, of geproduceerd na besmetting van het voedingsmiddel met een microorganisme. Toxische reacties onderscheiden zich van overgevoeligheids reacties door het feit dat iedere persoon last zal hebben van het in het voedingsmiddel voorkomende gif, mits de dosis hoog genoeg is. Daarentegen komen overgevoeligheidsreacties alleen bij bepaalde personen voor.
Voedselovergevoeligheid is een verzamelnaam voor niet-toxische ongewenste reacties op voedsel. Voedselallergie en voedselintolerantie vallen daar allebij onder. In het geval van een voedselintolerantie wordt de patient ziek van een bepaald voedingsmiddel zonder dat het afweersysteem daarbij een rol speelt. Een voorbeeld van een product waarvoor men een voedselintolerantie kan hebben is aardbei.
Men spreekt van een voedselallergie als het afweersysteem een duidelijke rol speelt in de reactie op voedsel. Redelijk veel mensen denken dat ze een voedselallergie hebben maar in het merendeel van de gevallen zal bij nader onderzoek blijken dat het gaat om een voedselintolerantie, of dat ze het voedingsmiddel gewoon niet lekker vinden, of dat de voeding om andere, veelal psychologische redenen, niet verdragen wordt. In het laaste geval spreekt men van een aversie.
Ongeveer 2% van onze bevolking lijdt aan een voedselallergie. Reacties zijn gericht tegen een onderdeel van het voedingsmiddel, meestal eiwit. De meest voorkomende voedingsmiddelen die eiwitten bevatten waarvoor een voedselallergie wordt waargenomen zijn koemelk, kippe-ei, pinda en soya. Daarnaast leiden ook vruchten en noten regelmatig tot klachten. Koemelk- en kippe-ei-allergie komen voornamelijk voor bij jonge kinderen die er vaak "over heen groeien". Een pinda-allergie daarentegen, lijkt over

op leveren door zeer ernstige reacties na het eten van pinda's door ee pinda-allergische patient. De symptomen van een voedselallergie kunne variëren van het opzwellen van de slijmvliezen van de keel en mondholt huiduitslag, astma, braken en diarree tot systemische anaphylactisch reacties, soms leidend tot shock of zelfs overlijden. Met name het eten vo pinda's door pinda-allergische personen kan, door een nog niet opgehe derde oorzaak, zeer ernstige gevolgen hebben waarbij de dood volgen op zo'n anafylactische shock helaas nog regelmatig voorkomt. Uiteraa zullen pinda-allergische personen het eten van pinda's vermijden ma toch worden ze soms onverwachts geconfronteerd met overgevoeligheid reacties tegen voedingsmiddelen waarin pinda-eiwitten zijn verwerkt zoo koekjes, soepen en sauzen.
Voor meer informatie betreffende overgevoeligheidsreacties kunt u terech bij het Landelijk Informatiecentrum Voedselovergevoeligheid (LIVC Postbus 84185, 2508 AD Den Haag.

In het bloed komen normaal diverse typen antilichamen voor, die gepro duceerd worden door een specifieke groep van witte bloedcellen, d zogenaamde B-lymfocyten of B-cellen. Bij allergische reacties komt ee bepaald type antilichaam, IgE genaamd, in het bloed voor. Zo wordt pi da-allergie gekenmerkt door een verhoogde hoeveelheid IgE gerich tegen pinda-eiwitten in het bloed van de patient. Pinda-specifiek IgE wor met name geproduceerd in de periode waarin de allergie zich ontwikkel Het pinda-specifiek IgE kan vervolgens binden aan gespecialiseerd cellen, de mest-cellen en basophielen, die overal in het lichaam voorko men. Bij een volgend contact met pinda-eiwit zal er binding plaats vinde van het pinda-eiwit aan het IgE. Als gevolg van deze binding kome diverse stoffen vrij uit de mestcellen die (mede) verantwoordelijk zijn voo de symptomen die zich bij allergische personen kunnen voordoenen. D productie van IgE door B-cellen wordt gecontroleerd door een ander groep witte bloedcellen, de T lymfocyten of T cellen. Na herkenning va pinda-eiwit worden de T cellen geactiveerd en zullen producten gepro duceerd worden door deze geactiveerde T cellen die, in het geval va allergie, de IgE-produktie stimuleren.

Het doel van het in dit proefschrift beschreven onderzoek was om mee inzicht te krijgen in het mechanisme van voedselallergie, waarbij pindo allergie als model is gekozen. Daarnaast werd de herkenning van pindo

eiwit door de B en T cellen van het afweersysteem bestudeerd alsmede de daaruit voortvloeiende sturing van de reacties binnen het afweersysteem. De vergaarde kennis kan inzicht geven in de mechanismen die een rol spelen bij het ontstaan van een voedselallergie alsmede de mechanismen verantwoordelijk voor de zeer ernstige reacties op pinda. Dit inzicht kan op den duur bijdragen aan de ontwikkeling van therapieën die de allergie verminderen en voor de verbetering van de diagnostische tests. Ook voor het ontwikkelen van een preventief middel kan de vergaarde kennis gebruikt worden. Juist voor een zo ernstige allergie als pinda-allergie, is het van belang om een goede therapie te ontwikkelen.

Hoofdstuk 1 bevat een algemene inleiding over ongewenste effecten van voedingsmiddelen en in het bijzonder voedselallergie met de daarbij voorkomende immunologische responsen zoals hierboven in het kort beschreven is.
In hoofdstuk 2 staat een studie beschreven die enerzijds als doel had het bestuderen van de symptomen van pinda-allergische patiënten op een kleine hoeveelheid pinda's. Anderzijds werden verschillende diagnostische testmethoden vergeleken. De symptomen na het eten van pinda's kunnen van patient tot patient er verschillen maar ook de individuele patient kan op verschillende tijdstippen anders reageren. Voor het stellen van de diagnose pinda-allergie zijn verschillende testen op de markt. Zoals wij met deze studie aantonen zijn de uitslagen van deze testen niet altijd in overeenstemming met elkaar. Dit wordt mogelijk gedeeltelijk veroorzaakt door het feit dat de testen niet gebruik maken van hetzelfde basis principe hetgeen het vergelijken van de testen moeilijk maakt. Maar ook binnen één soort test komen soms grote verschillen voor welke deels samenhangen met de herkomst van de gebruikte materialen van de verschillende fabrikanten. Met name grote verschillen in de pinda-extracten die voor deze tests gebruikt worden spelen hierbij een rol. Deze extracten zijn vaak slecht gekarakteriseerd en gestandariseerd wat tot gevolg kan hebben dat extracten verschillen in eiwit-samenstelling. De conclusie die uit deze studie getrokken kan worden is dat het voor een correcte diagnose van pinda-allergie nodig kan zijn meerder typen testen te gebruiken. In voorkomende gevallen zou het gebruik van meerdere extracten voor 1 type test waardevol kunnen zijn. Bovendien verdient de standarisatie van pinda-extracten aanbeveling.

Hoofdstuk 3 beschrijft de herkenning van pinda-eiwitten door IgE uit he bloed van 14 pinda-allergische patiënten. Ongeveer 10% van de Ig bevattende bloedmonsters van de patienten herkende bijna alle eiwitte in het door ons gebruikte pinda-eiwit. Zelfs 70% herkende 3 dezelfd eiwitten.

Zoals al eerder is gezegd spelen T cellen een belangrijke rol in d aansturing van B cellen tijdens het ontstaan van IgE-afhankelijke allergieën Deze sturing wordt in een belangrijke mate verzorgt door twee stoffen interleukine-4 (IL-4) en interferon-γ (IFN-γ) die tegengesteld werkingen hebben: IL-4 stimuleert de IgE produktie terwijl IFN deze remt. Het doel van de in hoofdstuk 4 beschreven experimente was om te bestuderen of een overmaat aan IL-4 productie ook b pinda-allergie een rol zou kunnen spelen. Het bleek inderdaad da pinda-specifieke T cellen van een pinda-allergische donor ve IL-4 en bijna geen IFN-γ produceerden. In het bloed van nie pinda-allergische personen konden echter nauwelijks pinda-spec fieke T cellen worden aangetoond waardoor de vergelijking met cellen van niet-allergische personen niet mogelijk was.

De herkenning van pinda-eiwit door pinda-specifieke T celle staat beschreven in hoofdstuk 5. Ook deze herkenning is zee divers, de verschillende T cellen (clonen) herkennen uiteenlo pende eiwitten van pinda.

Contact van B en T cellen met eiwitten die ze specifiek herkenne resulteerd over het algemeen in een versnelde celdeling. D delings-respons op pinda-eiwit van T cellen uit het bloed va allergische maar niet-pinda-allergische personen is gemeten e beschreven in hoofdstuk 6. De verwachting was dat deze cellen nie aanwezig zouden zijn in het bloed van allergische maar niet pinda allergische personen zoals dat het geval is bij niet-allergsich personen. Maar tot onze verbazing, bleek de gemeten respon vergelijkbaar met de response van T cellen van pinda-allergisch patiënten. Alhoewel verdere experimenten zijn uitgevoerd om mee inzicht te krijgen in het mechanisme van deze respons, kunnen w daar helaas nog geen uitspraak over doen. Dit fenomeen is pinda specifiek en is niet waargenomen bij experimenten met ander allergie-veroorzakende stoffen, zoals kippe-ei-eiwit, soya e kattehuidschilferallergeen.

Hoofdstuk 7 vormt een algemene discussie van de gevonden resultaten. In deze discussie wordt beschreven dat de diverse herkenning van pinda-eiwitten door zowel IgE als T cellen, de ontwikkeling van een goede therapie voor pinda-allergie wel eens zou kunnen bemoeilijken. Het onderzoek dat in dit proefschrift beschreven staat, levert een bijdrage aan het inzicht in het mechanisme van pinda-allergie.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 26 februari 1965 te Am
sterdam. Na het behalen van haar VWO-diploma aan het Calscollege
Nieuwegein in juni 1985, ging zij Biomedische Wetenschappen studere
aan de Faculteit Geneeskunde, RijksUniversiteit Leiden. Na een afstudee
stage aan de Universitá degli Studii di Siena in Italië met als titel "Bon
biopsies in culture: a model for humoral hypercalcemia of malignancy
onder begeleiding van Prof. G. Francini en Prof.Dr. G.J. Fleuren, behaald
zij op 26 februari 1991 haar doctoraaldiploma.
Op 1 september 1991 trad de auteur als assistent-in-opleiding in dienst va
de Universiteit Utrecht en werd gedetacheerd bij TNO-Voeding waar h
project "Mechanism of IgE-mediated food allergy" werd uitgevoerd ond
begeleiding van Dr. Th. Ockhuizen (tot 1994) Dr. A.H. Penninks, Dr. M.L. Ka
senberg en Drs. S. Spanhaak. Dit vondt plaats in het kader van het Utrech
Toxicologie Centrum (UTOX). Het UTOX is een samenwerkingsverband va
Universiteit Utrecht (RITOX), Rijksinstitute voor Volksgezondheid en M
lieuhygiëne (RIVM) en TNO-Voeding.
Dit werk heeft geleid tot het proefschrift "Food allergy, human lymphocy
responses to peanut proteins".

List of publications

E.C. de Jong, E.A. Wierenga, S. Spanhaak, B.P.M. MArtens, Th. Ockhuizen (1993). The mechanism of IgE-mediated food allergy. *Voeding* **54**: 31.

E.C. de Jong, S. Spanhaak, B.P.M. Martens, M.L. Kapsenberg, A.H. Penninks, E.A. Wierenga. (1995) Food allergen (peanut)-specific Th2 clones from the peripheral blood of a peanut-allergic patient. *J Allergy Clin Immunol*, In press.

E.C. de Jong, S. Spanhaak, H. Pellegrom, E.A. Wierenga, A.H. Penninks. (1995) Diverse protein specificity of peanut-specific Th2 clones from a peanut-allergic patient. *Submitted for publication.*

E.C. de Jong, S. Spanhaak, B.P.M. Martens, A.H. Penninks. (1995) Comparison of *in vivo* and *in vitro* reactivity to peanut extracts in peanut-allergic patients. *Submitted for publication.*

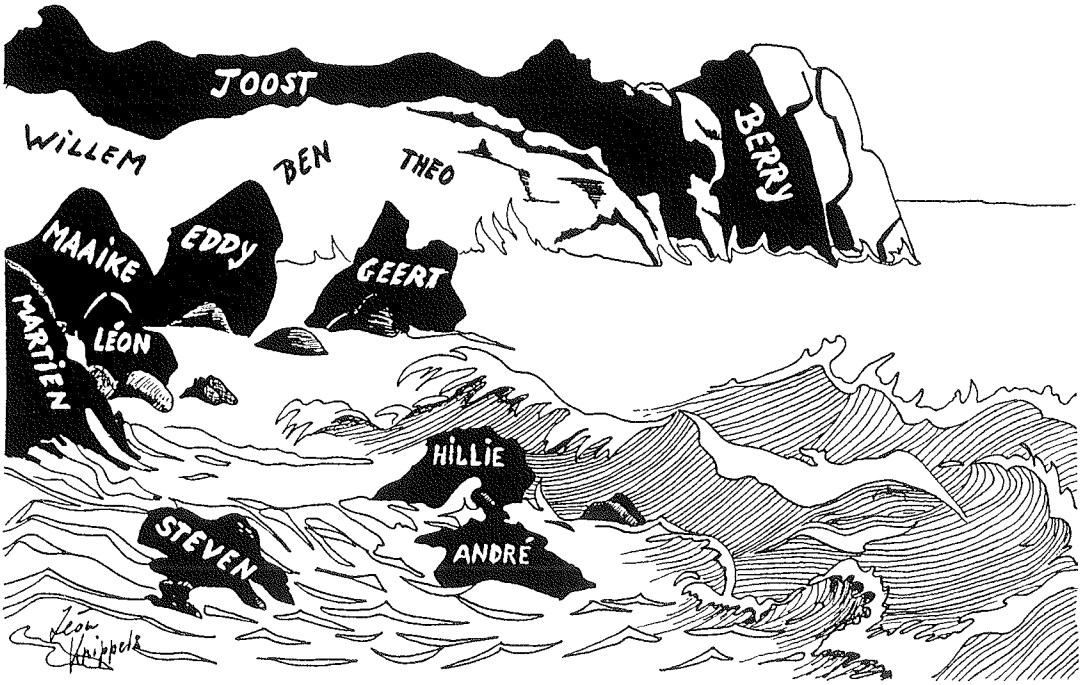
E.C. de Jong, M. van Zijverden, S. Spanhaak, H. Pellegrom, A.H. Penninks. (1995) Identification of multiple major allergens in peanut proteins. *Submitted for publication.*

E.C. de Jong, S. Spanhaak, H. Pellegrom, J.P. Bruyntjes, A.H. Penninks, M.L. Kapsenberg (1995). Enhanced proliferative responses of peripheral blood T cells of allergic donors to a non-relevant allergen, peanut protein. *Manuscript in preparation.*

E.C. de Jong, L.M.J. Knippels, S. Spanhaak, G.F. Houben, A.H. Penninks (1995). Does protein challenge influence human and rat lymphocyte responses? *Manuscript in preparation.*

Several poster/abstracts have been presented at national and international meetings

Mijn pinda-rotsen in de branding



Stellingen

1. Allergeen-specifieke Th2 cellen spelen een dominante rol in de voedselallergie.
   (Dit proefschrift)

2. De herkenning van pinda-eiwitten door zowel B als T lymfocyten van pinda-allergische personen is zeer divers.
   (Dit proefschrift)

3. Het verdient aanbeveling om de extracten gebruikt voor de huid-prik-test beter te karakteriseren en te standaardiseren.
   (Dit proefschrift)

4. De discrepantie tussen de hoeveelheid personen die denken te lijden aan een voedselallergie en het aantal bewezen gevallen, geeft aan dat voorlichting op dit gebied nodig is.

5. Een kleurstofovergevoeligheid die hyperactiviteit veroorzaakt bij kinderen is veelal gebaseerd op een aversie van de ouders.

6. Het aantal op de markt zijnde hypoallergene babyproducten, doet vermoeden dat ieder kind tegenwoordig allergisch ter wereld komt.

7. Van reactief opgeleide artsen kan niet worden verwacht dat zij goede, creatieve onderzoekers zullen zijn. Dit maakt de eis van een wetenschappelijke promotie voor een opleidingsplaats discutabel.

8. Het beursale systeem voor promovendi toont weinig respect voor de wetenschappelijk onderzoeker.

9. Niet voor iedereen geldt: Duyvis als er een fuif is!

10. Promoveren ..... Peanuts?!

Stellingen behorende bij het proefschrift:
Food allergy, Human lymphocyte responses to peanut proteins
Esther C. de Jong, 19 januari 1996