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Diagnosis and treatment of IgE-mediated allergy: new approaches using recombinant allergens

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**Denna avhandling är dedicerad till de fyras gäng,
som var och en är som solen om våren**

Clara

Linn

Lisa

&

Marie

ABSTRACT

More than 10% of the population in industrialized countries suffer from IgE-mediated cat allergy. Allergens produced by recombinant techniques offer new possibilites to diagnose and treat allergic patients but will also help to uncover the mechanisms behind the sensitisation. The aim of this thesis was to investigate the clinical usefulness of a recombinant form of the major cat allergen, Fel d 1, from construction of genes and characterisation to diagnosis of cat allergic patients. Furthermore, tools for treatment were developed using the Fel d 1 structure and the major allergen in timothy, Phl p 5.

Although Fel d 1 was cloned more than a decade ago, attempts to produce a tetrameric allergen by recombinant methods with structural features similar to the natural allergen have been only partially successful. A synthetic gene coding for direct fusion of the two chains of Fel d 1 was constructed. Expression resulted in a 30 kDa non-covalently associated homodimer. Biochemical and biological analysis showed that the overall fold and immunological properties were very similar to those of natural Fel d 1. The recombinant (r)Fel d 1 construct was subsequently used to determine the structure by X-ray crystallography at 1.8 Å resolution. The fold of Fel d 1 presents a striking resemblance to uteroglobin, a molecule with anti-inflammatory and immunomodulatory properties. An internal pocket and the surface localisation of three previously defined Fel d 1 IgE epitopes is presented.

We evaluated the diagnostic usefulness of IgE and IgG_4 antibodies to rFel d 1 in children and adults with doctors' diagnosis of rhinoconjunctivitis (RC) and/or asthma due to cat. All patients showed positive IgE responses to rFel d 1 by ELISA. Sera from children displayed higher IgE levels to rFel d 1 compared to the adult patients. There was a close correlation between IgE responses by CAP to rFel d 1 and the cat dander extract, however the IgE levels to the single rFel d 1 molecule were significantly higher. Among children with asthma, the IgE levels to rFel d 1 were higher in comparison to both the asthmatic adults and children with RC, whereas the IgG_4 levels were elevated in adults with allergic RC compared to adults with asthma.

The only curative treatment of allergic disease is allergy vaccination, however the patients face the risk of side effects. Allergens with decreased IgE-binding capacity, but retained T-cell reactivity (hypoallergens) have been tested in clinical trials. We present a new approach of how to generate hypoallergens using structural information and knowledge of B- and T-cell epitopes. The structure of the model allergen Fel d 1 was systematically altered by duplication of T-cell epitopes and disruption of disulphide bonds. Three derivatives displayed a marked reduction in IgE-binding capacity, induced a lower degree of basophil activation but stimulated T-cell proliferation equally well compared to rFel d 1, and are therefore promising hypoallergen candidates.

The occurrence of side effects caused by the aluminium hydroxide adjuvant (Alum) by allergy vaccination is frequently reported. In a mouse model using the recombinant version of the timothy pollen allergen Phl p 5, we demonstrated that carbohydrate-based particles (CBP) exhibit several potential advantages over aluminium-hydroxide as adjuvant for immunotherapy. CBP covalently bound to rPhl p 5b (CBP-p5) induced a strong antibody response, which cross-reacted with group 5 allergens from other grass species and exhibited characteristics of blocking antibodies. Alum-5b induced a preferential allergen-specific Th2 cytokine profile, whereas CBP-p5 induced a mixed

Th1/Th2 response. CBP-p5 yielded a stable vaccine formulation with preserved immunogenic features and, in contrast to Alum, induced no granulomatous tissue reactions.

In conclusion, the work presented in this thesis presents the development of a clinically relevant allergen, from idea, via biochemical, biological and structural characterization to diagnosis of cat-allergic patients and also the rational design of allergens and development of a novel adjuvant for therapeutic purposes.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numerals:

I Grönlund H, Bergman T, Sandström K, Alvelius G, Reininger R, Verdino P, Hauswirth A, Liderot K, Valent P, Spitzauer S, Keller W, Valenta R and van Hage-Hamsten M. Formation of Disulfide-bonds and Homodimers of the Major Cat Allergen Fel d 1 Equivalent to the Natural Allergen by Expression in *Escherichia coli*. *J Biol Chem. 2003 10;278:40144-51.*

II *Kaiser L, *Grönlund H, *Sandalova T, Ljunggren HG, van Hage-Hamsten M, Achour A, Schneider G. The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobin family. *J Biol Chem. 2003 26;278:37730-5.*

*These authors contributed equally to the work

III Grönlund H, Adedoyin J, Reininger R, Varga E-M, Fredriksson M, Kronqvist M, Szepfalusi Z, Spitzauer S, Grönneberg R, Valenta R, Hedlin G and van Hage-Hamsten M. Antibody responses to Fel d 1 in children with rhinitis and/or asthma to cat (*Felis domesticus)*. *Manuscript*.

IV Saarne T, Kaiser L, Grönlund H, Rasool O, Gafvelin G and van Hage-Hamsten M. (2004) Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin Exp Allergy. In press.*

V Grönlund H, Vrtala S, Wiedermann U, Dekan G, Kraft D, Valenta R, van Hage-Hamsten M. Carbohydrate-based particles: a new adjuvant for allergen-specific immunotherapy. *Immunology, 2002, 107, 523-529.*

CONTENTS

LIST OF ABBREVIATIONS

3D	three-dimensional
ALP	alkaline phosphatase
Alum	aluminium hydroxide
APC	antigen presenting cells
CBP	carbohydrate-based particles
CC10	Clara cell secretory protein
CD	cluster of differentiation
CDE	cat dander extract
cDNA	complementary DNA
CRD	component-resolved diagnostics
Cys	cysteine
DC	dendritic cell
DTE	duplication of T-cell epitope
E. coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FITC	fluorescein isothiocyanate
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IUIS	the International Union of Immunological Societies
MDDC	monocyte derived dendritic cells
MHC	major histocompatibility complex
MPD	2-methyl-2,4-pentanediol
n	natural
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phyco erythrin
r	recombinant
RAST	radio allergosorbent test
RC	rhinoconjunctivitis
r.m.s.d.	root mean square deviation
SEC	size exclusion chromatography
SDS-PAGE	sodium dodecyl sulphate polyacryl-amide gel electrophoresis
SI	stimulation index
S-S	disulphide bond
Th	T helper

INTRODUCTION

ALLERGY IN GENERAL

Allergy is a widespread and well-known hypersensitivity disease[1], affecting more than 20% in societies with a "Western" life style[2, 3]. Typical allergic symptoms are rhinoconjunctivitis (RC), asthma and eczema, caused by common environmental antigens, so-called allergens. Until the mid 20th century only a limited number of individuals suffered from allergic symptoms, but in recent decades a dramatic increase in prevalence has become evident[4, 5]. However, recent reports suggest that this trend has stabilized[5-7].

THE IMMUNE SYSTEM

The immune system has evolved to guard and clear the host from invading pathogens, be they bacteria, viruses, fungi or multi-cellular parasites. As a consequence, one main feature of the immune system is the ability to distinguish self from non-self. In the course of co-evolution many pathogens have also evolved means to circumvent, mimic or hide from the immune system. To cope with the various pathogens and invasion strategies, an intricate interplay of host organs, cells and signalling molecules has been established. The proper balance of this defence system depends on inherited and adopted traits as well as challenges of the commensal micro flora for proper function. When the fine balance of the immune system is disturbed the host is at risk of developing e.g. autoimmune or allergic diseases[8].

In evolutionary terms, the oldest part of the immune system is the so-called innate immunity. This early line of defence mechanisms is triggered immediately by the aggregation of constitutively up-regulated receptors on the surface of, most importantly, the antigen presenting macrophage and dendritic cells (DCs) in response to pathogen-associated molecular patterns, e.g. lipo-polysaccharides, bacterial DNA and certain carbohydrates from microorganisms. The innate system is present at all times but does not increase upon repeated exposure of a given pathogen. A very important function of the innate immune system is to send danger signals to the other important part, the adaptive immune system. This event will initiate a pathogen-specific learning (memory) process by the release of chemokines and cytokines, up-regulation of co-stimulatory molecules and display of processed antigens[8].

The adaptive immune system, a complex education system, activates lymphocyte populations to enable targeting of infections with high specificity. Antigen processed by antigen presenting cells (APCs) is presented to naïve T cells in secondary lymphoid tissues resulting in primed cytotoxic and T helper cells (Th). Recently, regulatory T cells have been rediscovered. These cells are thought to down-regulate T cell responses in a general or antigen-specific manner. Interactions between differentiated Th cells and B cells results in plasma cells producing five distinct classes of immunoglobulins: IgM, IgG, IgA, IgE and IgD. In contrast to the innate immunity, the adaptive immune system exhibits a capacity to amplify and refine antigen-specific responses as well as to keep long-lived memory cells for future use[8].

BASIC CONCEPTS OF IGE-MEDIATED ALLERGY

Hallmarks of human allergic inflammation are the allergen-dependent IgE activation[9] of mast cells and basophils[10] and tissue eosinophilia[11] in which cytokines play a major role[12]. In allergic individuals, allergen-specific IgE antibodies can be detected during the first few years after birth[13, 14]. These antibodies seem to be induced by early, postnatal allergen contact at mucosal surfaces[15, 16].

In a cascade of events, defining the sensitisation phase of the allergic reaction, antigens first enter through the mucosa where they are internalised by APCs, notably immature (i)DCs[17]. The antigens are further processed by the DC and presented as peptides on the cell surface, bound to the antigen cleft of major histocompatibility complex class II molecules (MHC II). The DC, now transformed into an efficient and mature antigen presenter may home to draining lymph nodes where the antigen is exposed to naïve $CD4^+$ Th cells. In direct cell contact, naïve $CD4^+$ Th cells with T cell receptors complementary to the MHC II-peptide complex will be activated and allowed to clonally expand. In cases when this event is not properly regulated these T cells may become allergy-inducing, i.e. differentiate into IL-4, IL-5 and IL-13 secreting Th2 cells[18, 19] (Fig. 1).

In a second line of events, soluble antigens encounter a second type of APC, the antibody-producing B cell carrying the B cell receptor (BCR). Antigens, which specifically bind to the antigen-binding site of the BCR, are internalised, processed and displayed on MHC II molecules on the cell surface. Upon encounter of an antigen-specific Th cell in the germinal centers of the regional lymph nodes the B cells differentiate and expand. Class-switching to IgE production occurs upon a second

encounter with antigen when B cells and Th2 cells interact in the presence of IL-4 and IL-13 (Fig. 1).

In the effector phase of the allergic reaction, soluble IgE antibodies subsequently bind to the high affinity IgE receptor on basophils and mast cells[20]. Upon encountering the corresponding allergen, IgE antibodies are cross-linked, thereby initiating an immediate degranulation of mast cells and release of pre-formed, granulae-stored mediators[8]. Typical IgE-mediated early phase allergic symptoms are itching, oedema, mucous secretion and bronchoconstriction and reactions like oedema and bronchoconstriction may also be experienced in a late phase, occurring 6-8 hours after the initial allergen challenge[21]. The late phase reaction consists of a selective recruitment of inflammatory leucocytes, including neutrophils, basophils, eosinophils, macrophages and T lymphocytes to the site of inflammation[21] (Fig. 1).

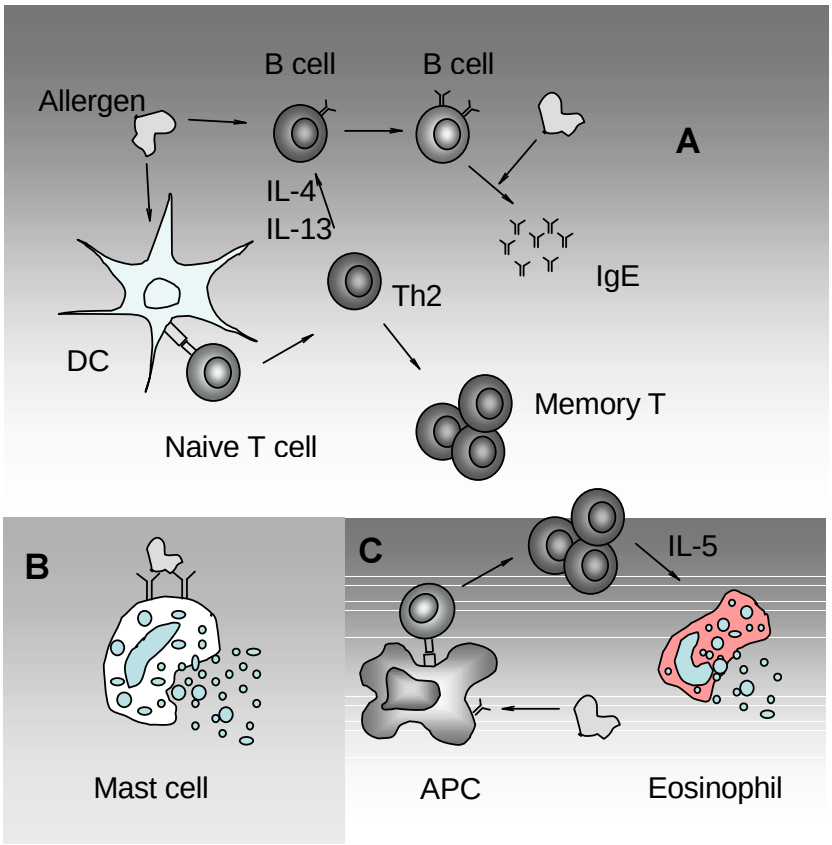


Figure 1. ***A*, Allergen sensitisation.** Mucosal DC:s take up antigen for presentation to naïve T cells, which may develop into allergy promoting Th2 cells and memory T cells. Th2 cells in contact with an allergen-presenting B cells and in the presence of IL-4 and IL-13 may induce a swich to IgE production. ***B*, The immediate allergic reaction**. Granulae-stored bioactive molecules are released upon allergen cross-linking of IgE bound to the surface of mast cells. ***C*, The late allergic reaction**, is caused by allergen presentation and activation of T cells to release proinflammatory cytokines. DC, dendritic cell; APC, antigen-presenting cell; IL, interleukin

Several hypotheses have been proposed to explain the development and pathomechanisms of allergy. It is evident that the symptoms result from a complex interplay of hereditary and environmental factors. Important factors in the sensitisation process are the amount of allergen and the site of exposure. In the latter case allergens may be adsorbed through the mucosa in the upper or lower airways, via the gut or through the skin barrier, causing sensitisation and subsequent local or systemic allergic reactions. In addition, in recent years environmental factors have been proposed to play an important role in the sensitisation process. The "hygiene hypothesis" attempts to explain the increase in prevalence of allergic disease that has been observed in "westernised" societies by investigating basic immunological mechanisms and comparing different lifestyles[22-25]. By this model, exposure to commensal micro-organisms during the first two years of life has been suggested to play a pivotal regulatory role[24, 26, 27]. In the context of allergen sensitisation, other environmental exposures such as exposure to cigarette smoke[28] or diesel exhaust particles[29] have also been discussed.

The genetic components in allergic diseases are well established by epidemiological and family studies[30, 31]. Genomic regions of implication for allergy and asthma have been linked to several chromosomes, namely 1, 2, 5, 6, 7, 11, 12, 13, 14[32-34]. Particular chromosome 5, where important cytokine genes have been found, such as IL-4, IL-5, IL-9, IL-12 and IL-13[35-37] but also G-protein-coupled receptor in chromosome 7[34, 38] and the high affinity IgE receptor located on chromosome 11[39, 40] have been studied.

ALLERGENS

The term "allergen source" defines the biological origin of allergens whereas an "allergen extract" represents a mixture of allergenic and non-allergenic molecules obtained by aqueous extraction of the allergenic source. An "allergen" is the molecular entity that is capable of binding IgE antibodies and eliciting an allergic reaction[41]. When more than 50% of patients in a population selected on the basis of sensitisation to an allergen source are sensitised to a particular allergen component, this component is called "major" allergen[42]. Allergens with less frequent IgE reactivity are minor allergens. The number of allergens varies from source to source. For example, the mould *Aspergillus fumigatus* contains more than 80 characterized allergens[43] whereas some sources like bee venom, birch pollen, cat dander and fish contain less than a dozen.

To keep track of allergens listed, International Union of Immunological Societies (IUIS) sponsor a catalogue containing more than 470 allergens with biochemical and clinical information included (www.allergen.org). An allergen is designated by the first three letters in the Latin genus name followed by one letter derived from the species name and a number given in order of appearance. Thus, the first allergen discovered in cat, (*Felis domesticus*), is termed Fel d 1[44].

Standardisation of allergen extracts

In most cases, allergens of natural origin are difficult, if not impossible, to completely standardise because time and mode of harvest[45], growth and isolation conditions[46, 47], contaminating allergen sources[48] and extraction procedures[49] will, in an unpredictable manner, influence the content of individual allergens and other molecules in the allergen extracts.

Biology and structure

An allergen is typically a 10-80 kDa protein or glycoprotein. The allergen structure is recognised by IgE antibodies via B cell epitopes and this binding is a key event for subsequent triggering of allergic symptoms. No apparent structural or molecular motifs have so far been found that can predict the IgE-binding nature of an allergen[50, 51] despite reports of dominant IgE epitopes[52, 53]. Information for recognition is also delivered to the immune system via short polypeptide stretches of sequestered allergen, T cell epitopes. T cells recognise APC-processed allergen peptides that can be scattered over the entire primary structure of the allergen[54-56]. Rather than just being associated with T- and B cell epitopes, the biological properties of the allergen themselves, such as proteolytic activity, have been suggested as contributing to allergenicity[57, 58].

Different allergen sources may contain evolutionarily conserved allergens, i.e. proteins with fold and surface topology largely maintained[59-63]. As a consequence, IgE antibodies may cross-react with allergens to which the patient was not primarily sensitised[64]. An example of this phenomenon is the birch pollen related syndromes[65-67], in which symptoms most often are associated with the major allergen, Bet v 1. Other allergens with similar cross-reacting behaviour are found in plants and food, e.g. profilin[68] and lipid transfer proteins[69] or in animal dander, the lipocalin family of proteins[70-72].

CAT DANDER ALLERGENS

Aqueous extracts of cat dander are used for both diagnosis and immunotherapy of allergic patients. Attempts to characterise the allergenic content have so far indicated that less than 10 components may bind IgE[73, 74], however of these only four are well documented.

Fel d 1

The most important and potent of the allergens derived from cat, Fel d 1, (originally termed Cat 1)[75], affects more than 80% of the cat-allergic patients[76, 77]. The dominance of Fel d 1 is emphasised by the fact that more than 60% of all IgE antibodies induced by cat dander are directed to this particular allergen[78]. Fel d 1, found in the saliva, skin and lacrimal glands of the cat[79-85], is a 35-39 kDaa acidic glycoprotein containing 10-20% N-linked carbohydrates[86, 87]. It is formed by two 18 kDa non-covalently linked hetero-dimers[86], each consisting of two anti-parallel 8 and 10 kDa peptides[88, 89] (chain 1 and chain 2, respectively) linked by 3 inter-chain disulphide bonds[87]. Fel d 1 was cloned in 1991[89] and the genes coding for the two polypeptide chains were mapped to different chromosomes[90]. Several isoforms of Fel d 1 have been described[87]. Over the years, Fel d 1 has been extensively characterized both by biochemical and immunological methods such as amino acid sequencing[86], epitope mapping by monoclonal antibodies[91-94], serological measurements of patients' IgE antibodies[77] as well as analysis of T-cell epitope repertoires[54, 95].

Other cat allergens

Although a dominating protein in cat dander extract (CDE), albumin or Fel d 2, a 43 kDaa acidic protein is a minor cat allergen[96-98] which reacts with IgE from 20-35% of cat-sensitised subjects[77]. Despite a relatively low level of sensitisation, the importance of Fel d 2 may lie in the fact that IgE antibodies raised against cat albumin are highly cross-reactive with albumin from other mammals[97].

Cystatin, or Fel d 3 is a minor cat allergen recognised by at least 10% of patients[99, 100]. This cysteine protease inhibitor belonging to the stefin protein family, like cat albumin, is evolutionarily conserved and may therefore contribute to cross-species sensitisation.

The latest addition to the list of cat allergens is the cross-reactive, approximately 20 kDaa lipocalin allergen Fel d 4. IgE responses to cat lipocalin are reported in 63% of cat allergic individuals although typically at low levels[101].

RECOMBINANT ALLERGENS

The complex nature of allergen extracts, containing molecules with unknown biological function and in many cases low content of allergens, has sparked the interest to produce recombinant allergens for investigation of biological pathomechanisms as well as for structural, diagnostic and therapeutic studies.

DNA technology

A wide range of recombinant techniques have been introduced over the years to enable isolation, cloning of cDNA encoding allergens and subsequent expression[72, 102-104]. The technique also allows for engineering desirable properties by construction of synthetic genes coding for allergens using overlapping oligonucleotides and polymerase chain reaction (PCR)[105]. One common feature of recombinant proteins is the addition of affinity tags used for protein purification, the most commonly used tag being 6 histidines[106, 107]. This tag promotes binding to immobilized Ni^{2+}, also under the harsh denaturing conditions used to dissolve inclusion bodies. This latter step requires a refolding process for the allergen to assume the structure and properties of the natural protein.

Biochemistry and production

The most commonly used expression system, for reasons of simplicity, time and cost is *E. coli*, although eukaryotic expression systems, such as the baculovirus system used for production in insect cells[108] or the yeast *Pichia pastoris*[109], have been tried as alternatives. Proteins expressed in *Escherichia coli* (*E. coli*) do not contain carbohydrates[110]. However, this lack may for several reasons be advantageous. Carbohydrate-containing proteins are known to be more difficult to crystallize for structural studies by X-ray crystallography. In addition, in the context of diagnosis of cat allergic patients, carbohydrate deficient recombinant (r)Fel d 1 may have two advantageous implications. First of all, the sugar moiety on chain 2 does not seem to influence the structure of Fel d 1 or react with IgE antibodies from cat allergic patients[88] and secondly, carbohydrate moieties expressed by an exogenous expression

system has been suggested to be involved in an increased frequency of non-allergen related serum IgG responses[111].

Cloned allergens

In the last 15 years most of the clinically important allergens have been cloned and produced in large quantities as functional recombinant molecules. As a consequence, it has become possible to reconstruct almost complete repertoires of the most relevant allergens and their epitopes[41, 112]. The first allergen to be cloned was the major mite allergen Der p 1 in 1988[103], soon followed by the major allergens in birch, Bet v 1[113], cat, Fel d 1[89] and timothy, Phl p 5[114]. Despite the fact that the two chains of Fel d 1 were cloned more than a decade ago, attempts to refold the separate chains into a native-like allergen were only partially successful[88, 115, 116].

Among grass pollen allergens in *Phleum pratense* extract[117], the major allergen Phl p 5[114, 118] reacts with IgE antibodies in serum from about 80 % of grass-pollen allergic patients[114, 119]. Proteins from the plant kingdom, like Phl p 5, contain structurally similar carbohydrates that may induce cross-reactive IgE antibodies in allergic patients. However, antibodies to these structures, named cross-reactive-carbohydrate determinants (CCD), may not be clinically important because CCD-reactive IgE antibodies seem less prone to activate mast cells and basophils than the protein structure itself[120].

ALLERGY TO CAT

Allergens derived from cat are a common cause of IgE-mediated allergic diseases in Europe and elsewhere [121-123], emphasised in several cohort studies[124-126]. About 10% of the population is being sensitized to cat[121, 126-128] and allergy to cat is suggested as the primary cause of childhood asthma in Sweden[129].

Allergy avoidance is considered the primary intervention for allergic patients[130]. This may not be feasible because cat allergens are very difficult to avoid[131-134]. Several studies have analysed cat allergens, notably Fel d 1, in dust samples and have correlated the clinical outcome of cat sensitisation[135-138].

In general, the role of natural IgG antibodies in the pathogenesis of allergic diseases is not clear. It has been postulated that a small portion of IgG antibodies may have anaphylactic properties since IgG_1 and IgG_3 but not IgG_4 may induce degranulation of

eosinophils via the FcεRII receptor[139]. Accordingly, high levels of allergen-specific IgG_4 antibodies have been associated with the induction of tolerance to cat[137].

DIAGNOSIS OF ALLERGY

In the majority of cases allergy is not life threatening, but the symptoms may be severe and reduce quality of life[140]. However, for a sub-set of patients with allergy to foods, drugs and insect stings, an allergen challenge may trigger an anaphylactic reaction, which in the worst case may prove fatal[140, 141]. It is therefore important to clarify the allergen source causing the allergic symptoms. Among several methods used to analyse allergen sensitivity[142-145], skin prick test (SPT) and allergen-specific serum IgE measurements are the most widely used in the allergy clinics. By SPT, a drop of an allergen extract is applied to the surface of the forearm, the skin is slightly punctured by a lancet and the area of the resulting weal is calculated and compared to a histamine positive control. The result can be recorded while the patient is still in the out patient clinic[146, 147].

In vitro diagnostics

Almost 40 years ago the discovery of immunoglobulin E initiated development of *in vitro* allergy diagnostic tests as well as a rapid progress in the field of allergology in general[148, 149]. Of particular importance, anti-IgE(ND) antibodies and crude allergen extracts conjugated to cellulose discs were used to develop the RAST technology, which allowed measurement of IgE antibodies in the serum of allergic patients[149]. Although modern quantitative *in vitro* allergy diagnostic systems, such as the World Health Organisation (WHO)/IgE reference calibrated CAP System®[150, 151] are particularly suitable to detect IgE antibodies to allergen sources, the analysis may be compromised. Non-allergen compounds in the extracts will compete for binding to the solid phase with the natural allergens, which can decrease the sensitivity of the test. Furthermore, interpretation of the IgE responses may be complex because the extract may contain cross-reactive allergens[60-62] or contaminants from other allergens sources[48].

Component-resolved diagnostics

In an effort to improve extract-based diagnostics, single recombinant allergens have been introduced[152, 153]. In cases when the content of certain important allergens are too

low, single allergens have been used to spike natural extracts for appropriate diagnostics in sera from sensitised patients[154]. More importantly, it has been demonstrated that cocktails of recombinant allergens can be assembled to match the complexity of the most important allergens in natural extracts[41] and that the single allergens may add important clinical information[155]. For example, it has been shown that IgE antibodies to rAsp f 4 and 6 were found exclusively in sera from cystic fibrosis patients with the allergic bronchopulmonary aspergillosis syndrome but were not detected in patients with asthma to *Aspergillus*[156, 157]. Importantly, component-resolved diagnostics, as demonstrated in allergen micro-array detection[158-160], will allow the precise selection of those molecules for specific immunotherapy to which the patient is actually sensitized[155].

ALLERGY VACCINATION

The only treatment of allergic diseases, introduced almost a century ago[161], that leads to long-lasting relief of symptoms is allergen-specific immunotherapy. The therapy is often recommended[130], but nevertheless the current method is time consuming and should be performed by trained personnel because of the risk for local[162, 163] or systemic reactions, in rare cases even with fatal outcome[130, 164]. Therefore there is a demand from allergic patients, and also for socio-economic reasons[165], to improve the current treatment protocols[130, 166].

Safety and efficacy

Although the clinical efficacy varies between allergen extracts, specific immunotherapy is generally considered safe and effective[167-169]. The treatment is commonly based on injections of low doses of allergenic extracts, comprising one or two weekly administrations in an up-dosing period, followed by maintenance doses every 4 to 8 weeks over 3-5 years[168]. The occurrence of severe anaphylactic side-effects has prompted development of new and safer vaccine formulations. More than 60 years ago allergen extracts adsorbed to aluminium hydroxide (Alum) particles were introduced, with the properties to simultaneously act as an allergen depot and immune stimulus for improved safety and efficacy[170, 171]. Alum is still today the most common adjuvant for human use, despite its Th2-promoting effect and granuloma-forming capacity.

The prevailing dogma state that high dose by allergen challenge is associated with successful immunotherapy[172, 173] while low dose is ineffective[172, 174]. A high dose may

on the other hand induce a high and unacceptable rate of systemic reactions[130]. In the context of safety, the properties of the allergens have also been considered. Reduction of anaphylactic activity may be accomplished by chemical cross-linking of extract allergens leading to diminished IgE-binding capacity, while retaining the immunogenic properties[175, 176]. Another concern is the finding that by natural extract (vaccine)[130] immunotherapy there is a risk of inducing allergen-specific IgE antibodies to which the patient was not previously sensitised[177-180].

Allergen formulations

Recent progress in biotechnology has brought new perspectives to allergy vaccination[112, 181-183]. Peptide chemistry and recombinant technology have allowed conversion of allergens into a variety of derivatives[184], among them allergen isoforms[185, 186], oligomers[187-189], fragments[190, 191], point mutations[185, 192, 193] and peptides[194-196], to be tested as safer and more effective candidates for treatment. So far, approaches have often been a result of empirical testing of hypoallergenic molecules[187]. However, with accumulated knowledge of for example three-dimensional (3D) structure, dominating T cell epitopes and major IgE binding structures, rational design of hypoallergens is now feasible. More recently, several new Th1-promoting adjuvants have been evaluated (e.g. liposomes[197], CpG DNA[197-199] and PLGA microspheres[200]) in both animal models and humans.

Mechanisms

The immunological mechanisms responsible for a successful outcome by allergy vaccination are still obscure and controversial[201]. The suggestion that T cells play a major role in allergic diseases is supported by several clinical trials with T cell peptide treatment[194, 202-204]. However, the assumption that successful immunotherapy may be associated with a shift in IL-4/IFN-γ production either as a consequence of down regulation of Th2 responses or shift to Th1 responses[205, 206] has been questioned[137, 207]. Lately, T regulatory cells have also attracted attention as possible target cells for therapeutic intervention[208-211]. Another mechanism by which immunotherapy has been suggested to act is via blocking of IgG antibodies[212], either by blocking IgE-dependent activation of mast cells[213] or via competition of IgE-facilitated allergen presentation by APC[214, 215].

AIMS OF THE THESIS

The objective of this thesis was to demonstrate the usefulness of recombinant allergens, from cloning and allergen characterisation to diagnostics and potential therapeutic applications.

The specific aims of the individual papers were:

I. To produce a recombinant version of the major cat allergen, Fel d 1, with the biochemical, immunological and biological properties of the natural allergen.

II. To determine the 3D structure of the rFel d 1 described in paper I and to localise B cell epitopes.

III. To investigate the diagnostic potential of rFel d 1 (described in paper I and II) among cat allergic children from Sweden and Austria in comparison with a commercial test based on cat dander extract.

IV. To evaluate a novel approach for rational design of hypoallergens for immunotherapy, based on information from the 3D structure and T and B-cell epitopes, using rFel d 1 (paper I and II) as a model allergen.

V. To produce and to test in mice a novel adjuvant for potential use in immunotherapy of IgE-mediated allergic diseases.

MATERIALS AND METHODS

In this section an overview of the methods used in this thesis is presented. A more detailed description of the methods is given in the individual papers.

SUBJECTS

[**I**], [**III**], [**IV**], [**V**] Sera from a total of 154 patients, either with doctors' diagnosis of allergy to cat or timothy or subjects sensitized to cat were included. [**I**], [**III**] In addition, sera from 95 healthy subjects or non-cat sensitised individuals were collected for control of experimental conditions or determination of cut-off values. [**I**], [**III**] Heparinized blood samples from 11 patients and 2 control subjects were prepared for T cell and basophil experiments.

IMMUNISATION OF MICE

[**V**] Six groups, each consisting of eight female BALB/c mice were immunised in the neck with 5μg of the allergen rPhl p 5b (p5) and the adjuvants CBP or Alum in 100 μl: CBP-p5, Alum-p5, CBP+p5, p5 alone, Alum alone and CBP alone. Immunizations were given and blood taken on days 0, 23, and 52.

CONSTRUCTION OF RECOMBINANT FEL D 1 AND DERIVATIVES

[**I**] Engineering of the genes coding for chain 1, chain 2 and the joined chains [**IV**] and duplication of T cell epitopes (DTE) constructions were accomplished by using overlapping primers and amplification by PCR. Disruption of disulphide bonds by site directed mutagenesis was achieved by point mutations of the codons coding for cysteine. The PCR fragments were blunt ligated into pT7 Blue vector, nucleotide sequences confirmed, cloned into the pET 20b vector using the restriction endonucleases Nde 1 and Xho 1, sequenced a second time and transformed into *E. coli* strain BL-21(DE3)pLysS. Expression of rFel d 1, the separate chains and the hypoallergenic derivatives were induced by isopropyl thiogalactoside in the presence of ampicillin and chloramphenicol.

PROTEIN PURIFICATION

[**I**], [**II**], [**III**], [**IV**] Recombinant Fel d 1 was expressed as a fusion protein with a C-terminal 6 histidine tag for Ni^{2+} chelate affinity chromatography. In a first step, rFel d 1 and derivatives thereof were isolated from bacterial lysates as inclusion bodies, followed by solubilization in guanidine and simultaneous purification and refolding on a Ni^{2+} chelate column. Further purification involved size exclusion and [**II**] anion exchange chromatography.

BIOCHEMICAL ANALYSIS

[**I**], [**IV**] Protein concentration was established by the BCA protein assay and by total amino acid composition analysis. Purity and molecular size of antigen preparations were demonstrated by sodium dodecyl sulphate poly-acryl-amide gel electrophoresis (SDS-PAGE) using 15% homogenous gels and [**I**] molecular mass was analysed by electro spray mass spectrometry. [**I**] Disulphide bond formation and localisation in rFel d 1 was demonstrated by a combination of deconvoluted electrospray and electrospray ionization (ESI) mass spectrometry after tryptic digestion. Circular dichroism was used to compare the secondary structures of natural (n)Fel d 1 and rFel d 1. The homodimer formation of rFel d 1 was analysed by size exclusion chromatography (SEC) and surface plasmon resonance using a BIAcore instrument.

ADJUVANT PREPARATION

[**V**] Spherical cyanogen-bromide activated agarose particles with a mean diameter of 2.1 μm, were incubated with rPhl p 5b allergen by end over end mixing and thoroughly washed. Alum adsorbates were prepared by mixing rPhl p 5b and Alum particles.

CRYSTALLISATION

[**II**] Conditions optimal for crystal growth were obtained by systematic testing of different parameters such as temperature, buffer, concentration, detergents and other additives by hanging drop vapour diffusion technique. Crystals were soaked in cryoprotectant before flash-drying in a cold nitrogen stream. X-ray diffraction data from wild-type rFel d 1 were collected at MAX-II, Lund and data for Seleno methionine substituted rFel d 1 were retrieved at ESRF, Grenoble.

IMMUNOBLOTTING

[**III**] Samples were separated by SDS-PAGE and electrophoretically transferred to Immobiline Transfer Membranes. Strips with separated allergens were blocked, incubated with patient serum followed by rabbit anti-IgE antibodies, alkaline phosphatase (ALP) anti-rabbit conjugate and visualised by addition of substrate.

HISTOPATHOLOGY

[**V**] Skin was excised exactly from the injection areas, cut into 4 mm thick strips, fixed in 7.5% formalin and embedded in paraffin. Five μm sections were stained with hematoxylin-eosin or Giemsa and analysed by microscopy.

ELISA

[**I**], [**III**], [**V**] Enzyme-linked immunosorbent assay (ELISA) detection of allergen-specific antibodies was performed either by a direct method (plate-bound allergen followed by serum sample, detection antibody, ALP-conjugate and substrate) or by [**I**], [I**V**] competition ELISA including an initial pre-incubation of a serial dilution of antigen mixed with a diluted serum sample. Subsequent steps followed the method described for the direct ELISA. [**V**] A variant of competition ELISA was performed in which the capacity of pooled sera from allergen-immunised mice to block subsequent binding of human IgE from timothy allergic patients was analysed.

[**V**] Measurement of the cytokines IL-4, IL-5 and IFN-γ after timothy grass pollen extract challenge in cell cultures of spleenocytes from mice immunized with different rPhl p 5 and control preparations was performed by ELISA according to the manufacturer's instruction.

CAP ANALYSIS

[III] Recombinant Fel d 1 was covalently conjugated to the solid phase ImmunoCAP (Miab, Uppsala, Sweden) and IgG_4 and IgE responses in cat allergic patients were compared to the available cat dander extract (CDE) ImmunoCAP.

BASOPHIL ACTIVATION

[**I**], [**IV**] The basophil surface and activation marker 203c[216] and the basophil granulae marker CD63[217] were assayed to determine basophil activation and degranulation. Blood from cat allergic patients were incubated with serial dilutions of rFel d 1 or

derivatives thereof, anti-IgE and PBS/medium. Washed cells were incubated with phycoerythrin-labelled anti-CD203c and [**IV**] fluorescein-conjugated anti-CD63 monoclonal antibodies. The erythrocytes were lysed and the basophils analysed by two colour flow cytometry.

LYMPHOPROLIFERATION

[**I**], [**IV**] Allergen-induced proliferation of peripheral blood mononuclear cells (PBMC) isolated from cat-allergic patients was analysed by [^{3}H]thymidine incorporation and stimulation indices calculated by the quotient of the counts per minute in allergen stimulated and unstimulated control cell cultures.

RESULTS AND DISCUSSION

RECOMBINANT FEL D 1 PREPARATION AND CHARACTERIZATION [I], [II]

[I] Following cloning in the early 1990s, several attempts to refold the separate chains of Fel d 1 were reported, with limited success however[88, 115, 116]. Because of the dominating role in patients allergic to cat, there was a need for a well-characterised and functional rFel d 1 preparation for diagnosis and immunotherapy. In addition, a soluble and stable rFel d 1 could form a basis for structural studies as well as studies of pathomechanisms in the sensitisation process. Based on several assumptions, we hypothesised that it would be possible to construct an rFel d 1 molecule with biochemical and immunological properties similar to the native molecule. The suggestion that the polypeptide chains of Fel d 1 were oriented in an anti-parallel fashion[88], that additional amino acids should be avoided because of possible unwanted diagnostic or therapeutic reactions and that the three disulphide bonds in Fel d 1 must be correctly formed, guided the construct of directly linked rFel d 1 fusion proteins (Fig 2).

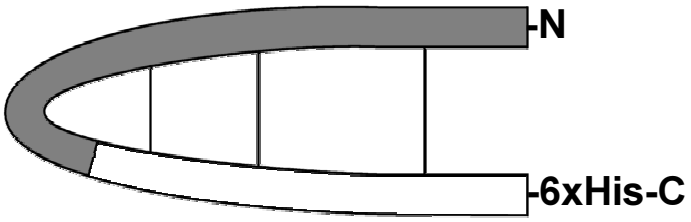


Figure 2. The rFel d 1(2+1) construct. Chain 2 (grey) is directly linked to the N-terminal of chain 1 (white) followed by a 6Histidine-tag.

In addition, the choice of expression system needed consideration. Although nFel d 1 itself is a glycoprotein[87], an expression system allowing post-translational additions of carbohydrates may for several reasons not be suitable. It was earlier shown that the N-linked carbohydrates in nFel d 1 did not seem to bind IgE or influence the structure of the molecule[218]. In contrast, proteins derived from a system expressing exogenous carbohydrate moieties may in fact recognise antibodies to the carbohydrates, as suggested by antibody responses to rFel d 1 in cat allergic patients[111]. In addition to clinical considerations[219], we used the prokaryotic *E. coli* expression system for

production of rFel d 1 because it yields high quantities of protein and it is inexpensive, fast and suitable for down-stream therapeutic applications[110].

In order to increase the likelihood of successful refolding, two constructs of rFel d 1 were produced, rFel d 1 (2+1) and (1+2), the latter with N-terminal chain 1 linked to C-terminal chain 2. Initial experiments revealed that the 2+1 construct exhibited greater capacity to inhibit IgE binding from cat allergic patients and therefore the 1+2 construct was initially not evaluated further.

After purification by Ni^{2+} chelate affinity and calibrated SEC, >95% pure 30 and 51 kDaa fractions of rFel d 1 were isolated. The 51 kDa fraction comprised inter-molecular disulphide bonds as demonstrated by SDS-PAGE under reducing and non-reducing conditions and was not further studied.

Next, we investigated whether rFel d 1 comprised non-covalently linked homo-dimers as was reported for the 36 kDa nFel d 1[220]. The similarity in quaternary structure between r- and nFel d 1 was supported by several experiments. Under denaturing conditions by SEC and by non-reducing SDS-PAGE, the 30 kDa rFel d 1 fraction from SEC exhibited a molecular size of about 15 kDa. Covalent binding of rFel d 1(2+1) to the sensor chip of BIAcore revealed a 53% reduction of bound protein and the dissociation constant was calculated to be $8.74\ 10^{-4}\ s^{-1}$, whereas a monomer control protein displayed stable binding to the surface. The apparent difference in molecular size (30 versus 36 kDa by SEC) between r- and nFel d 1 may be explained by the fact that nFel d 1 also contains 10-20 % carbohydrates[87, 92, 220].

The disulphide bonds in rFel d 1, previously determined for nFel d 1 at the sites ^{3}Cys(1)-^{73}Cys(2), ^{44}Cys(1)-^{48}Cys(2), ^{70}Cys(1)-^{7}Cys(2)[87], were analysed by mass spectrometry. First, experiments were performed to find out if free, un-paired cysteines could be found. Several experiments demonstrated that no free sulfhydryl groups were present, (1) by alkylation of non-reduced rFel d 1 using iodoacetamide, (2) by mass spectrometry showing a difference of 6 Da when comparing expected and experimental molecular masses of rFel d 1 (19 183 vs 19 177 Da, respectively) and (3) only SS-conjugated fragments were found after tryptic digestion of non-reduced rFel d 1. The data from the nano-ESI mass spectrometry was furthermore used to demonstrate the precise location of the disulfide bonds in rFel d 1, which were shown to be identical to those previously reported for nFel d 1[87].

The secondary structure of rFel d 1 was compared to that of nFel d 1 by circular dichroism spectroscopy. The spectra were essentially identical with two minima at 208

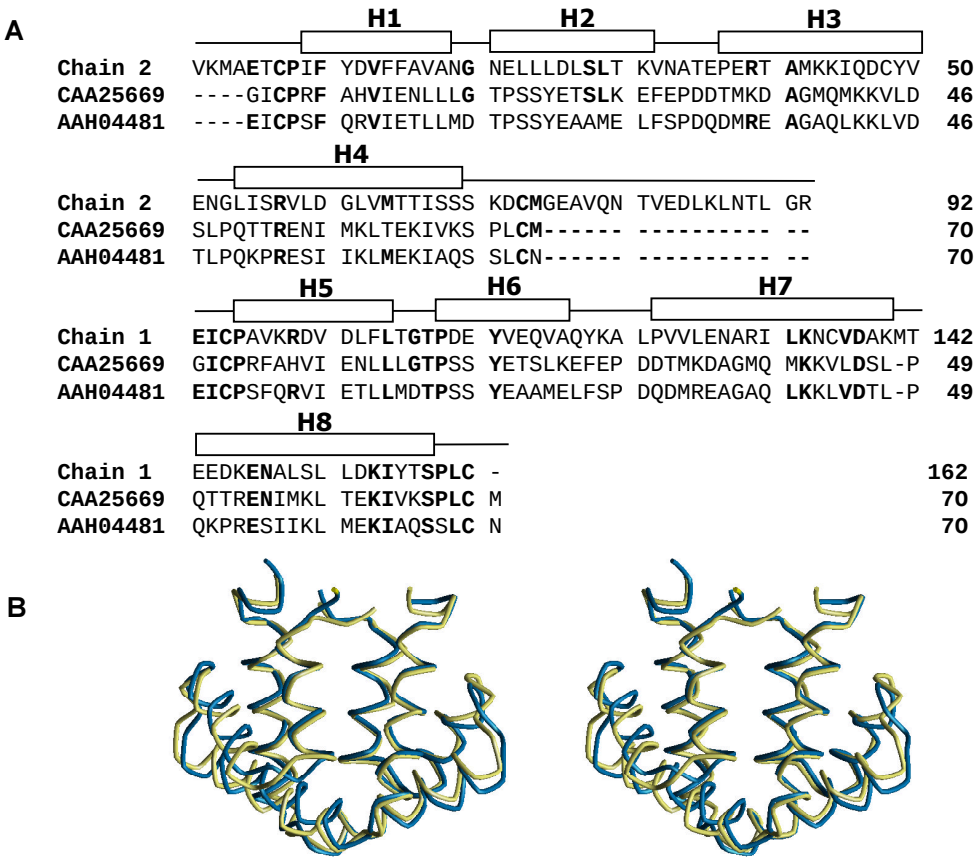
nm and 222 nm and a characteristic maximum at about 195 nm. The shape of the spectrum was indicative of a well-folded protein with significant α-helical secondary structure content.

[II] The primary structures of the two chains constituting Fel d 1 were revealed more than a decade ago[89], however no information about the tertiary structure was presented. Therefore experiments were performed to determine the 3D structure of the rFel d 1(2+1) allergen using X-ray crystallography.

Experiments were initially performed to find conditions for optimal growth of crystals by buffer screening using the hanging drop vapour diffusion technique in room temperature. The first crystals produced were of poor quality (spherulites) and therefore not suitable for X-ray diffraction. By an additional separation step, ion exchange chromatography and cold room conditions (+4ºC), crystals appeared using a 2-methyl-2,4-pentanediol (MPD) grid screen (10-65% MPD, pH 4-9). The quality and size of the crystals were improved by reduction of the protein concentration to 2.5 mg/ml and seeding the rFel d 1 crystals using a cat whisker. The condition for optimal crystal growth was found at 13% MPD, 0.1M sodium acetate pH 4.8[221]. For determination of phases, Seleno-methionine substituted crystals were grown under similar conditions. The 3D structure of rFel d 1 was solved by computer aided assistance at a resolution of 1.85 Å[222].

[I] As suggested by the circular dichroism spectrum, **[II]** the secondary structure of rFel d 1 consists of α-helix bundles only. Four α-helices in each chain converge to form a globular molecule of dimensions 40×30×40 Å, representing an allergen structure not hitherto described[51]. Three disulphide bonds, ^{7}Cys(2)- ^{162}Cys(1), ^{48}Cys(2)- ^{136}Cys(1) and ^{73}Cys(2)- ^{95}Cys(1), corresponding to those previously presented **[I]**, confirmed the anti-parallel orientation of the two polypeptide chains. Despite the fact that the two halves of rFel d 1 are coded by two different genes sharing only 9% amino acid identity, they are similar in structure, with an overall r.m.s.d of 1.7 Å for 67 of the corresponding Cα atoms. Interestingly, Fel d 1 belongs to the uteroglobin protein family[223] and chain 1 of Fel d 1 and rabbit uteroglobin share 30% sequence identity with its human homologue, the Clara cell secretory protein (CC10) [89]. However, the barely detectable identity (13%) between Fel d 1 chain 2 and human CC10 made the structural similarities unclear. Despite an overall sequence identity of only 20%, the 3D structure of Fel d 1 is strikingly similar to uteroglobin[224-233] (Fig. 3). The two halves of the rFel d 1 molecule form an internal cavity (480 Å^3). Residues lining the cavity

originate from practically all of the α-helices in the monomer. The strong residual electron density in the cavity cannot be attributed to side chains or ordered water molecules but indicate a ligand of unknown nature. Indeed, both the CC10 and Fel d 1 molecules are expressed in epithelial cells[83, 234] and are controlled and induced by steroids[235, 236]. The internal cavity of uteroglobin homologues has been suggested to carry small ligands such as androgen, progesterone, polychlorinated biphenyl, phosphatidyl inositol, phosphatidyl choline and retinol[227, 233, 237, 238]. However the shape and size of the pocket of uteroglobin is different from that of rFel d 1. The rFel d 1 pocket is smaller, asymmetric and the end of the cavity is lined with two aspartic acid residues, Asp-60 and Asp-101. The location of the conserved disulphide bonds ^{7}Cys(2)-^{162}Cys(1) and ^{73}Cys(2)- ^{95}Cys(1), the conservation of three-dimensional structures by structure-based alignment and the capacity of the pocket to harbour small ligands indicate a common evolutionary origin.



Figur. 3. Comparison of rFel d 1 and uteroglobin ***A:*** Structure-based alignment of rFel d 1 chain 2 and 1, and of uteroglobin from rabbit (accession no. CAA25669) and man (accession no. AAH04481). Identical residues are indicated in bold. The secondary structure elements are indicated above the sequence. ***B:*** Stereo view of a ribbon diagram of the main chain of rFel d 1 (blue) superimposed on rabbit uteroglobin (yellow).

[II] It was previously demonstrated that the tri-antennary carbohydrate moiety in the nFel d 1 molecule[87] did not contribute to IgE-binding[88]. This finding may be explained by the location of the carbohydrates to residue Asn33[87], which is located in the solvent-exposed loop connecting the H2 and H3 helices.

Fel d 1 occurs naturally as a tetramer[239], however, the contact area between the two monomers of rFel d 1(2+1) ranged from 420 to 470 Å^2, indicative of crystal contacts rather than a protein-protein interaction. The inability to form dimers may be explained by the conditions used during the crystallisation process, notably the high content of MPD solvent and low pH. Recently we have produced crystals from rFel d 1(1+2), using more physiological conditions. The initial data processing employing molecular replacement indicates formation of a true Fel d 1 dimer, which would allow an almost complete structural interpretation of the Fel d 1 molecule.

[I] High affinity antibodies are formed by a host in response to foreign, often soluble antigens[240]. These antibodies are complementary to surface-exposed structures and can be used to probe B cell epitopes[241]. IgE antibodies in serum from 15 cat-sensitized patients reacted in a similar, if not identical, fashion to r- and nFel d 1, whereas an equimolar mix of the separate chains 1 and 2 exhibited significantly lower IgE-binding. This result was in agreement with the competition ELISA experiments, which showed equal capacity of r- and nFel d 1 to inhibit binding to plate-bound nFel d 1 while a 25-fold lower blocking capacity was noted for the mix of the chains. Thus, the two ELISA experiments demonstrated that rFel d 1 shares all major IgE epitopes with the natural allergen and the mix may represent a not fully refolded preparation with fewer exposed epitopes.

Recognition of allergens by the immune system depends on antigen processing and display of antigen peptides to T cell by APC[8]. It also depends on recognition of the 3D structure by surface bound IgE antibodies[242], leading to cross-linking and activation of basophils and mast cells. We analysed the lymphoproliferation and basophil degranulation in two patients by analysis of the basophil activation marker CD203c[216] and compared the response of r- and nFel d 1. Both allergen preparations exhibited equally good activity to up-regulate CD203c and mounted similar proliferative responses in PBMC.

DIAGNOSTICS OF CAT ALLERGIC PATIENTS [III]

Accurate evaluation of clinical status is critical for the management and well-being of cat allergic patients[121, 122, 243]. One important tool for this purpose is serologic analysis of allergen-specific antibodies. Previously, Fel d 1 mixes of chain 1 and chain 2 and rFel d 1 produced by insect cells[111] have been tested as candidates for diagnosis of cat-sensitised subjects. However, despite considerable efforts, comparably low sensitivity was recorded for the mix of chain 1 and 2, possibly due to the mix not refolding completely [88, 115, 116]. We evaluated the IgE and IgG_4 antibodies to rFel d 1 in serum from cat-allergic children from Sweden (n=27) and Austria (n=41) and in a cat-allergic group of adults (n=31). Three age matched non-cat allergic control groups were assembled for comparison.

The clinical laboratory device most widely used for analysis of allergen-specific IgE is Pharmacia CAP System®[150, 151, 244], considered both reliable and sensitive. In our study 94% of sera among the 99 cat-allergic children and adult patients were recognized as IgE positive to CDE. Surprisingly, CAP responses to the single rFel d 1 molecule was significantly higher ($p<0.001$) and not only recognized one more positive patient, but, close to the 0.35 kU/L cut-off level, the responses were found to be two times higher compared to those recorded for the CDE CAP. This difference gradually decreased to 37% for the group of patients with values below 100 kU/L. The result was unexpected, because it has been claimed that the ImmunoCAP can adsorb all corresponding allergen-specific IgE antibodies in serum samples[151]. CDE may contain up to 8 different allergens[74]. Thus, the increased sensitivity may therefore be attributed to a higher antibody accessible rFel d 1 density of these CAPs. Furthermore, a high correlation of IgE antibody levels between rFel d 1 and CDE among the patients was registered ($r_s= 0.93$, $p<0.001$) which emphasises the dominant role of IgE antibodies to Fel d 1 in serum from cat-allergic patients[77, 78].

We previously demonstrated that there is a close correlation of IgE responses between the major allergen in *Aspergillus fumigatus* (rAsp f 1) bound to ImmunoCAP and the same recombinant protein used in a corresponding ELISA assay[245, 246]. Similarly, using the single rFel d 1 by ELISA, we unexpectedly demonstrated positive IgE responses in all 99 tested patients, whereas none of the 75 tested control subjects were reactive. This result should be interpreted taking into consideration the number of patients, control subject and selection criteria but, nevertheless, suggests that the 0.35 kU/L used by CAP System® is not optimal for single allergen determinations.

The children displayed significantly higher IgE responses to rFel d 1 compared to the adult patients. The reason for the difference is not clear. The difference in age may suggest that children generally have a more vigorous immune response, however this notion was not supported by concomitant higher IgG_4 levels.

Sensitization to cat allergens is suggested as the major risk factor for development of asthma in Sweden[247]. Among the children included in this study, 59% and 48% from Sweden and Austria, respectively and 68% of the adults were diagnosed as having asthma in contact with cats. Although no difference in IgE levels was observed between Swedish and Austrian children, the IgE responses to rFel d 1 was significantly increased among patients with asthma compared to those with RC only, suggesting a role for Fel d 1 in the pathogenesis of cat allergic asthma.

Antibodies of the IgG_4 subclass have been suggested as markers for tolerance to allergic disease[137], and have been implicated in immunotherapy as beneficial blocking antibodies[188, 212]. Conversely, low levels of allergen-specific IgG_4 have been implicated in asthma disease[248, 249] suggesting a role in the pathogenesis of the disease. IgG_4 antibodies were analysed among the patients with the aim to evaluate the importance, if any, of these antibodies in relation to IgE and asthma. IgG_4 antibody responses to rFel d 1 were detected in 84% of the children, whereas the number for adult patients was 67%. Furthermore, there was a weak correlation between IgE and IgG4 (r_s= 0.44) whereas no correlation was noted among the adult patients (r_s =0.11, p>0.05). Interestingly, the ratio of IgG_4 to IgE antibodies was significantly higher (p=0.042) in adult patients with RC compared to adults with asthma. A similar difference between children with asthma and RC was not noted. This result suggests that beside IgE, an allergen-specific Th2-driven IgG_4 antibody response may develop, possibly protecting against the development of asthma.

ALLERGEN VACCINATION [IV], [V]

Improved safety and efficacy of allergen preparations for allergen vaccination are often achieved by simultaneous reduction of allergenicity and enhancement of immunogenicity by addition of an adjuvant. The risk of side-effects is always present in the course of allergen-specific immunotherapy and safety aspects are therefore of utmost importance[130]. **[IV]** The aim of the study was to design a rational model for the construction of hypoallergens[182, 250, 251] by molecular engineering of rFel d 1 for safe and effective treatment of cat allergic patients **[II]**. The strategy was based on the

belief that it would be possible to identify and disrupt important IgE epitopes and at the same time maintain or even increase the capacity of the altered allergens to activate T cells. Vaccination with such a molecule should have the capacity to induce IgG antibodies that can act simultaneously by blocking allergen activation of mast cells and decrease IgE-mediated allergen presentation[215, 252].

[IV] T cell epitopes, previously shown to induce frequent T cell responses in cat-allergic patients[54], were duplicated separately on rFel d 1 chain 1 and chain 2, to form rFel d 1 with duplicated T cell epitopes (DTE). The two sequences selected for DTE were parts of solvent-exposed loops between helix 2 and 3 of chain 2, and helix 6 and 7 of chain 1, which were known to harbour IgE-binding epitopes[53]. To further increase the hypoallergen character, disulphide bonds in rFel d 1 and rFel d 1 (DTE) were disrupted by changing cysteine residues to serines. The point mutations were made in the codons at position 44 on chain 1, and at positions 7 and 48 on chain 2. Hence, molecules with one, two or three cysteines replaced by serines (rFel d 1 (I-III)) were created, generating seven hypoallergen candidates in total (Fig. 4).

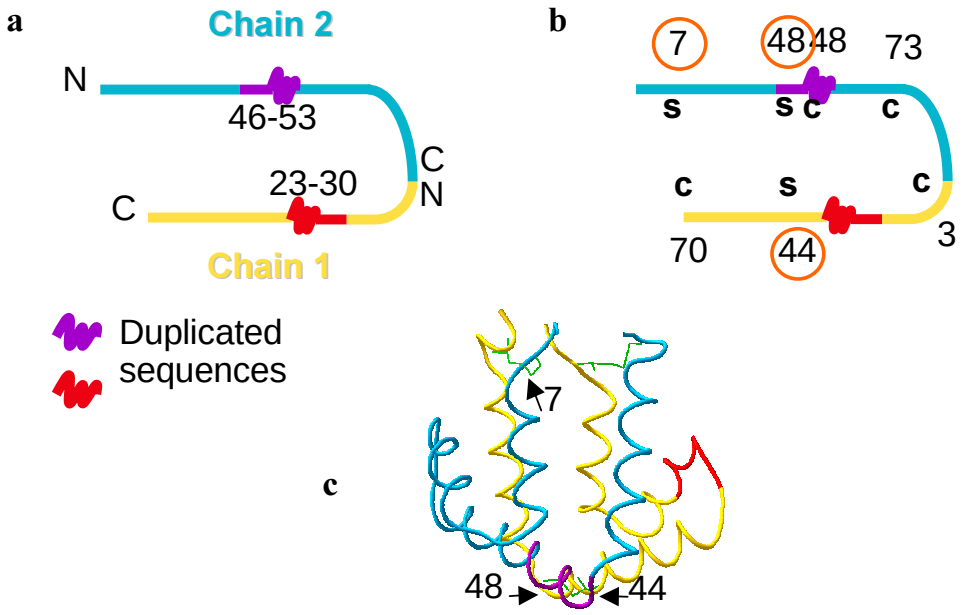


Figure 4. Rational engineering of rFel d 1 hypoallergens by the use of DTE and point mutations.
a: Duplications of amino acids 46-53, chain 2, and amino acid 23-30, chain 1, forming 2 DTE of rFel d 1. ***b***: Amino acid exchange by point mutations. Cys44 on chain 1 and Cys7 and Cys48 on chain 2 (only Cys48 in the parental sequence) were changed to serines. The construct with three cysteine mutations, rFel d 1 (DTE III), is exemplified. **c***:* A ribbon representation of the rFel d 1 (DTE) backbone displaying modifications of chain 1 and 2 shown in red and purple, respectively. Chain 1 (yellow), chain 2 (blue) and three disulphide bonds (green) are displayed. Cysteine residues subjected to mutagenesis are indicated by their respective numbers.

[IV] A simple first screening of the hypoallergenic nature of an allergen is often performed using competition ELISA comparing the hypoallergen with the natural allergen or the recombinant allergen wild-type. This method has the advantage of being quick, simple and can reliably judge the nature of a preparation in solution to a reference sample. As a surrogate for nFel d 1 we used rFel d 1, which was demonstrated to have the same structure and B cell epitopes as the natural allergen **[I, II]**. Three derivatives, rFel d 1 (DTE I-III), exhibiting 400 to 900 times reduced IgE-binding capacity compared to wild-type rFel d 1 were selected for further studies. The concentrations required for 50% inhibition of IgE binding to rFel d 1 were 21.8 µg/mL for rFel d 1 (DTE I) and 14.3 µg/mL for rFel d 1 (DTE II), while the highest concentration tested (30 µg/mL) resulted in 35% inhibition for rFel d 1 (DTE III). For rFel d 1, a concentration of 0.034 µg/mL was needed to reach 50% inhibition.

[IV] By dose-dependent activation of basophils, rFel d 1 and the three rFel d 1 derivatives were further evaluated for anaphylactic activity. The ability to induce degranulation of basophils in four patients was assessed using flow cytometry for hypoallergens with an increasing number of cysteine mutations (rFel d 1 (DTE I-III). In agreement with the ELISA inhibition experiments, the rFel d 1 with duplicated T cell epitopes and three disrupted disulphide bonds, (DTE III), showed the lowest capacity to activate basophils. A control allergen from the storage mite *Lepidoglyphys destructor*, rLep d 10^{253}, did not induce basophil degranulation in any of the patients. Furthermore, stimulation of cells from two non-atopic individuals with rFel d 1 and the derivatives did not result in basophil activation.

[IV] We hypothesised that the strategy of duplications of sequences spanning T cell epitopes would allow not only manipulation of the 3D structure, but would concomitantly allow display of the entire T cell epitope repertoire by APC. Therefore the lymphoproliferative activities of rFel d 1 and the three rFel d 1 DTE derivatives were assayed in PBMCs from nine cat-allergic patients. PBMC from seven patients were stimulated to proliferate (SI ≥ 2.0) in response to the different rFel d 1 proteins, while no proliferation was detected in PBMC from two of the cat-allergic patients. For the responding patients, the SI values obtained with rFel d 1 (DTE I-III) were at least as high as for rFel d 1. Only a weak response to rFel d 1 (DTE I) (SI 2.3), but not to the other rFel d 1 molecules, was observed in PBMC from a non-atopic control. In accordance with the aim of the rational engineering of the rFel d 1, these results suggest

that the modifications of the rFel d 1 derivatives did not impair the MHC antigen presentation and that the rFel d 1 derivatives were fully capable of stimulating Fel d 1 specific T cells.

[V] Side-effects experienced by allergy vaccination are not always linked to the allergen preparation as such. The most commonly used adjuvant, Alum, can cause granuloma lumps at the site of injection. In a mouse model we tested if a model allergen, in this case rPhl p 5 (p5), covalently coupled to carbohydrate based particles (CBP) potentially could be a better adjuvant than Alum. CBP is based on agarose, which is known to be well tolerated when exposed to a variety of cell types in tissue culture and as column matrix in clinical *ex vivo* treatments[254].

By immunohistochemical analysis of tissue sections at the site of injection, a mixed cellular inflammatory reaction with prominent eosinophilic granulocytes in the deep dermis was observed in mice treated with allergen-adsorbed Alum or with Alum alone. Additional granuloma formation could be seen with granular debris at the center of the injection site in the deep dermis and predominantly foam cells and eosinophils at the cellular outer rim. On the other hand, the inflammatory tissue reactions of mice immunized with CBP conjugated allergen or CBP alone as well as with p5 alone, were smaller and contained less granular debris than the Alum-treated mice.

[V] Antigen covalently linked to 2 μm particles, e.g. CBP, is not taken up and processed by B cells. Nonetheless, data document that antigens attached to particles are recognised in a very efficient way by APC[255-257]. We have recently shown that upon stimulation with particle-bound rFel d 1 of $CD1a^+$ cultured immature monocyte-derived dendritic cells (iMDDCs), the co-stimulatory molecule CD86 was up-regulated and increased levels of cytokine tumor necrosis factor and the chemokine IL-8 were released compared to cells stimulated with free rFel d 1 or CBPs only[256] (Fig 5).

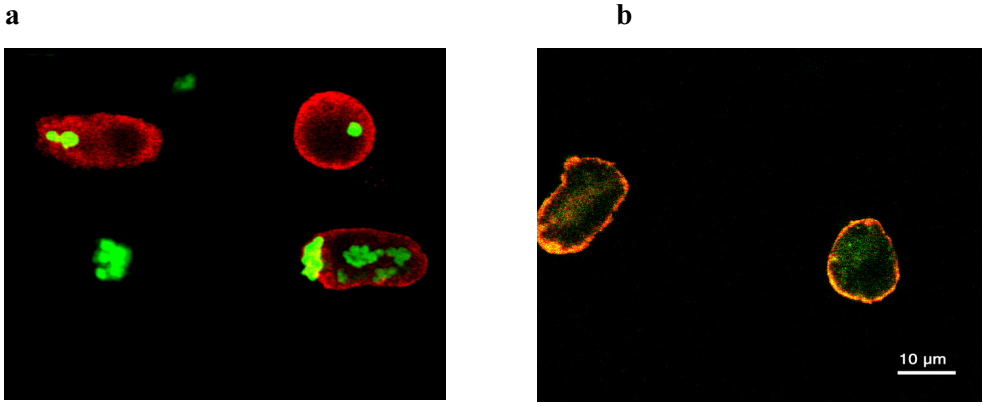


Fig 5. Uptake of CBP-rFel d 1-FITC and rFel d 1-FITC by immature MDDCs. MDDCs were incubated with FITC-labelled CBP-rFel d 1 or rFel d 1 (green) followed by staining with anti-CD1a PE (red) and analysis by Confocal Laser Scanning Microscopy (CLSM). CLSM shows iMDDCs internalization of CBP-rFel d 1 (a) and rFel d 1 (b).

The efficient antigen presentation of CBP was supported by our study because mice immunised with rPhl p 5 and in combinations with adjuvant showed increasing rPhl p 5b-specific antibody responses in the course of treatment. Preparations containing adjuvant showed significantly higher antibody titres than mice who had received rPhl p 5 alone. Interestingly, the group of mice immunised with CBP-p5 displayed a more vigorous IgG 2a/b response compared to Alum-p5, CBP+p5 or rPhl p 5 only. This result was also supported by grass pollen extract-stimulated splenocyte cultures, where the IFN-γ production in cultures of CBP-p5 immunized mice was significantly elevated. However, the elevated IgG1 antibody levels indicated that CBP-p5 immunisation created a balanced Th1 and Th2 profile. This was confirmed in the culture supernatants because, in addition to IFN-γ, strong reactivity was also recorded for IL-4 and IL-5 in the cultures. Alum-p5 and CBP+p5 on the other hand showed similar antibody and cytokine patterns, which were indicative of a more Th2-skewed response. The balanced T helper cell cytokine pattern produced by CBP-p5 immunized mice may be an advantageous feature. It has been demonstrated that there is a limited ability of antigen-specific Th1 responses to inhibit Th2 cell activity and, moreover, pure Th1 responses have been suggested to increase the suseptibility to allergic inflammation[207, 258, 259].

Several studies performed with Alum-adsorbed allergens provided evidence that immunotherapy-induced blocking antibodies possess desirable effects as they can inhibit the allergen-induced effector cell activation and the IgE-mediated presentation of allergens to T cells[27,28, 29]. Therefore, the capacity of mouse anti-rPhl p 5 antibodies produced by immunisation with CPB-p5 and Alum-p5 to block IgE binding to plate-

bound rPhl p 5 in sera from grass pollen allergic patients was investigated. We found that mouse serum from the two preparations demonstrated a similar and prominent blocking activity and that the mouse IgG anti-rPhl p 5 antibodies cross-reacted with group 5 allergens from various grass species.

CONCLUSIONS

We have demonstrated that a novel construct of the major cat allergen, Fel d 1, cloned into *E. coli*, can be expressed in high amounts and purified to homogeneity. The rFel d 1 allergen displayed almost identical biochemical, immunochemical and biological properties as the natural counterpart. Using the rFel d 1 construct, we solved the 3D structure of rFel d 1 and found a close resemblance to the immunomodulatory and cytokine-like human lung secretoglobin CC10. We conclude that the rFel d 1 molecule is a suitable candidate for diagnosis and treatment of cat allergic patients.

Diagnosis of cat-allergic patients using the rFel d 1 construct established that this single allergen may accurately diagnose 100% of patients using a simple laboratory ELISA in comparison to 94% detected by crude CDE in the frequently used Pharmacia CAP System®. Moreover, the rFel d 1 molecule exhibited significantly higher IgE responses to rFel d 1 CAP compared to the CDE ImmunoCAP. Therefore we conclude that a single allergen, rFel d 1, may be equal or even superior than CDE for diagnosis of cat-allergic patients.

Allergy vaccination involves the allergen itself an adjuvant component. Based on the knowledge of T and B cell epitopes and the 3D structure of rFel d 1, we successfully designed several hypoallergens, of which rFel d 1 (DTE III) showed the most promising properties as a candidate for treatment. We conclude that this novel approach is potentially applicable for the design of new hypoallergenic derivatives of allergens.

Alum, the only adjuvant approved for human use, has several less desirable features. Similar to Alum, CBP induced beneficial allergen-reactive blocking antibodies, but in contrast to Alum, immunization of mice with CBP induced a balanced Th repertoire and no granulomatous reactions under comparable treatment. We conclude that 2 μm allergen-conjugated CBPs exhibit many features of a successful adjuvant for allergy vaccination in humans.

FUTURE PERSPCTIVES

Although allergy diagnostic testing has been successfully practised for decades, there is room for both technical development and improved clinical utility. *In vitro* allergen extract tests are all based on a complex mix of allergens in a source of natural origin. Improved diagnosis will be achieved by means of micro-arrays containing large numbers of relevant single allergens, requiring minute amounts of serum or blood. The rFel d 1 produced and characterised in this thesis could be an important tool since we demonstrated that the single allergen may be a better indicator of sensitisation than the extract. The quantitative aspect of serum IgE measurements will be more important, which will increase the demand for more sensitive tests but also include probability of disease calculations. This trend will bring added clinical value for recombinant allergens. The challenge by IgE detection using allergen arrays will be to decipher the complex patient-specific sensitisation profiles into clinically relevant information. For this purpose, it is highly likely that database stored, region-specific information from large cohorts of clinically well-characterised patients will be readily available for the allergologist. Perhaps most importantly, information from allergy diagnostics will also form the basis for component-resolved allergen vaccination, thereby forming a logical link between diagnosis and optimal treatment.

Allergy vaccination has been conducted in the same way for more than 60 years, despite the high incidence of side-effects caused by the use of natural extracts and Alum. Biocompatible particles with sizes suitable for enhanced allergen uptake and presentation by APC and simultaneous elimination of allergen spreading into the tissue will be a candidate for the new generations of allergen adjuvant. To provide evidence for safety and efficacy of particle-based treatment, a mouse model for cat allergy based on rFel d 1 is being established. The aim of the cat allergy mouse model is to evaluate a rationale for treatment of allergic patients and perhaps also for prophylactic vaccination in children.

Allergens with modulated allergenic activity by molecular engineering, hypoallergens, has recently been evaluated in clinical trials. Although rational design of hypoallergen preparations as described in this thesis will provide new candidates for therapy, future allergy vaccines will have additional features. Engineering will allow properties like cell penetrating peptides and the invariant chain to be directly linked to the allergen for

enhanced antigen presentation. Such a construct with rFel d 1 as active substance is presently being prepared to allow treatment of cat-allergic patients by three injections, two weeks apart into draining lymph nodes.

The structure of the rFel d 1 presented here did not allow the contact site between the homo-dimers to be analysed. We have recently crystallised and solved the structure of the second construct presented in paper 1 (chain 1 N-terminal of chain 2). The results revealed that the rFel d 1(1+2) molecule indeed forms homodimers corresponding to the nFel d 1 tetramer (Fig. 6). The complete 3D structure of Fel d 1 and its structural similarity to the immunomodulatory human lung secretoglobin CC10 may provide significant insights into the sensitisation process by Fel d 1 in cat-allergic patients from which steps to prevent sensitisation to cat can be taken.

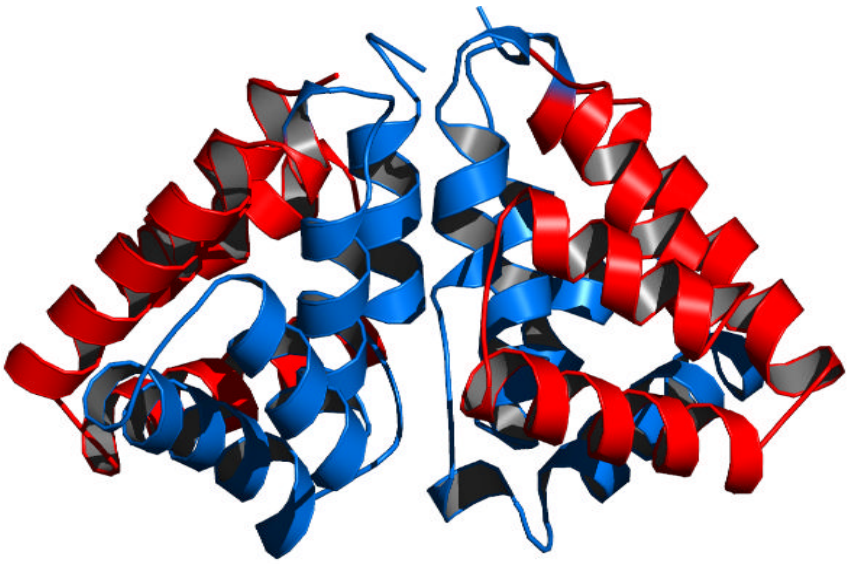


Figure 6. The α-helix ribbon structure of the rFel d 1 homodimer. The contact area between the two monomers is almost exclusively consiting of amino acids derived from chain 2 (blue). The solvent exposed chain 1 is depicted in red.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Allmänt om kattallergi

Allergi är en folksjukdom som drabbar mer än 25 % av befolkningen. Överkänslighet mot katt är en av de vanligaste allergierna och återfinns hos cirka 10 % av svenska befolkningen och är dessutom den vanligaste orsaken till astma bland barn i Sverige.

Allergi uppstår genom att luftburna partiklar med äggviteämnen (proteiner) från kattens päls kommer i kontakt med slemhinnorna (mastcellerna) i övre luftvägarna. Under vissa omständigheter, bland annat beroende på ärftliga faktorer och upprepad kontakt med dessa proteiner, överreagerar vi. Kroppens förvar mot främmande ämnen aktiveras och bildar en typ av försvarsproteiner, antikroppar av IgE-typ. När dessa IgE-antikroppar, bundna till vissa celler i slemhinnan, kommer i kontakt med kattens proteiner (allergen) uppstår de typiska allergiska sjukdomssymptomen såsom snuva, kliande ögon och astma.

De luftburna partiklarna från katt innehåller cirka 10 olika allergen, varav fyra stycken är välkända. Det allergen som flest människor är känsliga mot är ett protein som har givits namnet Fel d 1. Det utsöndras från celler som finns i kattens slemhinnor och sprids genom att katten slickar sig. Över 90 % av alla kattallergiker bär på IgE-antikroppar mot detta protein.

För att lyckas bota allergi behöver man med säkerhet kunna bestämma vad patienten är allergisk mot. Detta kan göras genom att mäta halten av allergiframkallande IgE-antikroppar i blodet mot olika kända allergen. När orsaken till allergin är klarlagd kan behandlingen påbörjas.

Vanligtvis behandlas allergi med mediciner som lindrar symptomen. Vill man däremot bota allergin kan man ge s.k. immunterapi. Vid denna behandlingsform strävar man efter att uppnå tolerans mot allergenet genom injektion med gradvis ökande doser av allergenextrakt. Allergenkällor som står till buds idag består av extrakt från naturliga källor, till exempel kattmjäll eller pollen från gräs och björk. Dessa preparat är inte möjliga att helt och hållet standardisera och därför varierar allergeninnehållet från sats till sats. Ett sätt att förbättra diagnostik och behandling kan vara att med hjälp av DNA-teknik låta bakterier producera önskade allergen som därefter renas och karakteriseras. Problem som orsakas av nuvarande behandling är att allergenet utlöser kroppens försvar med omedelbara biverkningar som följd. Dessa kan i sällsynta fall vara ytterst allvarliga. För att minska risken för en omedelbar reaktion och samtidigt höja kroppen

tolerans mot allergenet adsorberas det på aluminiumhydroxidpartiklar. Dessa partiklar har, trots att de ger en allergiliknande effekt vid injektion, använts på liknande sätt vid allergibehandlingar i över 60 år. Dessa partiklar kan dessutom ge upphov till biverkningar, så kallade granulom, som uppträder som förhårdnader under huden vilket kan leda till att behandlingen måste avbrytas.

Framställning av huvudallergenet från katt

Med hjälp av DNA-teknik har man försökt framställa huvudallergenet från katt, Fel d 1, i ren form. Försöken har inte varit helt lyckade eftersom de två proteinerna som utgör Fel d 1 inte har antagit en fullständigt naturlig form.

I det första delarbetet sökte vi ett sätt att skapa Fel d 1 med egenskaper som efterliknar det naturliga allergenet. Genom att sätta samman de två arvsanlagen (generna) som ligger till grund för Fel d 1 till en gen med DNA-teknik och därefter föra in konstruktionen i bakterier lyckades vi framställa Fel d 1 i ren form. Därefter vidtog en rad steg för att visa att bakterieframställt Fel d 1 var likt det naturliga proteinet. Det vi särskilt undersökte var att storleken var rätt, att vissa byggstenar, aminosyror, i Fel d 1 satt ihop på rätt sätt, men framför allt undersökte vi att IgE antikroppar från kattallergiska patienter reagerade på likartat sätt med vårt skapade protein och naturligt Fel d 1. Alla resultat pekade på att vi lyckats skapa ett protein med egenskaper som motsvarar det naturliga allergenet.

Bestämning av den tredimensionella strukturen hos Fel d 1

I delarbete två gick vi vidare med det renade Fel d 1 som vi framställt i arbete 1. Vi ville bestämma strukturen hos Fel d 1 med hjälp av en metod som kallas röntgenkristallografi. Proteiner byggs upp av 20 stycken olika aminosyror som kan sitta i vilken ordning som helst, som pärlor i ett pärlband. Ordningen bestämmer vilken form (struktur) och därmed vilken funktion ett protein har. Röntgenkristallografi tillåter att man med stor noggrannhet kan bestämma positionen hos varje enskild aminosyra i ett protein, till exempel Fel d 1. Först bestämdes förhållanden under vilka Fel d 1 bildade små kristaller. Dessa kristaller utsattes sedan för röntgenstrålar. Genom att mäta på vilket sätt strålarna sprider sig efter att ha passerat proteinkristallen kan man bestämma proteinets struktur. Detta lyckades med Fel d 1 och strukturen visade sig vara tidigare okänd i allergisammanhang. Fel d 1 liknar ett protein som finns i lungan hos människa och som påverkar lungans funktion. Likheten i struktur och funktion mellan Fel d 1 och

dess mänskliga motsvarighet kanske så småningom kan hjälpa oss att förstå varför Fel d 1 är ett så kraftfullt allergen.

Bestämning av allergi hos kattallergiska barn och vuxna

Frågan vi ställde i delarbete tre var hur pass bra Fel d 1 är när det gäller att bestämma om en person är allergisk eller inte. Detta låter sig göras genom att bestämma om det finns IgE-antikroppar i serum hos kattallergiker respektive kontrollpersoner. Vi samlade serum från 99 barn och 31 vuxna, alla med kattallergi och från 75 kontrollpersoner. Dessutom bedömde den behandlande läkaren om personen i fråga hade astma mot katt. Med ett kommersiellt test (ImmunoCAP) bestämdes att de flesta av kattallergikerna (94 %) hade IgE antikroppar mot kattmjäll. När samma personer analyserades på liknande sätt med en analysmetod som är vanligt förekommande på laboratoriet (ELISA) hade alla antikroppar mot Fel d 1. Det visade sig dessutom att astmatiker hade högre IgE-nivåer mot Fel d 1 än patienterna med bara snuva, och metoden skulle därmed i framtiden kunna vara ett sätt att förutsäga risken för att utveckla astma. Ytterligare ett intressant fynd var att de som var vuxna men som inte utvecklat astma hade högre nivåer av en annan typ av antikropp, IgG_4. Mätning av en kombination av dessa två antikroppar skulle således kunna ge värdefull information för vidare behandling.

Kan vi förbättra behandling av allergiska personer?

I arbete 4 och 5 undersökte vi olika aspekter av behandling mot allergi. För att möta problemet med en omedelbar allergisk reaktion i samband med injektionsbehandling skapade vi med hjälp av DNA-teknik tre Fel d 1 allergen med 400-900 gånger lägre IgE-bindningskapacitet än naturligt Fel d 1. Tillvägagångssättet vid framställningen av dessa så kallade hypoallergen var speciell såtillvida att vi använde tillgänglig kunskap om Fel d 1 för att på ett logiskt sätt bygga lämpliga kandidater för behandling. Genom att utnyttja information om strukturen hos Fel d 1 samt var patienters IgE antikroppar binder till Fel d 1 men även hur delar av Fel d 1 känns igen av vissa typer av vita blodkroppar kunde vi identifiera tre kandidater som lämpliga för immunterapi. Även om mycket återstår har vi nu redskap att på ett säkrare sätt behandla kattallergiska patienter.

Vårt mål med femte delarbetet var att skapa förutsättningar för en säkrare och effektivare behandling av allergi. Som alternativ till den vanliga bäraren av allergener,

aluminiumhydroxid, valde vi att pröva 2 mikrometer stora kolhydratbaserade partiklar (CBP). Denna storlek är speciellt lämpad att tas upp av kroppens ätarceller som därefter aktiverar kroppens försvarsmekanismer. Genom att kemiskt binda proteinerna så att de inte släpper från CBP presenteras allergenet, i detta fall ett gräspollenallergen, effektivt för kroppens försvarsceller. Vi kunde med en musmodell visa att CBP gav ett likvärdigt svar som aluminiumhydroxidpartiklar med den skillnad att CBP gav en bättre balans av celler som motverkar allergi. Vi kunde dessutom visa att antikroppar av ett allergiblockerande slag hade bildats samt att, till skillnad frånaluminiumhydroxid, inget granulom bildades vid injektionsstället. Sammanfattningsvis kan sägas att de föreslagna partiklarna har många fördelar som skulle kunna komma patienter tillgodo vid immunterapi.

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