CAT ALLERGY – DIAGNOSIS AND CHARACTERIZATION OF IMPORTANT ALLERGENS

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Stockholm 2008
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Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden
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ISBN 978-91-7409-039-0
To my woman and my boys.
Allergy affects about 25% of the population. Allergic diseases are manifested in many ways, from mild rhinitis to life-threatening anaphylaxis. Globally, more than 15% of children and adults are allergic to pets.

The focus of this thesis is important cat allergens. First we identified novel cat allergens, cat immunoglobulin (Ig)A and cat IgM. Analyses revealed that the IgE-reactivity in human sera was directed to carbohydrates of the immunoglobulins. We also detected IgE-reactivity to IgM in 7 of 9 animal species tested, but not to human immunoglobulins. Since our immunoassays were not optimized for testing allergens with carbohydrate epitopes, appropriate controls were performed. These controls revealed that the reaction was not mediated by IgE but by IgM in patient serum by cross-linking animal immunoglobulins and alkaline phosphatase. This IgM fits into the heterophile antibody classification and is directed to carbohydrates on animal immunoglobulins and on calf intestine alkaline phosphatase.

Sera from cat-sensitized patients were further analysed for IgE antibodies to purified cat IgA using the ImmunoCAP System. Thirty-eight percent of the cat-sensitized sera were positive. Indirect enzyme linked immunosorbent assay (ELISA) confirmed the IgE-reactivity to cat IgA and also to cat IgM, but not to deglycosylated cat IgA. Competitive inhibition ELISA was performed and strong inhibition of patient IgE to cat IgA was observed in all sera after pre-incubation with cat IgA and cat IgM. Thus a new group of cross-reactive allergens with carbohydrate epitopes has been identified. This is the first report of mammalian carbohydrate IgE epitopes. These epitopes are also targeted by IgM and may therefore interfere in immunoassays.

Recombinant variants of the major cat allergen Fel d 1 have also been studied in this thesis. We confirmed that recombinant (r)Fel d 1 (1+2), rFel d 1 (2+1) and natural Fel d 1 have equivalent biological and immunological properties. This confirmation was based on similar basophil stimulation, T-cell proliferation, direct and competitive inhibition ELISAs. We also present the crystal structure of the rFel d 1 (1+2) tetramer at 1.6 Å resolution. The crystal structure of tetrameric Fel d 1 reveals two different calcium-binding sites. The external calcium-binding site corresponds to a putative calcium-binding site previously suggested for uteroglobin. The second calcium-binding site lies within the dimerization interface, inducing important local conformational changes that directly govern the shape of two water-filled cavities. The crystal structure suggests a potential portal for an unknown ligand.

We evaluated IgE- and IgG4-binding to rFel d 1 among cat allergic children and adults suffering from asthma and/or rhinoconjunctivitis (RC) in populations from Sweden and Austria. A high correlation of IgE responses to rFel d 1 and cat dander extract in ImmunoCAP among the patients was demonstrated. Using ELISA, 98% of patients and none of the controls had IgE to rFel d 1 and there was a 3-fold increased risk of asthma for those children with the highest IgE levels. IgE responses to rFel d 1 among children with asthma were higher compared to children with RC and adults with asthma. Furthermore, children with asthma displayed higher IgG4 levels than did asthmatic adults. We demonstrated that a single recombinant molecule, rFel d 1, is at least as sensitive for in vitro diagnostics of cat allergy as the currently used extract-based test. We suggest that elevated IgE antibody levels to Fel d 1 is a risk factor for asthma in cat allergic children.
LIST OF PUBLICATIONS

I. Adédoyin J, Johansson SGO, Grönlund H, van Hage M.
   Interference in immunoassays by human IgM with specificity for the carbohydrate moiety of animal proteins.

II. Adédoyin J, Grönlund H, Oman H, Johansson SGO, van Hage M.
   Cat IgA, representative of new carbohydrate cross-reactive allergens.

    Structural characterization of the tetrameric form of the major cat allergen Fel d 1.

    Higher IgE antibody levels to recombinant Fel d 1 in cat allergic children with asthma compared to rhinoconjunctivitis

*These authors contributed equally to the work
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<tr>
<td>3D</td>
<td>3-dimensional</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>Bet v</td>
<td><em>Betula verrucosa</em> (birch) allergen</td>
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<td>CCD</td>
<td>Cross-reactive carbohydrate determinant</td>
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<td>CDE</td>
<td>Cat dander extract</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Der p</td>
<td><em>Dermatophagoides pteronyssinus</em> (house dust mite) allergen</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
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<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<td>Fc</td>
<td>Fragment crystallizable</td>
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<td>Fel d</td>
<td><em>Felis domesticus</em> (house cat) allergen</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>kDa</td>
<td>Kilodaltons</td>
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<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>n</td>
<td>Natural</td>
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<tr>
<td>Phl p</td>
<td><em>Phleum pratense</em> (timothy grass) allergen</td>
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<tr>
<td>r</td>
<td>Recombinant</td>
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<tr>
<td>RC</td>
<td>Rhinoconjunctivitis</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>Th</td>
<td>Helper T-cell</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<td>Treg</td>
<td>Regulatory T-cell</td>
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INTRODUCTION

THE IMMUNE SYSTEM

The term immunity originates from the Latin words immunitas and immunis which, by Romans referred to as the exemption of an individual from service or duty and later, in the Middle Ages, referred to as the exemption of the Church and its properties and personnel from civil control [1, 2]. Its first use in the context of disease has been traced to as far back as 500 B.C., describing immunity to the plague. It was not until the 19th century that the term became widely accepted thanks to Edward Jenner’s vaccination against smallpox. However, the concept was already used in ancient China where children were immunised by inhaling powder from lesions from smallpox survivors.

The human immune system comprises two parts, the innate and the adaptive systems. The innate system is rather primitive and is strikingly similar and conserved between organisms separated billions of years ago [3]. It serves as a first line of defence, clearing the pathogens or holding them off while the more sophisticated adaptive system is preparing itself to take over if the innate system is defeated.

Innate immunity exists even before the pathogens are encountered, like a preformed defence concept. Epithelia, anti-microbial substances, phagocytes, natural killer (NK)-cells, cytokines and complement system proteins are all part of the innate immune system. Innate immune cells have receptors, for example Toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns on pathogens, such as bacterial specific cell wall components, for example the endotoxin lipopolysaccharide (LPS). Binding of such compounds leads to triggering of signalling cascades for the pathogen-associated molecular patterns receptor-bearing cells which result in secretion of pro-inflammatory cytokines, chemokines and antimicrobial peptides [4]. Since the innate system is unable to adapt itself, evolution has led to pathogens being able to evade the innate immunity. The adaptive immunity has therefore evolved, and is further divided into two parts, cell-mediated and humoral immunity.

Humoral immunity was identified after the discovery that serum could transfer immunity from an immune to a non-immune animal, so-called passive immunization. The responsible molecules were identified as being antibodies. Immunoglobulins (Igs) are antibody glycoproteins composed of immunoglobulin domains [5]. They are produced by B-cells and exist as cell-bound B-cell receptors and as serum-secreted proteins. Antibodies can attach to pathogens and toxins and thereby neutralize or target them for different effector mechanisms.

The other part of the adaptive immunity encompasses cell-mediated immunity. It can be transferred by other blood components than those of humoral immunity, namely by cells, or to be more specific, by lymphocytes. It consists of T-cells that often in concert with other cells, such as phagocytes, can destroy virus and bacteria that have infected cells and that are therefore unavailable for antibodies. The adaptive immune system has some fundamental features that make it very efficient.

- Specificity and diversity make the antigen receptors only recognise specific epitopes of the antigen. There are a huge number of pre-made clones that can fight pathogens that have not earlier been encountered.
- Memory cells ensure that a second encounter to an antigen is met with a more rapid and effective response.
• Clonal expansion elicits proliferation of pathogen engaging clones.
• Specialization of different types of cells and molecules within the adaptive immune system makes a more powerful defence against the variety of microbes and their different stages.
• The expansion of cells must end and inflammation resolve after the microbes have been cleared, and homeostasis is therefore very important.
• Tolerance and non-reactivity to self-antigens is one of the most central tenets in the immune system and is maintained by several mechanisms.

The major histocompatibility complex (MHC) is a large genomic region containing genes encoding for important immunological proteins such as MHC molecules and different cytokines. MHC class I is used by all lymphocytes to present peptides that are obtained from internal proteins, which is crucial for clearing virus and intracellular bacteria. MHC class I-peptide complexes are recognised by CD8+ cytotoxic T-cells if a non-self peptide is displayed. CD8+ cytotoxic T-cells kill cells that display complexes that are recognised by their T-cell receptors (TCRs).

The humoral part of the adaptive immune system is triggered by antigen presenting cells (APCs), such as macrophages and dendritic cells. APCs activate T-cells by processing pathogens and presenting peptides from pathogens on (MHC) class II molecules. A subset of T-cells, CD4+ T-helper cells, have TCRs that recognise MHC class II-peptide complexes. If they have correct specificity the T-cell will become activated and can activate B-cells. These will differentiate into memory B-cells and immunoglobulin-secreting plasma cells.

**ANTIBODIES**

Antibodies can be of different classes or isotypes: IgA, IgD, IgE, IgG and IgM. IgD and IgM are the first antibodies to be produced in the early stage of an immune response, but the production can eventually switch to production of IgA, IgG or IgE depending on location and antigen. Historically, antibodies can structurally be considered as comprising two parts, the antigen-binding Fab (fragment antigen-binding), which is different for each B-cell clone, and the constant Fc (fragment crystallizable) part. A flexible hinge region in IgA, IgD and IgG connects the Fab and Fc regions, whereas IgE and IgM instead have an extra constant domain that connects Fab and Fc (Figure 1) [6].

Antibodies consist of two light chains and two heavy chains linked together by disulfide bonds. Each chain is made up by domains, one variable and one or more constant domains. κ and λ chains (light chains) have one constant domain whereas γ, α and δ chains (IgG, IgA and IgD heavy chains) have three and ε and µ (IgE and IgM heavy chains) have four. IgM make pentamers or hexamers when secreted, a glycoprotein called J chain (which is a part of the multimer) promotes the polymerisation. In human and other primates IgA is a monomer in serum but in other vertebrates the J chain supports dimer formation. IgA will appear as J chain supported dimers when transported through epithelia, with a secretory component attached.

Antibodies are glycoproteins (Figure 1). The glycans are of significant importance for effector functions and structure [6]. Each isotype has different types, numbers and locations of oligosaccharides. Glycosylation can also vary between subclasses. N-linked glycans are present on the Ig-domains whereas both N- and O-
linked glycans occur in the hinge region. The glycosylation ranges between 2-14% of the molecular weight of the antibody depending on isotype. IgG has a low degree of glycosylation whereas both IgE and IgM are highly glycosylated. The Fc part enacts the effector functions, binding to Fc receptors, activating complement and being responsible for immunoglobulin transportation.

Using enzymes and recombinant techniques, antibody fragments that are capable of binding antigens can be obtained. A Fab fragment consists of the variable and the first constant domain of a heavy chain attached to a light chain. Traditionally, digestion with papain can produce such fragments. Using this method an Fc fragment will also be produced, which is the remaining constant regions of the two heavy chains attached to each other. A F(ab’)2 fragment is the variable domains and the first constant domains of the heavy chains attached to the light chains and also the hinge region. F(ab’)2 fragments are produced by digestion with the enzyme pepsin. Through this digestion the Fc resides in small fragments and a larger fragment called pFc’. Single chain variable fragment (scFv) molecules are produced by recombinant techniques and are the smallest fragments that can routinely be constructed with preserved antigen-binding capacity. They basically consist of the variable domains from a heavy and a light chain linked together.

**IGE-MEDIATED ALLERGY**

A factor in serum crucial for allergic disease was already discovered in the beginning of the last century. But it was the work of Ishizaka and Johansson that identified a novel immunoglobulin class, IgE, as the contributing molecule [7, 8]. Allergy is triggered by an initial encounter with an allergen. Allergens are otherwise not harmful proteins that will cause sensitization, a process that initiates the disease (Figure 2). APCs, most importantly dendritic cells, process the antigen and present peptides via MHC class II molecules [9]. The APC present these peptides to CD4+ T-cells, which in turn activate allergen-specific B-cells [10]. The allergen-specific T-cells are helper T-cell (Th)2

**Figure 1.** Human antibodies of different classes and subclasses. Blue and yellow stars are N-linked oligomannose and complex glycans, respectively. Green stars are O-linked glycan clusters (modified from [6, 177]).
polarized, secreting interleukin (IL)-4, IL-13 and IL-5. These cytokines cause chronic inflammation together with the Th1 molecule interferon (IFN)-γ [11]. Both IL-4 and IL-13 promote a class switch to IgE production in B-cells, while IL-5 promotes amplification of eosinophil production and activation [12, 13].

IgE binds to mast cells and basophils via the high affinity IgE receptor FcεRI [14]. Re-exposure to the sensitizing allergen will lead to cross-linking of mast cell bound IgE, causing mast cell degranulation. The immediate response results in release of mediators such as histamine, tryptase, chymase, kininogenase, heparin, prostaglandin D2 and the sulfidopeptidyl leukotrienes [15]. Additionally, cytokines such as tumor necrosis factor (TNF) are transcribed during the immediate response. The associated symptoms include sneezing, rhinorrhea, conjunctivitis and wheezing. Late phase allergic reactions will peak 6-9 hours after allergen exposure and include symptoms such as wheezing, edema and swelling [10]. Late phase reactions are promoted by Th2-cell mediated release of IL-3, IL-4, IL-5 and other cytokines that promote IgE production, eosinophil chemoattraction and eosinophil survival, as well as mast cell recruitment [16]. Eosinophils release a number of different inflammatory mediators. These proteins can cause great damage to airway endothelial cells, extracellular matrix and neurons [17]. Neutrophils, lymphocytes and macrophages are also involved in the late phase allergic reactions, the neutrophils promoting damage to the endothelium and T-cells driving the inflammation. Macrophages recruit other inflammatory cells, but may also inhibit inflammation through the release of inhibitory mediators.

Regulatory T-cells (Tregs) are thought to play a role in allergy by suppressing undesired activity induced by effector Th2-cells [18]. Tregs secrete IL-10 and transforming growth factor (TGF)-β that suppress IgE production and induce IgG4 and IgA, respectively. These cytokines also suppress allergic inflammation induced by mast cells, basophils and Th2-cells. The suppressing function of Tregs is thought to be

![Figure 2](image-url). Important cells, cytokines and interactions in IgE-mediated allergy (modified from [18, 178]).
defective in allergic individuals [19]. Tregs are CD4+CD25+, which also specifically express the transcription factor forkhead box p3 (Fox p3) [20]. There are different subsets of Tregs, naturally occurring CD4+CD25+ Tregs originate in the thymus, and adaptive Tregs are induced in the periphery by encountering antigens. Another subset is the IL-10- producing type 1 Tregs [21].

Hygiene hypothesis

In an attempt to explain the increasing prevalence of allergic disease, the hygiene theory was introduced. It originally suggested that reduced infections, smaller family sizes, improved living standards and higher levels of personal hygiene might have led to an increased risk of developing allergies [22]. The hypothesis has since then evolved to a broader definition, pointing to the modern life style’s influence on the immune system. The hypothesis is controversial and studies supporting [23] and contradicting [24] the theory have been published.

Environmental factors and pollutants

Changed environmental factors may have had an impact on the increased incidence of allergy. Studies have pointed out pollutants, vaccinations and changed food intake as being responsible for a shift in the Th1/Th2 balance towards Th2 [25, 26].

Pollutants such as diesel exhaust particles increase IgE production, increase T-cell reactivity in asthmatics and act as an adjuvant to induce IgE, even to non-allergens [27-29]. Other pollutants such as O₃, NO₂ and SO₃ have been demonstrated to increase airway responses to inhaled allergens in susceptible persons such as those with rhinitis and asthma [25].

A decrease in childhood infections, both bacterial and viral, may increase the risk of developing allergies [26]. The food that we ingest may also influence the allergic demographic pattern. Fruit and vegetable ingestion leads to intake of anti-oxidants such as flavonoids and vitamin C, which may have a protective role against allergy [30-32]. Modern western world fat intake with a high omega-6/omega-3 ratio, up to 15/1, has increased from a ratio of around 1 [33]. This might adversely influence the allergy and asthma epidemic since omega 3 acids have anti-inflammatory properties [34-36]. However, the role of the diet in regard to asthma has been reviewed and the results are not always easy to interpret, but it seems beneficial to eat natural food, especially fruit and vegetables [37].

Genetic factors

The unclear genetic association also reflects the complex nature of allergic diseases. A number of candidate gene regions for allergy and asthma have been suggested as a result of several genome-wide studies and candidate-gene association studies [38, 39]. There are different groups of candidate genes associated with innate immunity and immunoregulation, Th2-cell differentiation and effector function, lung function or epithelial cells [40]. Many chromosomes, especially 5, 6, 11, 12 and 13, have been linked to atopy. Chromosome 5 interestingly harbours the Th2 cytokines IL-4, IL-5 and also one of the most studied of the candidate genes for asthma and allergy, IL-13, and chromosome 11 harbours FcεRI. Several other important susceptibility genes for
allergy and asthma have been identified such as a disintegrin and metalloproteinase 33 (ADAM33), neuropeptide s receptor 1 (NPSR1) and uteroglobin for asthma and IL-10 and TGFB1 for allergy [40-42]. It should be pointed out that some of the most promising genes have failed to show an association to allergy and asthma in several studies, but the reasons for this are debated [43, 44].

ALLERGENS

Allergens are proteins or glycoproteins, typically 10-70 kilodaltons (kDa) in size. Allergens are nowadays named according to a standardized nomenclature, adopted by the World Health Organization and maintained by the International Union of Immunological Societies. The names start with three letters indicating the genus followed by a single letter for the species and then a number indicating the order of identification [45]. Not all allergens are equally important, so allergens are divided into minor and major allergens. If a study shows that more than 50% of a group of patients sensitized to a certain allergen source, e.g. cat dander, are sensitized to a particular allergen within that allergen source, e.g. Fel d 1, then this is a major allergen. But if 5-50% of the patients recognize a certain allergen then this is considered a minor allergen. Allergens can generally be divided into 3 groups:

1. Indoor allergens
2. Pollen allergens
3. Plant and animal food allergens

No unifying structure or motif has been recognized among allergens [46]. However, epitopes are shared by multiple allergens and major allergen families have been identified [45, 47].

Recent advances in molecular biology, bioinformatics and modern analytical methods have radically increased the knowledge of allergen structures, functions and biological relevance [48]. Allergens have diverse biological functions. They may for example be enzymes, enzyme inhibitors or structural proteins. The structure of many allergens has been determined by nuclear magnetic resonance or X-ray crystallography. More than 40 are listed in the Structural Database of Allergenic Proteins. This has led to better understanding of the relationships between allergens and epitopes. For example, solving the structure of the major cat allergen Fel d 1 by X-ray crystallography revealed a striking 3 dimensional (3D) structural similarity to human uteroglobin, despite having only 20% sequence identity [49]. Detailed information about one of the dominant IgE epitopes of the major birch allergen Bet v 1 has been obtained by co-crystallization of an IgE blocking Fab fragment and the allergen [50]. Almost 30 proteins with determined IgE epitopes are listed in the Structural Database of Allergenic Proteins. As in the case of the identified Bet v 1 epitope, epitopes are mostly conformational and discontinuous. The epitopes might cover regions of the polypeptide chain that the secondary, tertiary, and quaternary structure of the protein have brought together, regions that are distant from each other in the primary structure [51]. T-cell epitopes, conversely, are short, 8-10 amino acids for MHC class I and 13-17 amino acids for MHC class II, non-conformational peptides of the protein. The epitopes are scattered throughout the entire amino acid sequence and are independent of the B-cell epitopes [52-55].
Evolutionarily conserved allergens can be found in different allergen sources, giving rise to cross-reactivity. Proteins can be called cross-reactive if at least one antibody binds both these different proteins. It has been reported that the main criteria for cross-reactivity is high similarity in the 3D structure, rather than high sequence homology [56, 57]. One of the cross-reactive group of allergens, lipocalins, often share less than 20% sequence identity but high 3D structure similarity [58]. Allergens can occur as different isoallergens or isoforms, which are allergens with only minor differences in their amino acid sequence [45]. However, those differences might be important, for example different isoforms of the major birch allergen, Bet v 1, induce different immune responses [59, 60].

When describing the affinity of protein-ligand complexes, the dissociation constant ($K_d$), is often used. It is the ratio between the dissociation rate and association rate. $K_d$ is usually $10^{-7}$-$10^{-11}$ molar (M) for antibody-antigen complexes and $10^2$-$10^6$ for MHC-T-cell receptor complexes. The affinity of IgE-binding to an allergen is usually very high, in the order of $10^{-10}$ M, whereas IgG-binding affinity to the same allergen has been reported to be $10^{-3}$ fold lower [61-63]. However, one study demonstrated much higher IgG-binding, between $10^{-8}$ and $10^{-12}$ M [64]. Most IgE-allergen binding occurs to the backbone of the protein, but there are exceptions. For example, the cross-reactive carbohydrate determinants (CCDs) are glycan IgE epitopes present on related and unrelated plant proteins [65-67]. IgE-reactivity has also been demonstrated to the glycan moiety in insect venoms [65, 68, 69] and seafood [70]. Ambiguous results have been demonstrated from histamine release experiments employing glycosylated allergens [71, 72], where they have been suggested to have low, or even no clinical relevance [71, 73]. Low binding affinity has been suggested as the reason for the poor biological activity of glycodeterminants since IgE-epitope affinity has been demonstrated to influence the efficiency of histamine release [74, 75]. Low affinity was also suggested as a reason for negative skin prick test results despite the presence of allergen-specific IgE [61]. However, a recent study has demonstrated that the affinity of IgE to CCD is as high as that to “normal” allergens [62]. Interestingly, the ratio of IgE-/IgG-affinity was much lower for CCD, suggesting better blocking capabilities for CCD-specific IgG compared to “normal” allergen-specific IgG. Immune reactivity to glycans on vertebrate proteins is rare, not surprisingly since most glycan structures are shared between vertebrates [76]. Human anti-Gal antibodies, directed to the α-gal epitope (Galα1-3Galβ1-4GlcNAc-R), are one of the biggest obstacles for successful pig-to-human xenotransplantation [77]. Chung et al. have recently demonstrated IgE antibodies directed to this same epitope present on a chimeric mouse-human IgG1 monoclonal antibody called Cetuximab [78]. Cetuximab is directed against the epithelial growth factor receptor and is used for treatment against colorectal cancer and squamous-cell carcinoma of the head and neck. These IgE antibodies have been demonstrated to cause severe allergic reactions.

**Cat allergens**

Cat allergens are one of the most powerful triggers of allergy [79, 80]. Allergens from cat abound in public facilities and are often transported by the clothes of pet owners, making these allergens difficult to avoid [81, 82]. Cat dander extract has suggested many IgE-binding proteins [83], but far from all have been identified and added to the official allergen list. Fel d 1 is the dominant major allergen [84]. It was identified in 1974 [84] and was originally named Cat 1. More than 90% of cat allergic patients are reactive to Fel d 1 [85] and up to 60% of cat dander-specific IgE is directed to Fel d 1 [86]. Fel d 1 is a tetrameric glycoprotein formed by 2 heterodimers [87, 88]. Fel d 1
was cloned in 1991 [89] and the 2 polypeptide chains (chain 1 and 2) originate from different chromosomes [90]. A recombinant variant (rFel d 1 2+1) with biological and immunological properties indistinguishable from the natural Fel d 1 (nFel d 1) has been produced [91]. The 3D structure of this recombinant variant has been solved and great structural similarities, despite low sequence match, was determined with uteroglobin [49].

Another major allergen is Fel d 4 (lipocalin) [92], although IgE responses to this allergen are generally low. Fel d 2 (albumin) and Fel d 3 (cystatin) are minor allergens. The former is cross-reactive with albumin from other mammals [93, 94], while the latter is potentially cross-reactive with other cystatin allergens since it belongs to the group of highly conserved cysteine inhibitors [95].

Cat immunoglobulins

Cat IgG was proposed as an allergen in 1976, but no concluding evidence was presented at the time [96]. The only IgG of listed allergens is the food related cow and milk allergen Bos d 7 [97]. IgA, IgG and IgM in cat were identified in 1968 by identification of similarities in immunoelectrophoretic patterns in cat, rabbit and human serum [98]. IgA in vertebrates, not including primates, is a dimeric molecule with an approximate molecular weight of 350 kDa. The IgA monomer consists of κ or λ light chains and α heavy chains joined by disulfide bonds. The dimers are formed by disulphide linked monomers and a J chain. IgG is monomeric, has an approximate molecular weight of 160 kDa and consists of κ or λ light chains and γ heavy chains. IgM is a pentameric molecule with a molecular weight of 800-1000 kDa. The IgM monomers consist of κ or λ light chains and μ heavy chains. Each pentamer consists of disulfide-linked monomers and a J chain. The concentrations of IgA, IgG and IgM in healthy adult cats is estimated at 2.6, 19.08 and 2.04 mg/ml, respectively, in serum and 0.54, 0.10 and 0.13 mg/ml, respectively, in saliva [99].

ALLERGEN CLONING

The first allergen, Der p 1, was cloned in 1988 [100] and since then numerous others have been cloned. Purified recombinant allergens have been crucial for studies at a molecular level and for evaluation of the immunological responses to the individual allergen components. It is essential to have complementary deoxyribonucleic acid (cDNA) containing the sequence for the desired cloning object. One method for cloning is to use reverse transcriptase polymerase chain reaction (RT-PCR). Knowledge of the DNA sequences from homologous proteins or from a short stretch of the allergen desired to clone is necessary in order to design the needed primers [101, 102]. Another approach is to use IgE immunoscreening of expression cDNA libraries [103, 104] or filamentous phage displayed cDNA libraries [105-107]. One of the major advantages of immunoscreening over RT-PCR is the possibility to screen for unknown allergens. It can be performed by using serum from patients with IgE to allergen extract from the source of interest. An advantage of filamentous phage display cloning lies in the physical linkage of each protein to the genetic material that it encodes. This is accomplished by fusing displayed proteins to phage-coated proteins. Even high throughput screening for rapidly obtaining recombinant allergens can be performed using phage display [108].
The cloned allergens can be expressed and a number of different systems are available for this purpose. The prokaryotic Escherichia coli is the oldest and most often used expression system for recombinant proteins [109]. Prokaryotic systems are cost-effective and may produce large amounts of protein. Many expression vectors and systems for purification, such as tags or fusion proteins, are commercially available. Improper folding and aggregation sometimes occurs and time consuming in vitro refolding may be necessary. Many eukaryotic expression systems such as yeast (Saccharomyces cerevisiae and Pichia pastoris), insect (by baculovirus infection), mammalian cell (Cos-1) and plant (by tobacco mosaic virus infection) systems have been used successfully for allergen production. The biggest advantage of eukaryotic systems is that post-translational modifications can be incorporated. However, the cost is often high compared to prokaryotic systems and the yields are often low.

Recombinant allergens often display identical biological functions as their native counterparts, which make them viable options for use in diagnosis and treatment [110].

ALLERGY DIAGNOSIS

The two methods for allergy diagnosis used today are in vivo skin prick test and in vitro IgE determination [111, 112]. The in vivo tests originate from experiments already performed in 1831. The common procedure for skin prick test is to measure the wheal and flare reaction after introducing allergen into the skin via a lancet, the size of the wheal and flare being graded [111]. The in vitro diagnostic tests were introduced in 1967 after the discovery of IgE. They measure IgE antibody levels in serum against an allergen component or allergen extract [113, 114]. One commercially available in vitro system is the CAP system (Phadia AB, Uppsala, Sweden), which provides solid phase coupled allergen extracts or single allergens. Crude allergen extracts or purified allergens from natural sources are used in both in vivo and in vitro methods. More recently, recombinant allergens have also been introduced into these protocols either separately or by spiking natural extracts [115]. Recombinant allergens can be produced as pure proteins which can be standardized, unlike allergenic extracts [104].

Future allergy diagnosis

Future diagnosis will most certainly be based on a microarray technology and utilise panels of recombinant or purified single allergens [116-120]. Microarray diagnosis allows screening for a range of allergens on a chip. In a short time and with a minimum amount of serum, a patient can be analyzed for IgE antibodies to a wide range of recombinant allergens in one single analysis [121, 122]. Since small amounts of allergens are spotted onto the chips the conditions are quite different from today’s in vitro system in which allergen excess is the goal. An excess of solid phase bound allergen has been proposed as being an advantage, but some consider that the condition in the microarrays is beneficial since it may more closely mimic the conditions in patients in which allergens are only present in very small amounts [121]. Microarrays may also be beneficial for epitope investigation, as has been demonstrated in a study with overlapping peptides derived from peanut allergens [123].

Component resolved diagnostics (CRD) make use of a number of recombinant or purified allergens to determine each patient’s sensitization profile. A great advantage is
the possibility to identify allergens that are considered minor but which in individual patients might be the major contributing allergen [124]. The allergens can be used separately or mixed as cocktails to resemble the IgE epitope complexity in the natural extracts [125].

**ALLERGY VACCINATION**

Allergy vaccination is the only curative treatment for allergic diseases. It has been practised for nearly one hundred years [126] and is still the only therapy giving permanent relief of allergic disease symptoms.

Allergy vaccination is a time consuming treatment that takes 3-5 years with up to 20 subcutaneous injections per year of crude allergen extract [127]. The protocols start with an up-dosing period with low doses of allergens that are gradually increased, followed by a maintenance period [128]. Injections should only be administered by trained personnel, since there are risks involved, such as severe side-effects which could ultimately be fatal [129]. Alternative routes for administrating the allergen extract today include the sublingual route [130]. This is a promising alternative to subcutaneous vaccination but it is still being evaluated [131].

Treatment of allergy by using humanised monoclonal anti-IgE is another relatively new option that has had some promise in clinical trials. However, the long-term effects have not been sufficiently evaluated [132].

**Allergen extract**

Crude allergen extracts are currently used for allergy vaccination. The allergen is coupled to an adjuvant, most often aluminium hydroxide (alum). Even though the recommendation from World Health Organisation is to use 5-20 µg of major allergen per maintenance shot [129], the concentration of the major allergens is not given for allergen extracts. The protocols are instead designed on the basis of inconsistent company specific units [133]. Large differences in allergen content have been discerned between different batches of extracts and between extracts from different manufacturers [133-136]. Additionally, large differences in the content of endotoxins and β-glucans have been discovered in allergen extracts, which may result in their different immunomodulatory properties [137, 138]. De novo sensitization is another concern, since the extracts also contain allergens that the patient was not originally sensitized to. Injecting these allergens might promote the production of IgE to the newly encountered allergens [139, 140].

**Mechanisms**

The mechanisms underlying successful allergy vaccination are not completely understood and are still debated [141]. A decrease of IL-4 and IL-5 and an increase of IFN-γ, representing a shift from a Th2 to more of a Th1 cytokine profile, have been demonstrated following successful allergy vaccination [18, 142, 143]. An increase of IL-10 production by APCs and an induction of IL-10 and TGF-β-secreting Tregs are also apparent and seem to play a part by suppressing IgE and increasing IgG4 production [144-146]. IL-10 also reduces pro-inflammatory cytokine release from mast
cells, down-regulates eosinophil function and activity and suppresses IL-5 from resting Th0 and Th2 cells [147-149].

Increase of IgG with the same specificity as the patient’s allergen-specific IgE, so-called “blocking antibodies” leads to a significant improvement of clinical symptoms and quality of life [150, 151]. The reasons for these positive effects are thought to be several. Blocking antibodies, which compete with IgE for allergen-binding, protect against histamine release from basophils and mast cells [152]. They also inhibit anaphylaxis by down-regulation via the FcγRIIb receptor [153]. Blocking antibodies are mostly IgG4 [154] and IgG1 [150, 151] with little or no amounts of IgG2 and IgG3.

New allergy vaccines

By use of modern biotechnology hundreds of recombinant allergens have been produced and tested. The use of these proteins in immunotherapy have numerous advantages over the crude allergen extracts currently used [110]. Vaccines with standardised, pure and controlled amounts of allergens produced according to Good Manufacturing Practices can be used [155]. Successful vaccination has been conducted with recombinant grass pollen allergens (rPhl p 1, rPhl p 2 rPhl p 5a rPhl p 5b and rPhl p 6) [151].

Different recombinant so-called “hypoallergens” have been created in order to reduce IgE-binding but maintain T-cell reactivity. Using hypoallergens, allergy vaccination could in theory lead to safe, fast and cost-effective treatment. Different techniques have been used to obtain these hypoallergens [156-160]. Allergy vaccination of birch pollen allergic patients using hypoallergens derived from Bet v 1 has shown promising results [161]. A rise in allergen-specific IgG and decline in allergen-specific IgE was observed.

T-cell epitope containing peptides have also been used in clinical trials and demonstrated to be successful for cat and bee venom allergies [162-165]. The initial studies were associated with late phase reactions [164, 166]. However, recent studies, using lower amounts of peptides have demonstrated clinical benefits with reduced side-effects [167].

Other more experimental methods have also been investigated and blocking antibodies have been successfully induced in mice after administering large amounts of mimotopes and B-cell epitope-containing peptides [168, 169]. The mimotopes were derived from phage display peptide libraries that were panned with anti-Bet v 1 monoclonal antibodies.

Thus the knowledge of how to produce better vaccines is already established.
AIMS OF THE THESIS

The main focus of this thesis was to characterize allergens from cat, one of the most important allergen sources worldwide and in addition to investigate the antigen-antibody interactions in immunoassays.

I. To identify and characterize novel cat allergens present in cat dander extract.

II. To characterize IgE-binding properties in candidates identified in paper I.

III. To crystallize a recombinant variant of the major cat allergen, Fel d 1, and compare its biological properties to the natural counterpart.

IV. To compare the use of a recombinant variant of the major cat allergen, Fel d 1, to a commercial cat dander-based test for diagnosis of cat allergic adults and children.
MATERIALS AND METHODS

BASOPHIL STIMULATION

Blood samples were collected in heparinised tubes and aliquoted into fluorescent-activated cell sorting (FACS) tubes [III]. Controls and serial dilutions of antigen were added for stimulation. Stimulation was stopped and fluorescein-conjugated anti-CD63 and phycoerythrin-conjugated anti-CD203c monoclonal antibodies were added. Erythrocytes were lysed and leukocytes were collected by centrifugation, washed and resuspended in PBS. The cells were analysed by two-colour flow cytometry using a FACSCalibur instrument. The basophil activation was calculated as the percentage of CD63+ events among the 200 events in the basophil gate (CD63/CD203c).

BIOCHEMICAL ANALYSIS

Protein concentration was measured using the bicinchononic acid (BCA) protein assay using bovine plasma gamma globulin or bovine serum albumin as standard [I, II]. Purity was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 5% and 15% homogeneous gels with low molecular weight markers under either reducing or non-reducing conditions. Purity was also analysed by size exclusion chromatography (SEC) and by the use of specific anti-cat protein antibodies in Western blots.

CAP ANALYSIS

Cat IgA [II] and recombinant Fel d 1 [IV] were covalently coupled to the solid phase of ImmunoCAP by MIAB (Uppsala, Sweden). IgE [II, IV] and IgG4 [IV] responses were measured to cat IgA and rFel d 1 ImmunoCAPs. IgE and IgG4 responses were also tested against commercially available cat dander ImmunoCAPs [IV].

ELISA ANALYSIS

Enzyme-linked immunosorbent assay (ELISA) was performed in 96-hole plates to evaluate specific antibody-binding to immobilized antigen [I, II, III, IV]. Sandwich ELISA assays were performed with serum or different anti-cat protein antibodies, either diluted in buffer or in buffer with added competitive proteins. Thereafter, appropriate secondary antibodies were added followed by conjugated tertiary antibodies and substrate. Washing was performed between each step.

PATIENT SAMPLES

Serum was obtained from cat-sensitized patients based on levels of allergen-specific IgE to cat dander extract [I, II], from cow- or dog-sensitized [I] and non-sensitized subjects [I]. Sera from patients with a doctor’s diagnosed allergy were obtained as well as from healthy or non-sensitized subjects [IV]. Blood samples from 4 cat allergic patients and one healthy control were also obtained [III].
PROTEIN PURIFICATION

Sera from cats (Statens Veterinärmedicinska Anstalt, Uppsala, Sweden) were pooled and used as source for protein purification [I, II]. Affinity purification of cat IgA was performed with either \((Z_{IgA1})_2\)-His\(_6\) affibody (kindly provided by Affibody AB) [170] or monoclonal anti-human IgA with cross-reactivity to cat IgA, while Protein A was used for cat IgG purification. Further purification was performed by SEC. Deglycosylation of cat IgA was performed using PNGase F (Sigma). Cat albumin was purified from cat serum using Blue Sepharose and Q Sepharose ion exchange chromatography [II].

WESTERN BLOTTING

Proteins were separated by SDS-PAGE and transferred to membranes to illustrate molecular sizes of antigens bound by specific antibodies [I, II]. Membranes were cut into strips and sandwich assays were performed with serum or different anti-cat protein antibodies, either diluted in buffer or in buffer with added competitive proteins. Thereafter appropriate secondary antibodies were added followed by conjugated tertiary antibodies and substrate. Washing was performed between each step.
RESULTS AND DISCUSSION

IDENTIFICATION AND CHARACTERIZATION OF NEW CAT ALLERGENS [I, II]

Only a small number of cat allergens have been characterized to date despite that several IgE-binding proteins have been observed by immunoblotting and other techniques. Knowledge of additional cat allergens would improve diagnosis and future treatment of cat allergy and increase the understanding of the mechanisms underlying the allergic disease.

Identification of uncharacterized IgE-binding proteins in cat dander extract [I]

In an attempt to characterize new IgE-binding proteins in cat dander we used 10 cat-sensitized sera. Heavily stained bands in the high molecular area, around 64 and 94 kDa, representing novel allergens were observed. The three previously confirmed cat allergens were Fel d 1 (20 kDa), Fel d 2 (albumin, 68 kDa) and Fel d 3 (cystatin, 11 kDa). Albumin as the binder of 64 kDa was ruled out since the band was slightly smaller and occurred more frequently. Earlier unpublished data prompted us to investigate if these bands could correspond to immunoglobulin α chain and µ chain.

Purification and analysis of cat immunoglobulins [I, II]

Immunoglobulin proteins were purified from serum (IgA and IgG) or commercially obtained (IgM) and demonstrated to be highly pure. All proteins exhibited a 28 kDa band in reduced SDS-PAGE, corresponding to immunoglobulin light chains (Figure 3). IgA exhibited an additional 24 kDa band, presumably corresponding to the J chain. The heavy chains migrated at the anticipated sizes, 55, 64 and 94 kDa for IgG, IgA and IgM, respectively. Furthermore, SDS-PAGE under non-reducing conditions and SEC verified the purity and correct sizes of the intact proteins, 150, 350 and 800-1000 kDa, respectively [171, 172]. Using affinity purified isotype-specific anti-cat immunoglobulin antibodies to probe immunoblotted proteins confirmed the proteins as being cat immunoglobulins. Only about 1% IgA and 1% IgG were present in the IgM preparation and less than 1% IgG and less than 10% IgM in the IgA preparation.

Investigation of IgE-binding to cat immunoglobulins [I]

We investigated the influence of cat immunoglobulins on the staining pattern of immunoblotted cat dander by competitive inhibition of patient sera. In a pool of 10 cat-sensitized sera we observed no effect after pre-incubation with IgG,
whereas pre-incubation with either IgA or IgM abolished both the 64 and 94 kDa bands (Figure 4). Pre-incubation with the control protein, Fel d 1, only affected low molecular weight proteins, corresponding to the two chains of nFel d 1. We also recorded elimination of both the 64 and 94 kDa bands in cat dander immunoblotting after pre-incubation with cat IgA in 10 individual patients. The immunoblotting experiments with cat dander extract suggested that that cat IgA and IgM seemed to be highly IgE-binding and potentially cross-reacting, but the proposed cat allergen IgG was of minor importance. To investigate this further, ELISA analyses were performed and we observed that 67 of 82 cat allergic patients and 9 of 19 controls were positive to cat IgA. Competitive inhibition ELISA revealed complete mutual cross-reactivity between IgA and IgM, whereas IgG had a 50-100 fold lower inhibitory capacity. Fel d 1 exhibited no inhibitory capacity. The results from the competitive inhibition ELISA and immunoblotting were intriguing since complete mutual cross-reactivity between different isotypes is unlikely.

We also investigated IgE-reactivity to IgM from other species by direct ELISA (Table I). We observed a high reactivity to IgM from 6 species whereas no reactivity was evident to IgM from 2 species. A high correlation between IgE-reactivity to cat IgM and to IgM from the 6 other reacting species was observed. These results indicated a common IgE-binding structure present on immunoglobulin M from many different species. We tested the hypothesis that these proteins had a common post-translational modification and glycosylation was the first structure we investigated.

Table I. IgE-reactivity to IgM from different species expressed as mean optical density (OD, 405 nm) for 20 individuals and correlated to reactivity to cat IgM.

<table>
<thead>
<tr>
<th>Species</th>
<th>OD-mean</th>
<th>Spearman R</th>
<th>p-level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dog</td>
<td>0.75</td>
<td>0.98</td>
<td>0.00</td>
</tr>
<tr>
<td>Pig</td>
<td>0.76</td>
<td>0.97</td>
<td>0.00</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.10</td>
<td>0.93</td>
<td>0.00</td>
</tr>
<tr>
<td>Horse</td>
<td>0.55</td>
<td>0.91</td>
<td>0.00</td>
</tr>
<tr>
<td>Cow</td>
<td>0.50</td>
<td>0.87</td>
<td>0.00</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.15</td>
<td>0.68</td>
<td>0.00</td>
</tr>
<tr>
<td>Hamster</td>
<td>0.07</td>
<td>0.60</td>
<td>0.01</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>0.09</td>
<td>0.10</td>
<td>0.67</td>
</tr>
</tbody>
</table>
When cat IgA was treated with PNGase F no IgE-reactivity was observed. Reactivity to non-treated IgA and sham-treated IgA was apparent in the same separate and pooled sera. Specific anti-cat IgA antibodies recognized deglycosylated cat IgA as well or even better than natural IgA. We therefore concluded that the protein structure had not been disrupted through deglycosylation. The IgE responses must thus be a result of the carbohydrates, existing on cat IgA, cat IgM and IgM from many other species. IgE epitopes in the glycan moiety have only previously been described in allergens from plants, insects and seafood, but not in mammalian allergens.

Control experiments of IgE-binding to cat immunoglobulins [I]

Since our systems had been set up in order to investigate IgE-reactivity to allergens with protein epitopes we performed extra controls to thoroughly verify our assays. The influence of allergen-specific human IgE was investigated. Myeloma-derived IgE was added in increasing amounts to the rabbit anti-human IgE step in the direct ELISA using a pool of sera. There was no dose-dependent decrease in reactivity, indicating that it was independent of IgE. Omitting rabbit anti-human IgE as a second control also only resulted in a small decrease in reactivity. These data supported the first control, suggesting an IgE-independent reactivity.

We hypothesized that a serum component could be responsible for cross-linking of the solid phase bound immunoglobulins and the conjugated antibodies. SEC of positive serum and addition of the different fractions to a cat IgA-coated ELISA plate revealed a high molecular weight factor causing the cross-linking. We observed that alkaline phosphatase (ALP)-conjugated goat anti-rabbit as well as ALP-conjugated streptavidin could be used, indicating that it was common carbohydrate structures on the immunoglobulins and on ALP that were cross-linked by a large serum component. The size of the cross-linking protein led us to suspect human IgM as being the interfering protein. Furthermore, a positive serum pool that was adsorbed over rabbit anti-human IgM removed most of the reactivity, and it could be detected in the eluate. No reduction of reactivity was recorded when using normal rabbit-IgG. We therefore concluded that human IgM with specificity for the glycan moiety of animal proteins was cross-linking the conjugate and coated proteins, and thereby created false positive signals in our study. To eliminate the cross-reactivity between the conjugate and animal immunoglobulins we evaluated a different conjugate, horseradish peroxidase (HRP). Since HRP originates from the plant kingdom, we assumed that cross-reactivity would be minimal. A pool of sera confirmed this assumption. Positive signals were apparent using both the ALP and HRP systems, although higher reactivity was noted using ALP. When the rabbit anti-human IgE antibodies were removed, we still observed positive signals using the ALP system but not using the HRP system, indicating that the IgE-reactivity was directed to cat IgA.

Study of IgE-binding to cat immunoglobulins using other systems [II]

To confirm the positive signals evident in ELISA using HRP substrate we next used the ImmunoCAP system, which utilizes β-D-galactosidase as substrate. In addition we used HRP in ELISA and immunoblotting assays. The ImmunoCAP system revealed that 38% of cat-sensitized sera (31 of 81) were positive to cat IgA (>0.2 kU/L). No
correlation between IgE-reactivity to cat IgA and cat dander extract was seen. This indicates that IgA is not a dominant allergenic component in cat dander.

In study I we demonstrated epitopes in the carbohydrate moieties. We next examined the IgE-reactivity of 21 cat IgA CAP positive sera to natural and deglycosylated IgA and IgM in ELISA using HRP as substrate. We determined that only 4 sera had reactivity to deglycosylated cat IgA. Furthermore, a high correlation between IgE to IgA and IgM was evident. Since carbohydrate epitopes were present on very diverse proteins in study I we wanted to examine if similar cross-reactivity could be demonstrated for this IgE-binding. To this end we performed inhibition ELISA using plate-bound cat IgA using sera from 9 separate patients and 5 different proteins (Figure 5). IgA and IgM gave almost complete inhibition, suggesting that cat IgA and cat IgM share carbohydrate epitopes. ALP was similarly inhibitory as cat IgA in 4 patients, gave 26% inhibition in one patient and no inhibition in the remaining 4 sera. This suggests that only partial epitope structures are shared between cat IgA and ALP.

Immunoblotting experiments demonstrated IgE-reactivity to cat IgA heavy chains in all cat IgA CAP positive patients tested. One patient also reacted to the light chain. Specific anti-cat IgA antibodies demonstrated the existence of cat IgA in 3 different batches of cat dander extract. No visible staining of blotted deglycosylated cat IgA in a pool of 21 cat IgA positive sera was observed, whereas the same pool stained natural cat IgA heavy chain.

**Figure 5.** Competition of IgE responses to cat IgA in 9 cat-sensitized patients using ELISA. Y-axis, (optical density 405 nm) after pre-incubation with buffer, rFel d 1, cat serum albumin (CSA), calf intestine alkaline phosphatase (CIP, same as ALP), IgM and IgA.
In conclusion [I, II], we identified both human IgM and human IgE antibodies directed to the same carbohydrate epitopes present on a variety of animal proteins including cat IgA, IgM and ALP. The heterophile IgM should be taken into account in allergy diagnostics whenever using human serum and ALP in immunoassays due to the risk of interference.

IN DEPTH CHARACTERISATION AND IMMUNOLOGICAL EVALUATION OF THE MAJOR CAT ALLERGEN FEL D 1 [III, IV]

A recombinant construct of Fel d 1 with chain 2 linked to chain 1 (Fel d 1 (2+1)) has earlier been crystallized [49] and demonstrated to have very similar structural, immunological and biological properties as nFel d 1 [91].

Recombinant Fel d 1 (1+2) mimics the biological and immunological properties of natural Fel d 1 [III]

In this study we compared rFel d 1 (2+1) with another recombinant Fel d 1 construct with chain 1 linked to chain 2 (rFel d 1 (1+2)). To establish that rFel d 1 (1+2) could mimic nFel d 1 in terms of biological and immunological reactivity, different analyses were performed. With lymphoproliferative stimulation of peripheral blood mononuclear cells derived from 4 cat allergic patients, rFel d 1 (1+2) could stimulate the peripheral blood mononuclear cells at all tested concentrations. When the T-cell stimulatory effect of rFel d 1 (1+2) and rFel d 1 (2+1) were compared by measuring proliferation of CD3⁺/CD4⁺ T-helper cells, we determined that they were equally active. It should be noted that the endotoxin levels were low in both preparations, less than 5 EU/mg protein. To evaluate IgE-binding, both direct ELISA and inhibition ELISA were performed with rFel d 1 (1+2), rFel d 1 (2+1) and nFel d 1. Similar IgE-reactivity and dose-dependent inhibition was noted. Finally, basophil stimulation was performed to compare the biological reactivity in vitro. All allergic patients displayed a nearly identical dose-dependent degranulation to all three preparations. Based on these results we could conclude that rFel d 1 (1+2) and Fel d 1 (2+1) mimic nFel d 1.

![Figure 6. 3D-structure of rFel d 1 (1+2) subunits (yellow and blue, respectively) superimposition demonstrates the high similarities except in the interface. Red balls represent calcium ions.](image-url)
The crystal structure of tetrameric Fel d 1 reveals calcium-binding sites [III]

The structure of rFel d 1 (1+2) was solved by molecular replacement using rFel d 1 (2+1) as a model. The final model consisted of 2 rFel d 1 (1+2) molecules with an internal and external calcium-binding sites and 270 water molecules. The electron density map was of high quality with well-defined polypeptide chains. The 3D structure revealed a tetrameric variant with 2 Fel d 1 (1+2) molecules at a resolution of 1.6 Å. Each chain of Fel d 1 consists of 4 helices, H1-H4 on chain 1 and H4-H8 on chain 2, which fold into a globular all helical structure. The dimerization interface mostly comprises of helices H5 and H8 in both subunits. It utilizes 65 residues with additional water molecules and a calcium ion present between the 2 subunits. A previously described cavity in Fel d 1 is located in the interface between the 2 subunits. A hydrophobic patch is made up of 6 residues and hydrogen bonds exist between the subunits. The calcium ion present in the interface between the 2 rFel d 1 (1+2) molecules in the tetramer causes local conformational changes. The calcium ion is coordinated by 7 oxygen ligands, a carbonyl group and 3 water molecules. The conformational changes make the cavities in the subunits non-identical. Due to the calcium ion many acidic residues are tolerated in the dimerization interface and it therefore probably stabilizes the tetrameric form of the molecule. As a result of the calcium-binding, one of the cavities is more than twice as large as the other (Figure 6).

The true ligand for the cavity is still unknown. Since the structure of Fel d 1 is 20% similar to that of uteroglobin, one could speculate that the ligand could be progesterone, as has been suggested for uteroglobin [173]. Interestingly, the external calcium-binding sites were located symmetrically on each side of the heterodimer, in the same way as has been described for uteroglobin. As a PLA2-inhibitory effect has been demonstrated for uteroglobin and determined to rely on the calcium-binding function, a similar function may therefore be suggested for Fel d 1.

Recombinant Fel d 1 is as good as cat dander extract in diagnostic use [IV]

The use of dander extract in diagnosis and therapy is well established. There are associated concerns, however, such as variations in amounts and quality of allergens. The use of recombinant allergens has therefore been proposed as an alternative diagnostic tool. Depending on the dominating allergen in the extract, even a single molecule can be used, such is the case with Fel d 1. We evaluated IgE and IgG4 levels to rFel d 1 using serum from cat allergic children and adults with

![Figure 7. Correlation between CDE and rFel d 1.](image-url)
rhinoconjunctivitis (RC) or allergic asthma from Sweden and Austria using ImmunoCAP and ELISA.

A total of 65 out of 68 children and 68 out of 72 adults were positive using rFel d 1 CAP, whereas 64 children and 71 adults were positive using cat dander extract (CDE) CAP. We determined a high correlation between CDE and rFel d 1, \( r_s = 0.85, p<0.001 \) (Figure 7). The IgE responses to rFel d 1 CAP were on average 30% higher than to the CDE CAP \( p<0.0001 \). This might be due to the approximately 3 times lower levels of Fel d 1 in the CDE ImmunoCAP compared to the rFel d 1 CAP. This could depend on the fact that Fel d 1 in CDE may have to compete for space on the solid phase with other molecules and is thus less abundant.

In another study in which cat positive sera were tested for IgE to a not fully refolded variant of recombinant Fel d 1 and nFel d 1, 96% and 94% were positive, respectively \[85, 174\]. Our study reveals that a fully refolded Fel d 1 identifies more allergic children than does the CDE CAP, and identifies 95% of the CDE CAP positive cat allergic adults.

Using ELISA all children and 69 of 72 adults were positive to rFel d 1, which demonstrates an even higher sensitivity and specificity. Addition of other allergens, such as Fel d 2-6, might improve the sensitivity and identify the rare cases of patients mono-sensitized to these other allergens. The IgE-reactivity to rFel d 1 in ELISA was closely related in comparison to rFel d 1 CAP and CDE \( r_s = 0.94 \) and 0.89, respectively, \( p<0.001 \).

Total IgE levels did not differ between patient nationality \( p>0.05 \) but differed between age groups \( p<0.01 \) (Table II). No difference in total IgE levels was noted between patients with asthma or RC \( p>0.05 \). Our observation that children have higher specific and total IgE levels than do adults is in agreement with previous observations and is suggested to be due to a generally less active immune system among adults \[175\].

Asthmatic children had significantly higher IgE-levels to Fel d 1 than did children with RC alone. Furthermore, children with asthma or only RC had significantly higher IgE levels to Fel d 1 (median 19.4 and 6.6 kU/L) than did adults (median 3.0 and 2.9 kU/L). Sensitization to allergens and especially to cat dander is suggested to be a risk factor for asthma \[176\]. Thus, high IgE levels to rFel d 1 among children seem to be associated with an increased risk of asthma either as a secondary phenomenon or as a pathogenic factor.

### Table II. Comparison of antibody responses in serum from cat allergic Swedish and Austrian children and adult patients.

<table>
<thead>
<tr>
<th>Cat allergen and test system</th>
<th>Ab</th>
<th>Children</th>
<th>Adults</th>
<th>p-value&lt;sup&gt;6&lt;/sup&gt; (Children vs adults)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sweden</td>
<td>Austria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median, range</td>
<td>Median, range</td>
<td>Median, range</td>
</tr>
<tr>
<td>CDE, CAP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>IgE</td>
<td>6.7, &lt;0.35-230</td>
<td>12.8, &lt;0.35-730</td>
<td>2.3, &lt;0.35-38.0</td>
</tr>
<tr>
<td>rFel d 1, CAP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>IgE</td>
<td>10.6, &lt;0.35-258</td>
<td>18.2, &lt;0.35-473</td>
<td>3.2, &lt;0.35-73.4</td>
</tr>
<tr>
<td>rFel d 1, ELISA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>IgE</td>
<td>0.69, 0.071-2.50</td>
<td>0.73, 0.067-2.20</td>
<td>0.25, 0.057-2.19</td>
</tr>
<tr>
<td>Total IgE CAP&lt;sup&gt;5&lt;/sup&gt;</td>
<td>IgE</td>
<td>665, &lt;150-7200</td>
<td>360, &lt;150-7380</td>
<td>190, &lt;150-5700</td>
</tr>
</tbody>
</table>

<sup>1</sup> Allergen-specific antibodies, measured in: ^2 kU/L, ^3 OD at 405 nm ^4 µg/L and ^5 kU/L.

<sup>6</sup>Mann-Whitney U test.
CONCLUSIONS

I. We have demonstrated a high prevalence of heterophile IgM antibodies to the carbohydrates on IgA and IgM from many animal species. The carbohydrates do not appear to be limited to immunoglobulins, however, as they are present on alkaline phosphatase, a commonly used substrate in immunoassays. Heterophile antibodies can therefore generate false positive signals in immunoassays.

II. We have demonstrated IgE antibodies to cat IgA and that the epitopes are located in the glycan moiety of the protein. To our knowledge these are the first IgE carbohydrate epitopes described in mammalian proteins and presumably the same carbohydrates as the heterophile IgM antibodies identified in paper I. Complete mutual cross-reactivity was demonstrated to cat IgM and partial cross-reactivity to other proteins such as calf intestine alkaline phosphatase, which implies an even broader cross-reactivity. The biological implications of these epitopes are unknown, but the biological and clinical relevance of other carbohydrate IgE epitopes are debated.

III. Crystallization of the recombinant variant of Fel d 1 (1+2) revealed a homodimer with two calcium-binding sites. One site is on each side of the tetramer, whereas the other lies in the interface between the 2 dimerized molecules. The biological reactivity of this recombinant variant was demonstrated to be equal to the natural variant and rFel d 1 (2+1). This makes rFel d 1 (1+2) a good candidate for use as a diagnostic tool and for allergy vaccination.

IV. Allergen extracts from natural source material are not consistent concerning quality and quantity of contained allergens. We demonstrated that a single recombinant allergen is at least as sensitive for diagnosing cat allergy in vitro as are currently used extract-based tests. Elevated IgE levels to rFel d 1 are associated with asthma in cat allergic children.
FUTURE PERSPECTIVES

Future allergy diagnosis will be based on high throughput screening, presumably using the microarray technology and panels of recombinant allergens. Sensitization profiles for individual patients can be made after a rapid analysis using a minimum of serum. Some allergens are more important than others, as for example the recombinant Fel d 1 used in this thesis was at least as effective as a diagnostic tool as was crude cat dander extract. However, larger panels of allergens also including minor allergens would likely improve diagnosis even further.

In an attempt to identify and characterise new allergens in cat dander we identified a cross-reacting carbohydrate epitope present on multiple proteins, even unrelated proteins from other species. The epitope was presumably the same for both IgM and IgE. Identification of these carbohydrates would be of great interest and could lead to a better understanding of how our immune system works. Antibodies to carbohydrates on organs from pigs are one of the factors preventing successful xenotransplantations. Thus our results indicate that not only IgG but also immunoglobulins from other classes are involved in reactions to mammalian carbohydrates and as such are interesting. The biological effects of IgE anti-mammalian carbohydrate antibodies should be investigated for obvious reasons. Even if biological effects can be enacted by IgE anti-mammalian carbohydrates, it will be of importance to investigate their clinical relevance since IgE anti-plant carbohydrates have revealed biological reactivity but poor clinical relevance. If clinical relevance can be established and the carbohydrates can be characterized, these carbohydrates should be included in future allergen chip arrays.

Further characterization of Fel d 1 is also of interest since we demonstrated that the recombinant variant could mimic the biological reactivity of the natural counterpart. This protein can be useful for diagnosis and as a basis for hypoallergenic modifications in strategies for new treatments of cat allergy. We also could demonstrate that a recombinant variant produced a homodimer, which mimicked the tetramer of nFel d 1. The cavity between the 2 dimers is most probably a pocket for ligand-binding since both human and rabbit secretoglobin bind hydrophobic molecules. However, neither of these ligands can be modelled into Fel d 1. Knowledge of the ligand might shed some light on why Fel d 1 is so highly allergenic.
Allergi är en sjukdom som drabbar en fjärdedel av befolkningen. Den orsakar allt från mindre irritationer till livshotande besvär hos de drabbade och medför även stora samhällskostnader. Allergi mot katter finns hos ca 10 % av befolkningen och är den vanligast förekommande allergin hos barn med astma i Sverige.


I det första delarbetet var målet att identifiera flera kattallergen. Trots att Fel d 1 är så dominerande är det viktigt att försöka få en klar helhetsbild av allergenpanoramat. Med hjälp av serum från kattsensibiliserade personer analyserades IgE-bindande proteiner från katt. Kattmjällsextrakt separerades med avseende på storlek och serum från kattallergiska patienter sattes till. Vi identifierade 2 vanligt förekommande proteiner av stor storlek och vi kunde bestämma att dessa var katt-IgA och katt-IgM. Ytterligare försök visade att det var kolhydrater på dessa proteiner som patienternas IgE band till. Detta var oväntat eftersom IgE mot kolhydrater på allergen från ryggradsdjur inte tidigare har påvisats. Därför utfördes noggrannare kontrollexperiment. Dessa visade att det framför allt var IgM-antikroppar som reagerade med kolhydrater på katt-IgA och katt-IgM samt på enzymer i våra detektionssystem. Resultaten har stor betydelse för ELISA-baserad diagnostik.

Vår första studie indikerade att det även fanns IgE mot katt-IgA och IgM bland kattsensibiliserade patienter. Därför fortsatte vi att studera IgE mot dessa kattproteiner, men med andra enzymer och detektionssystem. Vi fann att drygt 30 % av kattsensibiliserade patienter hade IgE-antikroppar mot katt IgA. Vi såg också att det förelåg en hög korsreaktivitet mellan katt-IgA och katt-IgM. Återigen kunde vi visa att det var kolhydrater på dessa och även andra, totalt obeväxta proteiner, som IgE-antikropparna var riktade mot.

I det tredje arbetet kristalliserades en rekombinant variant av huvudallergenet hos katt, Fel d 1 (1+2). Den 3-dimensionella strukturen av proteinet bestämdes med hjälp av kristallografi som visade att Fel d 1 även binder kalciumjoner. De biologiska och immunologiska egenskaperna hos rekombinant (r)Fel d 1 (1+2) jämfördes med en annan variant, rFel d 1 (2+1), samt naturligt Fel d 1. Ingen skillnad mellan de tre proteina kunde ses.

I det fjärde arbetet utvärderades rFel d 1 som diagnostiskt verktyg. Idag används kattmjällsextrakt som består av en blandning av många olika allergena och icke-allergena proteiner. I och med att så många kattallergiska personer är sensibiliserade mot just Fel d 1 skulle allergeneten kunna vara en bra kandidat för att ersätta dagens extrakt. Serum från kattallergiska barn och vuxna från Sverige och Österrike med...
hösnuva och/eller astma testades mot rFel d 1 och kattmjällseextrakt i det kommersiella in vitro-systemet som används i Sverige idag. Vi såg att rFel d 1 är minst lika bra som kattmjällseextrakt för att identifiera kattallergiska personer i detta system. När vi använde rFel d 1 i ELISA så var det ännu bättre än det kommersiella testet. Vi såg även att höga IgE-nivåer mot Fel d 1 är en riskfaktor för astma hos barn.
ACKNOWLEDGEMENTS

I would like to acknowledge the people who have helped me during the years that have led to this thesis, both those at work and outside in the real world. In particular, I would like to acknowledge the following persons who have contributed to the work in different ways:

My supervisor professor Marianne van Hage for believing in me and accepting me as your PhD-student. For great encouragement and teaching me so much about immunology and medical research.

My co-supervisor Hans Grönlund for sharing your incredible enthusiasm and broad as well as detailed knowledge.

My co-supervisor professor Per-Åke Nygren for challenging questions and valuable input from an outside perspective.

Professor S.G.O. Johansson for saving my first projects and invaluable scientific as well as non scientific discussions together with Anna Nopp.

All present and former members of Marianne’s group, Hans, Lotta, Sarah, Theresa, Tiitu, Guro, Jeanette, Klas and of course B.Ola! Neda for your great assistance in the lab, Gerd for all your help with bureaucracy, orders, invoices etc.

All the people at L2:04 that I have had the pleasure to work with for creating a stimulating research atmosphere.

All co-authors, who have assisted my work.

My roommates, Christine, Erika, Sarah, Helen, Maria and Monica.

Professor Reto Crameri for letting me work at your lab at SIAF in beautiful Davos. Claudio Rhyner and Michael Weichel for your excellent supervision.

My labmate Zihao Cheng, my roommate Rodrigo Barderas and the rest of the CSHL phage display group of 2006 for a great time.

The “gangs” that have helped me take my mind off research from time to time, the Katrineholm crew, the KTH crew, the Soul crew and the Biking crew.

Ann-Louise and Holger for always having room for my family and me. David and Emma, you are going to be great babysitters.

All my relatives scattered around the globe.
My fantastic parents, dad Baffe and mum Tina, for your endless support, love, generosity and your big welcoming house. My great siblings, bro Kunlé and sis Yemisi and your families, Annika, William, Ulf, Rasmus and Casper, for making our gatherings so nice.

My cats, strange dog-like scared Vincent and crazy red dot-addicted meowing Cornelius. You are mostly fun!

My amazing son Theodor, you are such a cool boy, already the greatest! Lots of love!

And above all, my lovely Eva, for all your love and your support for my “projects”! You are the world’s best “langare”! I love you!
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