MASTERARBEIT

Titel der Masterarbeit
„From mechanisms of food allergy
to new forms of diagnosis and treatment“

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**Introduction**

**Adverse reactions to food**

Abnormal reactions resulting from the ingestion of food can have many different origins. When reactions are non-immune-mediated the condition is termed food sensitivity or food intolerance. Food sensitivities include lactose intolerance, which is the most common cause of adverse reactions to milk. Moreover, food toxicity and pharmacological reactions for example to food containing histamine are also regarded as food sensitivities.\(^1\) On the other hand, reactions to food that are immune-mediated are termed food allergy.\(^2\)
**Food allergy**

Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly to a given food. The definition includes immune responses that are IgE-mediated, non-IgE-mediated, or a combination of both.³

The most common abnormal immunological reactions to food are IgE-mediated, so-called “type I or immediate type hypersensitivity” reactions. The accompanying symptoms range from gastrointestinal and skin reactions (e.g. urticaria) to asthma, rhinitis and anaphylactic shock. Moreover, this form of food allergy may be followed by delayed reactions characterized by infiltration of inflammatory cells into tissues.¹

Non-IgE-mediated pathological conditions, however, include eosinophilic gastrointestinal disorders (eosinophilic esophagitis, eosinophilic gastroenteritis) and food-protein induced disorders like allergic proctocolitis, enterocolitis syndrome and enteropathy like celiac disease.¹

Furthermore, certain fruits and vegetables are causes of allergic contact dermatitis, which characteristically belongs to “type IV or delayed type hypersensitivity” reactions. In this case, the allergens are flavoring substances like isothiocyanate in horseradish or limonene in oranges and lemon peels.⁴ These chemicals may penetrate into the skin and react with self-proteins. This eventually results in a hapten-specific cell-mediated immune response that is characterized mostly by effector mechanisms of T cells. The subsequent allergic symptoms are limited to skin reactions like redness, edema and pruritus.⁵

This thesis focuses on IgE-mediated food allergy. Therefore, subsequently the term food allergy refers to IgE-mediated food allergy.
IgE-mediated food allergy

IgE-mediated food allergy represents a serious and increasing problem in developed countries. A recent meta-analysis of mostly European studies found the prevalence of food allergy to constitute 3% to 35% in studies of self-reported food allergies and 2% to 5% in studies of patients with allergic symptoms and sensitization confirmed by doctors. Besides mild symptoms of the oral cavity, food allergy can also lead to life-threatening anaphylactic reactions. Furthermore, nutritional deficiencies especially concerning calcium and growth delay are consequences of the recommended restricted diets. Compared to patients with insulin-dependent diabetes mellitus, food allergic patients report even worse health-related quality of life.

According to the Austrian Nutrition Report 2012, the average daily intake of protein in Austria is 1.1 g per kilogram body weight and is derived from a big variety of mammals, birds, fish, fungi and plants. Nevertheless, eight food protein sources are the worldwide major causes of food allergy, which are peanut, tree nuts, egg, milk, fish, crustacean shellfish, wheat and soy (although more than 170 foods have been reported to cause IgE-mediated food allergy). Especially in Europe also celery, mustard, sesame, lupine and molluscan shellfish increasingly cause symptoms in food allergic patients.

Some of these allergies are known to be outgrown. This is mostly the case for egg, milk, wheat and soy. In comparison to that, spontaneous clinical tolerance cannot be expected in patients allergic to peanut, tree nuts and seafood. So it seems that the pathophysiology of food allergy depends on the eliciting allergens and some food allergens may be generally more allergenic than others.
Food allergens

Food allergens are specific components of food that are recognized by IgE antibodies and allergen-specific immune cells and elicit specific immunological reactions that result in characteristic symptoms.3 Regarding IgE-mediated food allergy, this means that a food allergen is a substance in food that causes allergic symptoms after allergen contact (e.g. by consumption, inhalation) as it is recognized by patients’ IgE. A complete allergen is able to induce sensitization to the food-derived allergen and to elicit allergic reactions after subsequent allergen contact, which is for example the case for Ara h 2, a major allergen from peanut and Cyp c 1, the major carp allergen. However, some allergens like the major allergen from apple, Mal d 1 are so-called incomplete allergens.15 Regarding Mal d 1 and other plant-derived allergens, pollen allergens may be the sensitizing agent. But they can cause the oral allergy syndrome due to cross-reactivity of their IgE epitopes with the sensitizing pollen allergens (birch pollen allergens). The oral allergy syndrome is characterized by type I allergic symptoms in the oropharyngeal mucosa of pollen allergic patients shortly after consumption of plant-derived food.16

Most food allergens belong to only a few different protein families. They share some common characteristics, which may contribute to their allergenicity. First of all, bioavailability of many food allergens is high, as they are mostly relatively stable during food processing and digestion. This does not apply to birch pollen cross-reactive food allergens like the apple allergen Mal d 1, which are destroyed by digestion or cooking.15 These allergens cause the oral allergy syndrome by resembling the sensitizing pollen allergens.16

Furthermore, food allergens are usually of relatively small molecular weight, in general < 70 kDa and water soluble. As the mucosal IgE immune system evolved for the host defense against intestinal metazoan parasites, which are
highly glycosylated, it is not surprising that certain food allergens are also glycosylated.\textsuperscript{14}

Moreover, some food allergens are lipid-binding molecules. This protects them from degradation and facilitates the absorption in the gastrointestinal tract.\textsuperscript{15}
Mechanisms of food allergy

The first phase in the allergic immune responses is the allergic sensitization. This means that food allergens are presented to naïve T cells, which leads to a food allergen-specific Th2 profile in food allergic patients. The Th2 cells produce interleukin-4 (IL-4), IL-5 and IL-13 which promotes B cell class switch to IgE. IgE binds to the high-affinity receptor on mast cells and basophils in tissues like the skin and the gut. Just after re-exposure the distinct allergen elicits allergic symptoms by cross-linking IgE on the mast cell surface, which causes mediator release. This second phase of allergic immune responses occurs within minutes after allergen contact.

Even though a single exposure to a food allergen may cause allergic sensitization, emerging evidence suggests that at a critical point of time early in life of an infant the exposure to a proper dose of antigen is necessary to train appropriate immune responses to food. Therefore, early strategies of delayed weaning patterns were not successful in preventing food allergies. Some studies even found increased prevalence associated with late introduction of food. Murine animal models reveal that different allergen doses either sensitize or tolerize animals, but it is questioned if these doses can be extrapolated to humans. Additionally, the route of allergen exposure may be an important feature. Oral administration of food usually results in oral tolerance induction. However, other routes of exposure like the skin may also be a site of sensitization (and elicitation). Epidemiological and murine studies support the idea of a dual-allergen exposure hypothesis, which says that low-dose cutaneous exposure favors sensitization, whereas early consumption favors oral tolerance. When considering factors like timing and dosage, defining new recommendations for the introduction of different potentially allergenic food remains challenging.
Oral tolerance

Oral tolerance describes the phenomenon that orally administered antigens, for example food allergens, suppress subsequent immune responses. This immunological mechanism was first described already more than 100 years ago.\textsuperscript{25,26} The gastrointestinal tract as the largest immunological organ in the body is exposed to huge amounts of exogenous antigens coming from our diet. Different mechanisms have been proposed to contribute to oral tolerance induction. In healthy individuals the antigens are taken up into Peyer’s patches or the lamina propria and may enter the bloodstream. In the liver, the peripheral lymph nodes or the spleen the antigens are presented in the absence of costimulation to naïve T cells, resulting in anergy. Anergic T cells are unresponsive to their specific antigen and their main characteristic is that they loose their ability to produce IL-2 and therefore do not proliferate or differentiate in response to antigen.

Furthermore, antigens are transported from the lamina propria into the mesenteric lymph nodes by CD103+ dendritic cells. Under homeostatic conditions this results in an active regulatory response.\textsuperscript{27} Active regulation is performed by T cells with a regulatory phenotype, so-called regulatory T cells (Tregs).\textsuperscript{28} These cells suppress other T cell subsets and therefore prevent excessive responses of the immune system.\textsuperscript{15} Some of these regulatory cells origin from the thymus, the so-called natural regulatory T cells (nTregs), whereas others are induced in the periphery, the so-called induced regulatory T cells (iTregs).\textsuperscript{28} nTregs are responsible for suppression of autoreactive T cells and are not important for oral tolerance induction.\textsuperscript{29} On the other hand, iTregs are indispensable, which was shown in mouse models.\textsuperscript{30} In healthy individuals they are selectively induced by CD103+ dendritic cells in the mesenteric lymph nodes. But if food allergy arises from a defective iTreg cell response is not yet clear.\textsuperscript{28}
Oral tolerance is considered as a potential tool for the prevention of food allergy. In mouse models it could already be seen that oral administration of whey proteins or ß-lactoglobulin leads to a state of ß-lactoglobulin-specific hyporesponsiveness. It was suggested that this kind of allergy prevention works by locally decreasing the specific IgE response and by reducing mucosal mast cell activation.\textsuperscript{31}

**Hygiene hypothesis**

In 1989 it was proposed that microbial exposure has an impact on the development of allergy.\textsuperscript{32} This hypothesis was later supported by studies showing that the increasing prevalence of allergy is mostly restricted to developed countries, whereas in some developing countries there are almost no food allergic patients.\textsuperscript{33} However, for the hygiene hypothesis in combination with food allergy only limited data exist.\textsuperscript{24}

Originally, the hypothesis suggested an association between reduced exposure to infectious diseases and the increasing prevalence of allergy. This concept has changed and nowadays suggests that a balance of microbial signals during development of the immune system is needed for prevention of both Th1- and Th2-driven diseases like autoimmune diseases and allergy.\textsuperscript{34} Supporting this notion, studies show that long-term and early-life exposure to microbes in farms and farm milk protects against allergy.\textsuperscript{35,36} However, environmental changes like usage of antibiotics, changed dietary habits and cesarean delivery disrupt the microbial balance and might be a reason for the increasing prevalence of allergy. Therefore, the human intestine with its commensal microbiota has become an important topic of research.\textsuperscript{37}

In this regard, a study revealed that allergic children have similar patterns of colonization in the gut with reduced lactobacilli and increased coliforms.\textsuperscript{38} Another study showed that atopic children have a reduced ratio of bifidobacteria to clostridia in their stools.\textsuperscript{39} Furthermore, a meta-analysis showed an odds ratio
of 1.32 (CI 1.12-1.55; six studies) for cesarean delivery and the risk of food allergy or food atopy. Therefore, natural delivery causing early colonization of the infant with colonic microbiota seems to have a protective effect.⁴⁰

Human studies are reinforced by studies using germ-free mice. These mice are not colonized with bacteria at birth and grow in a totally sterile environment. This results in impairment of their immune system including disorganized and poorly developed mucosal and secondary lymphoid structures and disturbed antibody responses. All together, they are not able to develop oral tolerance.¹⁴

Most murine models of food allergy use the C3H/HeJ mouse strain. These mice cannot signal through the receptor for bacterial lipopolysaccharides (Toll-like receptor 4), which is needed for the recognition of gram negative bacteria by immune cells. It is speculated that the commensal microbiota is the source of Toll-like receptor 4 ligand. Therefore, the susceptibility of this strain to food allergen-specific IgE production shows the relevance of the intestinal commensals for tolerance induction.⁴¹ It was also shown that this strain shows a reduced colonic Treg/IgA compartment, which could explain the origin of allergy susceptibility.³⁷
Diagnosis

The clinically most relevant test for diagnosing food allergy is the double-blind placebo-controlled food challenge. Its big advantage is that it can distinguish sensitization from clinical allergy. However, patients who have had life-threatening reactions recently should not undergo a challenge with that food.\(^\text{13}\)

Furthermore, allergen-specific IgE can be detected by skin prick tests or immunoassays measuring allergen-specific serum IgE levels. However, the results of these tests always have to be combined with the patient’s clinical history.\(^\text{3}\) Actually, the correspondence between these IgE sensitization results and clinical phenotypes of IgE-mediated allergic diseases may in some patients be very poor.\(^\text{42}\)

On one hand, skin prick tests and especially IgE binding assays with whole allergen source extracts may cause false-positive results.\(^\text{42}\) This is mostly because allergen sources usually contain several molecules that exhibit a wide range of allergenic activity. While some allergens are highly specific for a given allergen source, other allergens show cross-reactivity with allergens from many unrelated sources.\(^\text{43}\)

On the other hand IgE measurements may also produce false-negative results, which is a particularly important clinical problem in case of potentially high risk allergies. Regarding false-negative results the patients may for example have local mucosal IgE production and reactivity in the respiratory or gastrointestinal tract, which is not detectable in the serum or with skin prick tests.\(^\text{42}\) Moreover, allergen extracts may contain limited amounts of some allergenic molecules due to the extraction method from the allergen source. Especially concerning plant food, some important allergens may also become degraded.\(^\text{44}\)

Relevant factors that affect the relationship between allergen-specific IgE measurements and clinical immediate hypersensitivity have been summarized as affinity, specificity, clonality and specific activity (ratio to total IgE) of IgE responses. Moreover, apart from factors regarding IgE, end-organ sensitivity to
mediators of allergic inflammation and variations in effector cell reactivity and specific IgG may be important factors.\textsuperscript{42}

**Component-resolved diagnosis**

Molecular cloning technologies have enabled the production of allergens in a recombinant form. This gave rise to the development of *in vitro* and *in vivo* diagnostic tests based on recombinant allergens, epitopes and peptides.\textsuperscript{43} Diagnostic tests using recombinant allergens, usually referred to as component-resolved diagnosis, are able to determine IgE reactivities to individual allergens. This may for example reveal sensitization to an allergen with cross-reactive potential and, therefore, may predict possible reactions to allergen sources that contain immunologically related allergens.\textsuperscript{44}

**Cross-reactivity**

As an example for highly cross-reactive allergens, the muscle protein tropomyosin is present in shrimps, crabs, crawfish and lobster as well as house dust mites.\textsuperscript{45} Furthermore, apples, stone fruits, celery, carrots, nuts and soybeans contain cross-reactive allergens to the major birch pollen allergen Bet v 1. These allergens belong to the so-called pathogenesis-related (PR)-10 protein family. Other cross-reactive allergens, related to the birch pollen minor allergen Bet v 6, are found in plant food like apples, peaches, oranges, lychee fruits, strawberries, zucchini and carrots. But in many cases the occurrence of these cross-reactive IgE antibodies is not correlated with the development of symptomatic food allergy.\textsuperscript{46} Furthermore, there is cross-reactivity seen between different fruits containing lipid transfer proteins (LTPs). LTPs have been identified as triggers of severe food allergy especially for fruit allergic patients in the Mediterranean area.\textsuperscript{47} As the apple LTP Mal d 3 is highly homologous to peach LTP, apple allergy is common among peach LTP-sensitized patients.\textsuperscript{48}
However, cross-reactive IgE antibodies to carbohydrate structures have been shown to be mostly clinically irrelevant. These N-glycans are present in many plant foods like peanuts, but also food of invertebrate origin like mollusks, shrimps and snails and are recognized by patients usually sensitized to pollen or insect stings.

This lack of clinical relevance might be caused by monovalent IgE recognition and low antibody affinity. In diagnostic tests based on natural extracts, these IgE antibodies cause many false-positive test results especially for plant foods like cereals and legumes. Fortunately, this can be overcome by component-resolved diagnosis and recombinant allergens that do not carry N-glycans. IgE antibodies against N-glycans frequently occur in combination with antibodies against profilin. More than 100 profilins have been described as allergenic that are found in plant-derived food and pollen. Similar to N-glycans, the clinical relevance of profilin is limited especially for the population of pollen-allergic patients. But for food allergic patients, N-glycans and particularly profilin may potentially be of clinical relevance.

Therefore, better understanding of cross-reactivities is a big advantage of component-resolved diagnosis. For the management of allergies, it is important to know whether a patient is cosensitized to a variety of unrelated allergens in different allergen sources or is cross-sensitized to few cross-reactive allergens.

Moreover, as component-resolved diagnosis allows the identification of IgE reactivity patterns, it might lead to a better knowledge of the relationship between basic immunological mechanisms and clinical symptoms.

**Predicting severity and persistence of food allergy**

Regarding food allergic patients, component-resolved diagnosis may also predict the severity of allergic symptoms. A study showed that Pru p 3-sensitized peach allergic patients are less prone to develop severe symptoms
when they are also sensitized to Pru p 1 and Pru p 4. Furthermore, in patients with severe symptoms, Pru p 3-specific IgE was significantly higher than in patients with mild symptoms and it was a significantly better indicator for severe symptoms than peach-specific IgE.\textsuperscript{53}

Regarding egg allergy, component-resolved diagnosis of allergic patients may determine if patients can tolerate heated egg.\textsuperscript{54} Component-resolved diagnosis in combination with an assessment of the allergenic activity of individual allergens using a basophil activation assay may provide additional information for the diagnosis of milk allergy. It has been shown that the basophil activation assay may distinguish between patients with or without clinical milk allergy and it showed different results for patients who outgrew their allergy and patients who did not.\textsuperscript{55}

The persistence and severity of food allergy in affected patients may also be determined by peptide microarray assays. These assays test the diversity and affinity of patients’ IgE binding to sequential epitopes on major food allergens. A study on milk allergy found that milk allergic patients have increased epitope diversity compared to those, who have outgrown their allergy or tolerate heated milk. Moreover, competitive peptide microarray assays show that patients who have outgrown their allergy or tolerate heated milk have primarily low-affinity IgE binding.\textsuperscript{56} Another study identified four peanut-derived peptide biomarkers that can predict the outcome of double-blind placebo-controlled peanut challenges.\textsuperscript{57}

Taken together, component-resolved diagnosis, basophil activation assays and peptide microarray assays will offer the possibility to know more about a patient’s allergy and to base therapeutic decisions on the results.
New forms of treatment

The ultimate goal of every therapy in food allergy is to achieve tolerance in the food allergic patient. In relation to food allergy, tolerance means a state, in which a person can consume a food without any allergic symptoms also after weeks, months or even years of cessation of regular exposure to the food antigen. Desensitization, however, means that regular exposure is a prerequisite for maintaining clinical nonreactivity.3

Therapeutic approaches can be divided into food allergen-specific and non-specific. Allergen-specific approaches include subcutaneous, oral and sublingual immunotherapy, whereas non-specific approaches are for example humanized monoclonal anti-IgE and probiotics.6,58

The decision, which approach might be best suitable for a specific patient, could depend on his or her phenotype of food allergy. Food allergic patients can be divided according to three phenotypes: persistent food allergy, transient food allergy and food-pollen oral allergy syndrome. Increasing evidence suggests that these forms of food allergy result from different immunological mechanisms and therefore ask for different therapeutic approaches.58

Patients with persistent food allergy are more likely to suffer from adverse reactions on therapy and failure of desensitization, whereas transient food allergy could be seen as not requiring therapy. But accelerated development of tolerance and therefore improved quality of life and nutrition pleads for also treating these patients. Approaches for oral allergy syndrome might include pollen immunotherapy.58
Non-specific therapy

Humanized monoclonal anti-IgE
Humanized monoclonal mouse anti-IgE IgG\textsubscript{1} antibodies bind to the constant region of IgE antibodies preventing IgE from binding to its receptors.\textsuperscript{58} A study with peanut allergic patients suggests that the antibody could offer some level of protection against unintended ingestion.\textsuperscript{59} Additionally, anti-IgE has been investigated in combination with allergen-specific immunotherapy to decrease the risk of adverse reactions.\textsuperscript{60}

Probiotics
Already 1907, Metchnikoff first introduced the hypothesis of an association between the consumption of probiotics or fermented milk products and longevity through repopulating the intestine with the correct balance of favorable microbes. Lactic acid bacteria, especially Lactobacillus species, have therefore been a focus of research.\textsuperscript{37} Even though a beneficial effect of probiotics on the prevalence of atopic dermatitis has been shown in one study, similar effects on food allergy have not been seen.\textsuperscript{61} A probiotics study on cow’s milk allergic children showed no difference between the probiotic and the placebo group.\textsuperscript{62}

Allergen-specific therapy

Allergen-specific immunotherapy (SIT) is the only specific and disease-modifying approach for the treatment of allergy.\textsuperscript{63} The first trial was published already 1911. Grass pollen allergic patients were treated with subcutaneous injections of grass pollen extracts and achieved improvement of allergic symptoms.\textsuperscript{64} In 1935 a protective allergen-specific factor in patients’ sera after SIT was found, which inhibits the binding of IgE antibodies to allergens.\textsuperscript{65}

Nowadays, this factor, later on referred to as blocking IgG antibodies, is still seen as a major mechanism of SIT as the antibodies suppress allergen-induced
mast cell degranulation and therefore immediate allergic inflammation. Furthermore, cellular mechanisms during SIT are under investigation.\textsuperscript{66} Generally, there are two different explanations for the beneficial effects of SIT. The allergic reaction can either be inhibited by blocking antibodies or by a shift from Th2 to Th1/Treg, but actually SIT should aim at the induction of both pathways.\textsuperscript{8}

SIT for food allergy is used via different approaches, subcutaneously, orally and sublingually, presently using natural allergen extracts.\textsuperscript{6} However, it has been shown in clinical trials using recombinant allergens of birch, grass and ragweed pollen that extracts could effectively be replaced by single recombinant allergens.\textsuperscript{67,68}

**Subcutaneous immunotherapy**

Already in the 1930s, it was shown that subcutaneous immunotherapy consisting of rush inoculations could induce desensitization in a fish-allergic patient. Daily consumption of cod liver oil could even maintain this state.\textsuperscript{69} For the treatment of aeroallergens and bee venom allergy, subcutaneous immunotherapy is a well-established practice and efficacy and long-lasting effects were shown in different clinical trials.\textsuperscript{70-72} Therefore, subcutaneous immunotherapy for the treatment of peanut allergy was investigated in a double-blind placebo-controlled trial using aqueous peanut extract. The study revealed that the therapy was effectively decreasing symptoms in the following double-blind placebo-controlled food challenge and reduced skin prick test reactivity.\textsuperscript{73} Unfortunately, the study had to be stopped after an accidentally administered dose of peanut extract tragically caused the death of a placebo-treated subject. Although the study showed promising results regarding the efficacy of subcutaneous immunotherapy, significant adverse reactions discouraged further trials of subcutaneous immunotherapy.\textsuperscript{6}
Oral immunotherapy

Oral immunotherapy (OIT) is an actively studied approach for the treatment of food allergy. The term describes regular oral administration of gradually increasing amounts of food followed by continued daily ingestion of tolerated doses in the maintenance phase.\textsuperscript{58}

Despite promising results in some studies, achieving long-term tolerance after oral immunotherapy remains elusive.\textsuperscript{6} Furthermore, a meta-analysis published in 2011 found no significant difference between treatment and avoidance groups in the included studies.\textsuperscript{74} An explanation could be that most studies had focused on transient food allergies whereas little research had been done on persistent food allergies like peanut or fish allergy. Unfortunately, also OIT causes significant adverse reactions, which especially present safety concerns when maintenance doses are ingested at home.\textsuperscript{6}

Sublingual immunotherapy

In the case of sublingual immunotherapy (SLIT), smaller doses of allergen (micrograms to milligrams) of extracts are used, which are administered under the tongue. There it should be held for a defined period and then swallowed or spit.\textsuperscript{6}

Due to the difference in the amount of allergen, systemic side effects are less likely than during OIT. However, also the efficacy of SLIT seems to be lower than that of OIT, which was tested in studies of SLIT continued by SLIT or OIT.\textsuperscript{75} The optimal use of SLIT and OIT still has to be determined.\textsuperscript{6}

Recombinant, hypoallergenic food allergen derivatives

The major disadvantages of allergen-specific approaches using allergen extracts is that they can elicit severe and even life-threatening allergic reactions in the course of immunotherapy, as they contain allergens which display allergenic activity. In addition, natural allergen extracts may have unpredictable compositions and lack important allergens.\textsuperscript{63}

Since the isolation of allergen-encoding cDNAs became possible in the late 1980s, research was performed regarding the identification of primary and
three-dimensional structures of allergens. Furthermore, IgE and T cell epitopes of many important allergens could be identified.\(^{43}\) For food allergens, IgE epitopes often appear to be continuous epitopes if allergens are digested before they sensitize the patients, whereas aeroallergens contain primarily conformational IgE epitopes.\(^{76}\)

This knowledge enabled the development of recombinant hypoallergenic allergen derivatives, so-called hypoallergens. Such derivatives overcome several problems of extract based allergy vaccines. They can be produced in a reproducible way as defined recombinant molecules. Moreover they show reduced IgE reactivity to decrease IgE-mediated side effects of SIT, whereas T cell epitopes were preserved.\(^{77}\) This could be achieved using different strategies including point mutations\(^{78}\), denaturation\(^{79}\) and fragmentation\(^{80}\) reassembly of sequence elements\(^{81}\) and oligomerization\(^{82}\). But not all of them have been applied to food allergens so far.

Hypoallergens are selected in immunization studies for their ability to induce robust IgG antibody production. As these antibodies recognize also the wild-type allergen, hypoallergens are suitable for therapeutic approaches based on the induction of blocking IgG antibodies. As higher doses can be used in comparison to the wild-type allergens, the number of administrations can be reduced.\(^{43}\)

The first project that aims at the development of a hypoallergenic allergen-specific therapy for food allergies is the FAST project (food allergy specific therapy, http://www.allergome.org/fast/) funded by the European Union. The two targets of the project are allergies to fish and peach, as they represent severe and persistent allergies prevalent in the European Union. Furthermore, avoidance of the food has negative impact on the nutritional status.\(^{8}\)

In terms of peach allergy, the focus lies on the major peach allergen, the non-specific lipid transfer protein Pru p 3. Therefore, peach was chosen as a representative of all fruit allergies linked to lipid transfer proteins.\(^{8}\) On the other
hand, carp parvalbumin is the focus of research regarding fish allergy, which contains most IgE epitopes of saltwater and freshwater fish.\textsuperscript{8,83} A hypoallergen of parvalbumin, the major carp allergen Cyp c 1, was produced by site-directed mutagenesis of two functional Calcium-binding sites. This resulted in loss of three-dimensional structure and 95\% reduction of IgE reactivity shown in dot blot assays and immunoblot inhibition experiments. Furthermore, the hypoallergen was investigated in skin prick tests, which approved the in vitro results.\textsuperscript{78} Concerning the peach hypoallergen, different mutants of Pru p 3 are produced and purified in FAST.\textsuperscript{8} Mutations of disulfide bridges forming cysteins were shown to reduce the allergenicity of Pru p 3 significantly.\textsuperscript{79} This was seen also with the major LTP Par j 1 in \textit{Parietaria} weed.\textsuperscript{84} Therefore, candidates for the peach hypoallergen include mutants of disulfide bridges but also mutants of surface-exposed amino acids and chemically modified wild-type molecules.\textsuperscript{8}

After evaluation the hypoallergens will be used in clinical studies. Clinical centers in six countries will participate, for peach allergy trials especially centers in Spain, Italy and Greece, whereas for fish allergy trials also centers in Denmark, Iceland and Poland. The hypoallergens will be administered subcutaneously because of an expected higher efficacy compared to oral or sublingual administration. Adsorption to the adjuvants aluminium hydroxide also increases efficacy and furthermore increases safety due to its depot effect. Moreover, administration in clinical centers will be safe and will bring along better compliance.\textsuperscript{8}

Taken together, this promising project relies on a huge effort done on basic and clinical research on the topic of food allergy. Therefore, it may soon offer new efficient and safe treatment strategies for fish and peach allergy. At best, the strategies could be transferred to other food allergens improving quality of life of many patients suffering from diverse food allergies.
**Aim of the master thesis**

The master thesis contributes to the FAST project mentioned before.

First, I analyzed skin prick tests, which were performed in Greece with fish allergic children. In this regard, the aim was to compare wheal and flare reactions in response to Cyp c 1 and the hypoallergenic mutant. In addition to that, I measured different antibody levels in the patients using different methods.

The second aim was the development of a mouse model of fish allergy and the evaluation of the hypoallergenic mutant of Cyp c 1 in the model. Therefore, rabbits were immunized with the mutant and a therapeutic passive immunization model with mutant-induced rabbit antibodies was established. Moreover, methods like ELISA, RBL and immunoblotting were used to analyze the mouse model and the mutant-specific rabbit antibodies.
References


75. Keet CA, Frischmeyer-Guerrerio PA, Thyagarajan A, Schroeder JT, Hamilton RG, Boden S, et al. The safety and efficacy of sublingual and


Manuscripts

In vivo allergenic activity of a hypoallergenic mutant of the major fish allergen Cyp c 1 evaluated by skin testing

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ABSTRACT

Background: IgE-mediated fish allergy frequently causes severe anaphylactic reactions. A recombinant mutant of the major allergen from carp, Cyp c 1, with reduced IgE-reactivity and in vitro allergenic activity was constructed for specific immunotherapy of fish allergic patients.

Objective: To compare the in vivo allergenic activity of recombinant wildtype Cyp c 1 (wtCyp c 1) and the recombinant Cyp c 1 mutant (mCyp c 1) by skin testing of fish allergic patients.

Methods: Recombinant proteins were expressed in Escherichia coli and purified. Fish allergic children were tested for Cyp c 1-specific IgE by ImmunoCAP and ISAC, and for Cyp c 1-specific IgM, IgG, IgG1-4, and IgA by ELISA. IgE-cross-reactivity to parvalbumins from other fish species was investigated by IgE-immunoblotting. In vivo allergenic activity of wtCyp c 1 and mCyp c 1 was analyzed by skin testing of fish allergic children and controls.

Results: Fish allergic children displayed comparable IgE reactivity to rCyp c 1 in ImmunoCAP and ISAC whereas mCyp c 1 showed no relevant IgE reactivity. Skin testing of fish allergic children demonstrated a highly significant (p<0.001) reduction of the in vivo allergenic activity of mCyp c 1 compared to wtCyp c 1. No positive skin reactions to mCyp c 1 and wtCyp c 1 were observed in non-fish allergic individuals.

Conclusion: wtCyp c 1 allowed specific skin test identification of fish-sensitized patients. mCyp c 1 showed a highly significant reduction of allergenic activity compared to wtCyp c 1 and may be used for SIT of fish allergy.

Clinical implications: wtCyp c 1 may be used for skin testing of fish allergic patients and mCyp c 1 for the development of hypoallergenic vaccines for the treatment of fish allergy.

Capsule summary: Skin test evaluation of recombinant wtCyp c 1 and mCyp c 1 shows that wtCyp c 1 may be used for skin testing in fish allergic patients and mCyp c 1 for the development of hypoallergenic vaccines for the treatment of fish allergy.
Key words:
Food allergy, recombinant allergen, specific immunotherapy, recombinant hypoallergen

Abbreviations:
wtCyp c 1:  Recombinant wildtype Cyp c 1
mCyp c 1:  Recombinant Cyp c 1 mutant
SIT:  Allergen-specific immunotherapy
INTRODUCTION

Fish represents one of the most frequent allergen sources, responsible for IgE-mediated food allergy. One of the first descriptions and analyses of the mechanisms of fish allergy was the classical experiment performed by Prausnitz and Küstner in 1921. By transferring serum of a fish allergic patient into the skin of a pollen-allergic individual they could transfer allergic sensitization and subsequently induce an allergic skin reaction by the administration of fish extract. This experiment elucidated the three components required for an IgE-mediated allergic reaction long before IgE was characterized, i.e. a tissue factor present in all individuals (i.e., mast cells), an allergen-specific serum factor (i.e., IgE) and the allergen. Today, the structure of the major fish allergen, parvalbumin, a small calcium-binding protein, has been elucidated and recombinant parvalbumins from several fish species have been produced as recombinant allergens. In particular, recombinant carp parvalbumin, rCyp c 1, was found to contain the majority of fish-specific IgE epitopes and to show broad cross-reactivity with parvalbumins from a variety of fish species and therefore is a marker allergen for the diagnosis of fish allergy. While food allergies are frequently outgrown, allergy to fish represents a persistent form of food allergy with high prevalence in countries where fish consumption is common. Since parvalbumin is a highly stable protein resistant to cooking and digestion, ingestion of fish often causes severe and life-threatening anaphylactic reactions in sensitized patients.

Currently several approaches for allergen-specific immunotherapy of food allergy are evaluated, including different forms of administration and recombinant allergen-based forms of treatment. Recently, a hypoallergenic derivative of carp parvalbumin has been developed for SIT of fish allergy. The IgE reactivity and the ability of this protein to induce activation of patients’ basophils in vitro was reduced by introducing point mutations in the calcium-binding domains of the major carp allergen Cyp c 1. Furthermore it was shown that immunization of animals with the Cyp c 1 mutant induced IgG antibody responses specific for the wildtype Cyp c 1 allergen, which blocked fish allergic patients' IgE-binding to Cyp c 1. Moreover, the approach of introducing point
mutations was found to be suitable to reduce IgE reactivity of a variety of parvalbumins from different fish species indicating that the mutation strategy may be a general approach for improving the safety of SIT for fish allergy.\(^\text{15}\)

In this study we have analyzed the \textit{in vivo} allergenic activity of the Cyp c 1 mutant by comparing it with the wildtype allergen regarding the induction of immediate type skin reactions in fish allergic patients. Our results demonstrate a highly significant reduction of the \textit{in vivo} allergenic activity of the Cyp c 1 mutant protein in patients suggesting that it may be useful for SIT of fish allergy.
METHODS

Recombinant wildtype and mutant Cyp c 1, allergen extracts, SDS-PAGE

Recombinant wildtype Cyp c 1 (wtCyp c 1) and the recombinant Cyp c 1 mutant (mCyp c 1) were expressed in *E. coli* and purified as described previously. The purity of the recombinant proteins was evaluated by SDS-PAGE and Coomassie brilliant blue staining. For this purpose, purified recombinant wtCyp c 1 and mCyp c 1 were separated under reducing conditions by 16% SDS-PAGE. The protein concentrations were determined with a Micro BCA kit (Pierce, Rockford, IL) using BSA as a standard. The identity of the proteins was demonstrated by immunoblotting with specific antibody probes. The absence of host DNA was confirmed by application of the protein preparations to ethidium bromide plates using DNA as a standard. Proteins were dissolved in distilled water (wtCyp c 1) or 10 mM Tris-HCl, pH 9.0 (mCyp c 1), filter sterilised (0.2 µm) and stored at -20°C until use.

Fish extracts (cod, sardine) for skin prick testing were purchased from Stallergenes (Antony, Hauts-de-Seine, France). Fish extracts (carp, cod, and tuna) for immunoblot experiments were prepared as described.

Fish allergic children

Twelve fish allergic children between 3 and 14 years of age were studied. Each of the children was sensitized to fish, as measured by ImmunoCAP (Thermo Fisher, Uppsala, Sweden), had a positive skin prick test to fish extract, and had experienced immediate clinical symptoms which could be unambiguously attributed to ingestion of fish (i.e., isolated fish ingestion). Fish-induced symptoms were graded according to Sampson *et al.* Since the clinical symptoms could be unambiguously attributed to fish consumption no double-blind, placebo-controlled food challenges were performed in the children. The demographic and clinical characterization of the fish allergic children is summarized in Table I. Sera from a non-allergic individual and a fish-allergic patient containing Cyp c 1-specific IgE were included in the serological assays for control purposes. Serum samples from the fish allergic children were
analyzed in an anonymized manner with permission from the ethics committee of the Medical University of Vienna, Austria (EK565/2007).

**Measurements of allergen-specific antibodies by ELISA, ImmunoCAP, and ISAC**

Cyp c 1-specific IgM, IgA, total IgG, and IgG subclasses (IgG_{1-4}) were determined by ELISA. ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with wtCyp c 1 (5µg/ml) diluted in 100mM bicarbonate buffer (pH 9.6). Sera from fish allergic patients were diluted 1/50 in PBS, 0.05% v/v Tween 20, 0.5% w/v BSA for the measurement of allergen-specific IgG, IgA and IgM. For detection of IgG a HRP-conjugated sheep anti-human IgG antibody (GE Healthcare, Fairfield, USA) was used (1/5000 dilution). IgG subclasses, were detected with mouse anti-human IgG_{1}, anti-human IgG_{2}, anti-human IgG_{4} monoclonal antibodies (BD Pharmingen, San Diego, USA, 1/1000 dilution) or a mouse anti-human IgG_{3} monoclonal antibody (Sigma-Aldrich, St. Louis, USA, 1/5000 dilution). Allergen-specific IgA and IgM were traced with mouse monoclonal anti-human IgA_{1}/A_{2} and anti-human IgM (BD Pharmingen), respectively. The mouse detection antibodies were then detected with HRP-labelled sheep anti-mouse IgG (GE Healthcare, Fairfield, USA, 1/2000 dilution). All measurements were performed as triplicates. Except allergen-specific IgG measurement all experiments were done on one plate to avoid plate-to-plate variability. Serum from a non-allergic individual and buffer alone were included in all measurements as negative controls.

Quantitative measurement of specific IgE levels for wtCyp c 1 was performed with ImmunoCAP f355 (Thermo Fisher, Uppsala, Sweden) following the manufacturer's instructions. IgE values were expressed as kU\_A/L. The sensitization profile of fish allergic patients and IgE levels specific for rCyp c 1 and rGad c 1 were determined using ImmunoCAP® ISAC 103 (Thermo Fisher). The testing procedures were carried out following the manufacturer's instructions. IgE values were expressed as ISAC Standardised Units (ISU) by interpolating the mean fluorescence value with a previously established
IgE dot blot and immunoblot assays, SDS-PAGE

IgE-reactivity of sera of the fish-allergic children to recombinant wtCyp c 1 and mCyp c 1 were determined in dot blot experiments. For this purpose, 0.5 µg of wtCyp c 1 and mCyp c 1 were dotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Dotted proteins were exposed to the sera of the fish allergic patients. As controls sera of a fish allergic adult patient (Co), a non-allergic individual (N), and a buffer control (B) were included. Sera were diluted 1/10 in PBST (PBS (pH 7.5) containing 0.5% v/v Tween 20). Bound IgE antibodies were detected with 1/15 diluted 125I-labeled rabbit anti-human IgE antibodies (IBL, Hamburg, Germany) and visualized by autoradiography.

IgE-reactivity of the patients’ sera to natural carp, cod and tuna protein extracts was analysed by IgE immunoblot experiments. Fish protein extracts were separated by SDS-PAGE, and blotted onto nitrocellulose. After blocking in PBST 1% BSA, nitrocellulose strips were incubated with the sera from the fish allergic patients, diluted 1:5 in PBST or with controls (positive serum from a fish allergic patient, Co, serum from a non-allergic person, N, buffer control, B). Bound IgE antibodies were detected using a 125I-labeled anti-human IgE antibody (IBL, Hamburg, Germany) and visualized by autoradiography.16

Skin prick testing

Skin prick tests with natural allergen extracts and purified wtCyp c 1-wt and mCyp c 1 were performed during regular follow up of the patients at the Allergy Department, 2nd Pediatric Clinic, University of Athens, ‘P&A Kyriakou’ Children’s Hospital, with approval of the Institutional Ethics committee, after written informed consent was obtained from the parents and oral consent from the patients. Skin prick tests were performed on the forearms using histamine hydrochloride (positive control), physiological saline (negative control), sardine extract, cod extract (Stallergenes) and four concentrations (1 µg/ml, 4 µg/ml, 16 µg/ml and 32 µg/ml) of purified wtCyp c 1 and mCyp c 1. As the molecular
masses of wtCyp c 1 and mCyp c 1 are almost the same, the same concentrations of wtCyp c 1 and mCyp c 1 could be used to yield equimolarity in the skin test experiments.

In addition to the fish allergic children (Tables I, II), skin prick tests were performed in 11 control children (7 male, 4 female, 6-12 years), which were atopic to various food and/or inhaled allergens typical for the Mediterranean area, other than fish.

The skin reactions were recorded by marking the wheal contours with a pen and transferring the surrounded wheal with a tape to paper. The paper sheets were scanned and transformed into electronic images. The wheal sizes were then determined by digital planimetry (Image J software, NIH, open source). Mean values of 3 digital drawings of the same wheal were used for further evaluation. Pixel values were converted to mm² according to the formula for 200dpi: (pixel) x 0.01613 = (mm²). A wheal area greater than 7.1 mm² (corresponding to a mean diameter of >3mm) was considered positive.

**Statistical analysis**

Repeated measure ANOVA was used to fit the mCyp c1 and wtCypc1 wheal curves. The area under the curve (AUC) was calculated using integral approximation (trapezoidal rule). The distribution of AUC was tested with the Kolmogorov-Smirnov test. It was found that these parameters do not follow normal distribution. Therefore, the sign rank test was used to examine their association.
RESULTS

Fish allergic children show strongly reduced IgE reactivity to mCyp c 1

Twelve fish allergic children (age 3-14 years) who had experienced grade 2-4 reactions according to the grading system by Sampson which could be unambiguously attributed to fish ingestion, were studied for IgE reactivity to wtCyp c 1 and mCyp c 1. Table I provides a demographic and clinical characterization of the fish allergic children. All of them had experienced skin symptoms (i.e., urticaria and/or flushing) upon fish ingestion. Respiratory and gastrointestinal symptoms were present in 8 cases each (Table I). Each of the children showed positive skin prick test reactions to cod fish as well as sardine extract and had IgE antibodies specific for cod fish extract, the major cod fish allergen Gad c 1 and to the major carp allergen, Cyp c 1 (Table II, III). Cyp c 1-specific IgE levels could be measured by ImmunoCAP in 8 patients and ranged between 2 and >100 kUA/l (Table II). There was no apparent association between the severity of clinical symptoms according to the Sampson grading and the levels of cod fish-specific IgE levels in the children for whom allergen-specific IgE levels were available (Table I, II). Cyp c 1-specific IgE determined by ImmunoCAP ISAC measurements showed a good correlation with Cyp c 1-specific IgE levels determined by ImmunoCAP (R²=0.9558) (Figure 1). The fish allergic patients showed varying levels of Cyp c 1-specific IgG, IgM and IgA. Similar as found for respiratory allergens, patients showed varying IgG₁, IgG₂ and IgG₄ reactivity to Cyp c 1 but not IgG₃ (Table II). No apparent association of Cyp c 1-specific IgG responses with the type or magnitude of symptoms was observed.

All fish allergic patients had additional sensitizations to other food and/or inhalant allergens (Table I). ISAC measurements could be performed in 8 patients and revealed a sensitization pattern, which was typical for the Mediterranean area. For example 50% of the tested sera showed sensitizations to the major peach allergen Pru p 3 and to the inhalant allergens from olive and Parietaria pollen (Figure 2).

Recombinant wtCyp c 1 and mCyp c 1 were purified to homogeneity (Fig 3A). mCyp c 1 showed strongly reduced IgE reactivity (i.e., only three children
showed weak IgE reactivity to mCyp c 1) when compared to wtCyp 1 by IgE dot-blotting in the 12 fish allergic patients (Fig. 3B). A similar result was obtained for serum from an adult fish allergic patient who was tested as positive control and no IgE reactivity was seen for serum from a non-allergic person (Fig. 3B).

Cross-reactivity of fish allergic patients with parvalbumins from different fish species
Next we tested the fish allergic children for IgE cross-reactivity using nitrocellulose-blotted extracts from carp, cod, and tuna (Figure 4). Interestingly, all patients reacted with natural Cyp c 1 at approximately 10 kDa whereas only 10/12 patients reacted with Gad c 1 and only 5/12 patients showed weak IgE reactivity to the major tuna allergen, Thu a 1 (Figures 3A-C). This result was in agreement with the ImmunoCAP ISAC and ImmunoCAP measurements (Table II) which showed that patients had higher IgE levels to rCyp c 1 than to rGad c 1. Patients 7 and 8 showed low IgE levels to Gad c 1 (i.e., =/< 2 kUA/L).

Skin testing of fish allergic patients shows a highly significant reduction of the allergenic activity of mCyp c 1 compared to wtCyp c 1
Four concentrations of recombinant wtCyp c 1 and mCyp c 1 were included in the skin test diagnosis of the 12 fish allergic patients in addition to fish allergen extracts (Table II, Figure 5, Table III). Cod fish and sardine extract induced positive skin reactions in each of the 12 patients yielding mean wheal areas between 7.22 mm² (patient #10) to 114.28 mm² (patient #6) (i.e., codfish) or 30.46 mm² (patient #1) to 116.38 mm² (patient 2) (i.e., sardine) (Table III). Histamine induced positive reactions ranging from 16.52 mm² (patient 10) to 59.29 mm² (patient 6) whereas sodium chloride did not induce any reaction in any of the tested children (Table III). Table III shows the skin reactions to the four different concentrations of wtCyp c 1 and mCyp c 1 (i.e., 1, 4, 16 and 32 μg/ml). Six patients showed a positive reaction (i.e., wheal area > 7.1mm² in grey) to wtCyp 1 (1μg/ml), four were positive at 4 μg/ml and two at 16μg/ml (Table III). Figure 5 shows that skin reactions to mCyp c 1 were significantly
(p<0.001) lower than to wtCyp c1. In fact, the mean (SD) of AUC for mCyp c1 and wtCyp c1 was found to be 148.5(117.9) and 1059.73 (730.42), respectively (p<0.001).

No positive skin reactions to codfish, sardine, saline, wtCyp c1 and mCyp c1 were found in the 11 non-fish allergic children (data not shown). No local late reactions or generalized reactions were observed.
DISCUSSION

Fish allergy frequently induces strong systemic allergic reactions, which can be even life-threatening. While many food allergies developed during childhood are often outgrown, allergy to fish represents a form of food allergy which often remains persistent even in adulthood. The avoidance of the offending allergen in food allergy is currently the only formally indicated possibility for treatment, but this may diminish the quality of life and unintended allergen ingestion might occur and cause severe reactions. Therefore, the development of allergen-specific therapies such as SIT represents an important area of current research. Fish allergy is an excellent model for the development of recombinant allergen-based SIT approaches because it is caused mainly by one major allergen which shows extensive cross-reactivity among various fish species. Here we have evaluated the in vivo allergenic activity of a mutant of the major carp allergen, Cyp c 1, which has been shown to exhibit reduced IgE-reactivity and in vitro allergenic activity. Our study provides a comprehensive comparison of mCyp c 1 with wtCyp c 1 and natural allergen extracts by skin testing in allergic patients. We found a highly significant reduction of the in vivo allergenic activity of mCyp c 1 compared to wtCyp c 1. Even in those children in our study who had shown the most severe systemic allergic reactions to fish according to the grading of Sampson (i.e., patients 5, 6, 9, 10, 11; Tables I and III) mCyp c 1 induced only mild or no skin reactions up to the highest tested dose of 32µg/ml. By contrast, wtCyp c 1 and natural fish extracts induced stronger skin reactions in the tested fish allergic children. In fact, a concentration of 4µg/ml of wtCyp c 1 induced specific skin reactions in each of the fish allergic children but not in the non-fish allergic children indicating that wtCyp c 1 can be used as a standardized allergen for diagnostic skin testing of fish allergic patients. The analysis of the skin reactions induced by wtCyp c 1 versus mCyp c 1 in all tested patients showed a highly significant and consistent reduction of the in vivo allergenic activity of mCyp c 1. It should therefore be possible to formulate vaccines for fish allergy based on mCyp c 1 which allow the administration of much higher doses compared to vaccines based on wildtype allergens and thus it should be possible to reach therapeutic
maintenance doses with less side effects, particularly when the immunogen is adsorbed to an adjuvant which keeps it bound in the local tissues. The current study thus provides important safety information for the development of an adjuvant-adsorbed fish vaccine for subcutaneous immunotherapy based on mCyp c 1 which is currently under development in the European Union project FAST.\textsuperscript{11} According to the so far available \textit{in vitro} and experimental animal models and the current results from skin testing, it is anticipated that mCyp c 1-based injection immunotherapy will induce a robust allergen-specific IgG antibody response. These IgG antibodies should then inhibit allergen-induced mast cell and basophil degranulation and thus the major cause of life-threatening anaphylaxis and eventually also IgE-facilitated allergen presentation to T cells and thus late phase symptoms. Since the primary sequence of the Cyp c 1 allergen has been changed by only four amino acids, one can envisage that mCyp c 1 can also be used for sublingual or oral immunotherapy for induction of T cell tolerance.

Our study thus provides an important result documenting the reduced \textit{in vivo} allergenic activity of mCyp c 1 in allergic patients which is a prerequisite for the further development of mCyp c 1-based immunotherapy strategies for fish allergic patients.
REFERENCES


FIGURE LEGENDS

FIG 1. Correlation of Cyp c 1-specific IgE levels measured by ImmunoCAP (x-axis) and ISAC (y-axis). (R²: coefficient of determination)

FIG 2. IgE sensitization profile of the fish allergic children determined by ISAC. Sera from 8 of the patients were tested for IgE-reactivity to 103 different allergens by ISAC. The number of patients (y-axis) displaying IgE-reactivity to the allergens (x-axis) is shown.

FIG 3. (A) SDS-PAGE of purified recombinant wildtype Cyp c 1 (wt) and mutant Cyp c 1 (mu). A molecular weight marker (M; kDa) is shown on the left side. (B) Comparison of dot-blotted wtCyp c 1 with mCyp 1 regarding IgE reactivity as tested with allergic patients sera (Co, #1-12), serum from an non-allergic subject (N) or buffer alone (B).

FIG 4. IgE-reactivity to natural fish allergens. Nitrocellulose-blotted natural allergen extracts from carp (A), cod (B), and tuna (C) were incubated with sera from 12 fish-allergic children (1-12; Co: positive control serum of a fish allergic patient), serum from a non-allergic individual (N) or buffer alone (B). Bound IgE was detected with 125I-labeled anti-IgE antibodies and visualized by autoradiography. Molecular weights (kDa) are displayed on the left margin.

FIG 5. Reduced allergenic activity of mCyp c 1 compared to wtCyp c 1 demonstrated by skin testing of fish allergic patients. Twelve patients were pricked with increasing concentrations (x-axis: 1, 4, 16, 32 µg/ml) of wtCyp c 1 (wt) and mCyp c 1 (m). Displayed are box plots representing the wheal areas (y-axis: mm²) for the patients with the median indicated by horizontal lines and outliers are represented by asterisks. Significant differences (***, p<0.001) between wtCyp c 1 (wt) and mCyp c 1 (m) are displayed for each tested concentration.
TABLE I. Demographic and clinical characterization of fish allergic children.

TABLE II. Serological characterization of fish allergic children.

TABLE III. Wheal areas (mm²) in response to wtCyp c 1, mCyp c 1, cod, sardine, and histamine.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
### TABLE I. Demographic and clinical characterization of fish allergic children.

<table>
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<tr>
<th>Patient no.</th>
<th>Sex</th>
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<th>Other allergic symptoms</th>
<th>Therapies</th>
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<th>SPTs to inhalant allergens</th>
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Sex: male (M) / female (F)

Family history for allergies: yes (Y) / no (N)

Fish allergy symptoms: skin (S): urticaria, flushing; respiratory (R): sneezing, nasal rhinorrhea-congestion, cough, wheezing, dyspnea; gastrointestinal (G): throat pruritus, nausea, emesis, diarrhea; cardiovascular (C): dizziness, hypotension;

Fish allergy systemic reaction; grades according to Sampson (17): 1-5

Other allergic symptoms: atopic dermatitis (AD), rhinitis (R), asthma (A), urticaria (U)

Therapies: inhaled corticosteroid (ICS), nasal spray corticosteroid (n/s CS), oral antihistamine (AH), topical corticosteroid (TCS)

sIgE and SPTs to common inhalant allergens (d1, d2, g2, g6, m2, m3, s9, w19): at least one positive (+) / negative (-) / not done (n.d.)

Food allergies (other than fish allergy): egg (E), wheat (W), shellfish (S), peanut (P), tree nuts (T), fruit (F)
TABLE II. Immunological characterization of fish allergic children.

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<th>Pat. no.</th>
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<th>Gad c 1 ISAC (ISU)</th>
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<td>n.d.</td>
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<td>n.d. 0.03 0.03 0.50 0.02 0.03 0.02 0.02</td>
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N: non-allergic adult
n.d.: not done
TABLE III. Wheal sizes (mm²) in response to wtCyp c 1, mCyp c 1, cod, sardine, and histamine (pos. values shown in grey)

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Blocking antibodies induced by immunization with a hypoallergenic parvalbumin mutant reduce allergic symptoms in a mouse model of fish allergy

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ABSTRACT

Background: Fish is a frequent elicitor of severe IgE-mediated allergic reactions. Beside avoidance, there is currently no allergen-specific therapy available. Hypoallergenic variants of the major fish allergen, parvalbumin, for specific immunotherapy based on mutation of the two calcium-binding sites have been developed.

Objective: To develop a mouse model of fish allergy resembling human disease and to investigate whether IgG antibodies induced by immunization with a hypoallergenic mutant of the major carp allergen, mCyp c 1, protect against allergic symptoms in sensitized mice.

Methods: C3H/HeJ mice were sensitized with wildtype recombinant Cyp c 1 by intragastric gavage. Antibody, cellular immune responses and epitope specificity of sensitized mice were investigated by ELISA, RBL assay, T cell proliferation experiments using wtCyp c 1 and overlapping peptides spanning the Cyp c 1 sequence. Rabbit anti-mCyp c 1 antibodies were tested for their ability to inhibit IgE recognition of Cyp c 1, Cyp c 1-specific basophil degranulation and Cyp c 1-induced allergic symptoms in the mouse model.

Results: A mouse model of fish allergy mimicking human disease regarding IgE epitope specificity and symptoms was established. Administration of antibodies induced by immunization with a hypoallergenic Cyp c 1 mutant inhibited IgE binding to Cyp c 1, Cyp c 1-induced basophil degranulation and allergic symptoms caused by allergen challenge in sensitized mice.

Conclusion: Antibodies induced by immunization with a hypoallergenic Cyp c 1 mutant protect against allergic reactions in a murine model of fish allergy.

Clinical implications: IgG antibodies induced by vaccination with a hypoallergenic Cyp c 1 mutant may protect against fish allergy.

Capsule summary: Vaccination with a recombinant hypoallergenic Cyp c 1 mutant induces Cyp c 1-specific IgG antibodies which block IgE-binding to Cyp c 1 and protected against the symptoms of fish allergy in a mouse model.
**Key words:**
fish allergy, hypoallergenic parvalbumin mutant, specific immunotherapy, blocking antibodies

**Abbreviations:**
wtCyp c 1: recombinant wildtype Cyp c 1
mCyp c 1: recombinant hypoallergenic Cyp c 1 mutant
SIT: allergen-specific immunotherapy
i.p.: intraperitoneal
ELISA: enzyme-linked immunosorbent assay
RBL: rat basophil leukemia
OD: optical density
INTRODUCTION
Fish represents an important elicitor of food allergy causing severe allergic reactions which are often life-threatening.\textsuperscript{1} The prevalence of fish allergy ranges from 0.2% up to 10% depending on the population and is particularly high in countries with high fish consumption.\textsuperscript{2,3} While many food allergies are diseases of early childhood which are often outgrown, allergy to fish often persists up to adulthood.\textsuperscript{4}

Allergen-specific immunotherapy (SIT) is highly effective for respiratory forms of allergy and insect venom allergy.\textsuperscript{5} There are also several approaches pursued for SIT of food allergy including oral, sublingual, epicutaneous and subcutaneous administration of allergens or modified allergens.\textsuperscript{6,7} A recent review of clinical studies in oral allergen-specific immunotherapy for food allergy indicated that outcomes of treatment may be different for different allergens.\textsuperscript{8} Despite the variability of SIT regarding clinical outcome for different food allergens, studies performed for different allergens suggest that besides alterations at the cellular level, an induction of allergen-specific IgG antibodies may be important for the success of SIT in food allergy.\textsuperscript{9,10}

At present SIT is not available for fish allergy although parvalbumin, a protein containing calcium-binding sites, has been characterized as a cross-reactive allergen in many fish species and recombinant fish parvalbumins mimicking the immunological properties of the corresponding natural allergens have been produced.\textsuperscript{4,11} Based on the observation that the depletion of calcium leads to a substantial loss of IgE reactivity of fish parvalbumins\textsuperscript{12} we have developed a recombinantly expressed hypoallergenic variant of the fish allergen Cyp c 1 from carp by mutation of the calcium-binding sites in the protein as a candidate molecule for SIT of fish allergy.\textsuperscript{13} We recently also demonstrated that the strategy of introducing point mutations into the calcium-binding sites of fish parvalbumins can be used to reduce the allergenic activity of the major allergens from a variety of fish species.\textsuperscript{14}

In this study we aimed to establish a murine model of fish allergy which closely mimics fish allergy in patients. For this purpose, mice were orally sensitized with the major fish allergen Cyp c 1 and the development, epitope-specificity and
biological activity of specific IgE antibodies was determined by ELISA, basophil degranulation experiments as well as by *in vivo* provocation testing and assessment of allergic symptoms. In order to answer, whether IgG antibodies induced by immunization with the recombinant Cyp c 1 mutant (i.e., mCyp c 1) can protect against fish allergy we performed passive immunization of fish allergic mice with mCyp c 1-specific antibodies before oral provocation. The results obtained demonstrate that mCyp c 1-specific antibodies can protect against fish allergy and thus indicate that blocking antibodies might represent a major mechanism in SIT with mCyp c 1.
METHODS

Recombinant allergens, synthetic peptides

Recombinant wildtype Cyp c 1 (wtCyp c 1) and recombinant Phl p 1 were obtained from Biomay AG (Vienna, Austria). The recombinant Cyp c 1 mutant (mCyp c 1) was expressed in *E. coli* and purified as described. Overlapping peptides spanning the Cyp c 1 sequence were synthesized on a peptide synthesizer Apex 396 (AAPPTec, Louisville, USA) as described. The biochemical characteristics and positions of the peptides within the amino acid sequence of Cyp c 1 are summarized in Table I and Figure 2C.

Rabbit antisera, sera from fish allergic patients

For the generation of mCyp c 1-specific antisera, two female New Zealand rabbits were immunized at Charles River Laboratories (Sulzfeld, Germany) four times subcutaneously with 200 µg mCyp c 1 using Freund's adjuvant (complete Freund's adjuvant / incomplete Freund's adjuvant). A rabbit antiserum obtained by immunizing a rabbit with rPhl p 1 in the same way served as a control. Sera were obtained from patients with a positive case history of IgE-mediated allergy to fish, who had experienced at least one of the typical clinical symptoms (dermatitis, urticaria, angioedema, diarrhea, rhinitis, asthma, or a systemic anaphylactic reaction) after consumption of fish. IgE-mediated fish allergy was verified by determination of specific IgE antibodies using the ImmunoCAP System (Thermofisher/Phadia, Uppsala, Sweden).

Preparation of fish extracts and immunoblotting

Allergen extracts were prepared from fresh filets of carp, cod, mackerel, salmon, sardine, swordfish and tuna. Ten gram aliquots of each fish were frozen in liquid nitrogen, crushed to powder and dissolved in 50 ml ice-cold phosphate buffered saline (PBS). Extraction of the fish powder was performed overnight at 4°C. After centrifugation at 14000rpm and 4°C, supernatants were filtered through a 0.22µm filter and protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, USA). Aliquots of eighty microgram of total protein of each fish extract were used for immunoblotting. Extracts, 1µg
purified wtCyp c 1 (positive control), and the grass pollen allergen rPhl p 6 (negative control) were separated by 16% SDS-PAGE\textsuperscript{16} and blotted on a nitrocellulose membrane (Whatman Protran nitrocellulose membrane, Sigma-Aldrich, St. Louis, USA).\textsuperscript{17} After blocking blotted proteins were incubated with mCyp c 1-specific rabbit serum diluted 1:1000 overnight at 4°C. Then membranes were washed and bound IgG antibodies were detected with \textsuperscript{125}I-labeled goat anti-rabbit IgG (PerkinElmer, Waltham, USA) and visualized by autoradiography.\textsuperscript{18}

**Development of a mouse model of fish allergy**

Six to eight weeks old, female C3H/HeJ mice were obtained from Harlan (San Pietro Al Natisone, Italy). All experiments were approved by the local review board of the Medical University of Vienna, and were performed in accordance with national and international guidelines of laboratory animal care. The mouse model of fish allergy was established according to the protocol in Figure 1. Mice (n=24) were sensitized 5 times in weekly intervals with 100µg wtCyp c 1 and 20µg Cholera toxin (Sigma–Aldrich, St. Louis, USA) in 200µl 0.2M bicarbonate buffer (pH 9) by intragastric gavage. After sensitization mice were randomized in two groups of 12 mice each with comparable Cyp c 1-specific IgE antibody levels. On day 48, the therapy group received 500µl of heat-inactivated mCyp c 1-specific rabbit antiserum and the control group received 500µl of heat-inactivated Phl p 1-specific antiserum intraperitoneally (i.p.). Blood samples were taken on the day before each sensitization and before and after rabbit serum application. Mice were then challenged by intragastric gavage on day 51 with 100µg wtCyp c 1. Symptoms were recorded by two independent observers according to the symptom score published by Li X. M. et al. (0: no symptoms, 1: scratching and rubbing around the nose and head, 2: puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate, 3: wheezing, labored respiration, 4: no activity after prodding or tremor and convulsion 5: death).\textsuperscript{19} For control purpose naïve mice were also included in the study. Results shown are representative for two independently performed experiments.
Measurement of wtCyp c 1-specific and peptide-specific antibodies in mouse and human sera

Mouse IgM, IgA, IgG₁, IgG₂a, IgG₃ specific for wtCyp c 1 were determined by ELISA as previously described.²⁰ ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with wtCyp c 1 (5µg/ml). Mouse sera were diluted 1:50 (for IgM, IgA, IgG₂a and IgG₃) and 1:500 (for IgG₁). IgM, IgA, IgG₁, IgG₂a and IgG₃ were detected with monoclonal rat anti-mouse IgM, IgA, IgG₁, IgG₂a and IgG₃ antibodies, respectively (GE Healthcare, Fairfield, USA, 1:1000 dilution) followed by a HRP-labeled goat anti-rat IgG (minimal cross-reactivity) antibody (BioLegend, San Diego, USA, 1:2000 dilution).

To measure human and mouse IgE specific for wtCyp c 1 and Cyp c 1 peptides, ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with wtCyp c 1 or the individual peptides (5µg/ml). Mouse and human sera were diluted 1:10. Human IgE was detected with a HRP-labeled goat anti-human IgE antibody (KPL, Maryland, USA, 1:2500 dilution), mouse IgE with a rat anti-mouse IgE antibody (GE Healthcare, Fairfield, USA, 1:1000 dilution) and HRP-labeled goat anti-rat IgG (BioLegend, San Diego, USA, 1:2000 dilution).

wtCyp c 1-specific rabbit IgG antibodies were measured in mouse sera, which were obtained before and after i.p. application of mCyp c 1-specific or rPhl p 1-specific rabbit antisera to the mice. For this purpose, ELISA plates were coated with wtCyp c 1 (5µg/ml). Mouse sera were diluted 1:50,000 and rabbit IgG was detected with HRP-labeled anti-rabbit IgG from donkey (GE Healthcare, Fairfield, USA, 1:2500 dilution). All measurements were performed in duplicates. OD values shown are mean values of the different mouse groups.

Titration and epitope mapping of mCyp c 1-induced rabbit IgG and inhibition of human IgE-binding to wtCyp c 1

ELISA plate-bound wtCyp c 1 (5µg/ml) was incubated with serial dilutions of the mCyp c 1-specific rabbit antiserum (diluted 1:8000 - 1:512000). Rabbit IgG was detected with a HRP-labeled anti-rabbit IgG antibody from donkey (GE Healthcare, Fairfield, USA, dilution: 1:2500). For the epitope mapping of rabbit anti-mCyp c 1 antibodies, ELISA plates were coated with wtCyp c 1 or Cyp c 1
peptides (5µg/ml) and exposed to the 1:50,000 diluted mCyp c 1-specific rabbit antiserum.

For IgE inhibition experiments, ELISA plate-bound wtCyp c 1 (1µg/ml) was pre-incubated with serial dilutions of the mCyp c 1-specific rabbit antiserum (1:20 – 1:100000) or, for control purposes, with the pre-immune serum (1:20). After washing 1:10 diluted patients’ sera were added. Human IgE was detected with a HRP-labeled goat anti-human IgE antibody (KPL, Maryland, USA, 1:2500). All measurements were performed as duplicates. The OD values shown are mean values with a deviation of less than 5%.

Rat basophil leukemia cell degranulation experiments
RBL cells were grown in 96-well cell-culture plates (Costar, Corning, Tewksbury, USA) for 20 hours at 37°C and 5% CO₂. Five µl of mouse sera were added and the cells were incubated at 37°C, 5% CO₂ for 2 hours and washed 2 times with Tyrode’s Buffer (Sigma-Aldrich, St. Louis, USA). Degranulation of RBL cells was induced by adding 100 µL of wtCyp c 1 (0.3µg/ml) in Tyrode’s Buffer and incubation for 30 minutes at 37°C, 5% CO₂. In the inhibition experiments wtCyp c 1 (0.3µg/ml) was added together with 10% v/v mCyp c 1-specific heat-inactivated rabbit antiserum or pre-immune serum. Beta-hexosaminidase in culture supernatants was detected with 0.16 mM 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide (Sigma-Aldrich, St. Louis, USA) and fluorescence was measured at λex:360/λem:465 nm using a microplate reader (VICTOR™ Plate Reader, Perkin Elmer, Waltham, USA). Results are reported as percentages of total β-hexosaminidase released after cell lysis by addition of 10% Triton X-100. All measurements were performed in triplicates.

Proliferation assays
Spleens were removed from mice on day 62 under aseptic conditions and homogenized. Single cell suspensions were filtered through a 70 µm nylon cell strainer to remove remaining tissue. Erythrocytes were removed by adding ice cold red blood cell lysing buffer (Sigma–Aldrich, St. Louis, USA). Cells (2 × 10⁶ cells/ml) were cultivated in 96-well round-bottom plates (Nunclon Delta Surface,
Roskilde, Denmark) in the presence of wtCyp c 1 or mCyp c 1 (2μg/well), or synthetic Cyp c 1 peptides (0.36 μg/well). Concanavalin A (0.5 μg/well) (Sigma-Aldrich, St. Louis, USA) was used as positive control and medium as a negative control. The plates were incubated at 37°C, 5% CO₂. On day five 0.5 μCi ³H thymidine ([methyl-³H] thymidine, Perkin Elmer, Waltham, USA) per well was added to cells. After 18 hours cells were harvested and thymidine incorporation was measured in a beta counter (Beta scintillation liquid, Wallac Micro Beta TriLux Luminescence Counter, Perkin Elmer, Waltham, USA).

**Statistical analysis**

Differences between mouse groups were determined using a Mann-Whitney U-Test. P-values < 0.05 were considered as significant (*), p-values <0.01 as highly significant (**).
RESULTS

Development of a mouse model of fish allergy

In order to develop a mouse model of IgE-associated fish allergy which resembles the human situation as closely as possible, C3H/HeJ mice were sensitized by repeated intragastric gavage with the major fish allergen, Cyp c 1, from carp, which shows extensive IgE cross-reactivity with parvalbumins from various fish species. The application of recombinant wtCyp c 1 together with the mucosal adjuvant cholera toxin resulted in the induction of a Cyp c 1-specific IgE response which was accompanied by a specific IgG1, IgG2a and IgM response (Fig. 1, 2A-D). No relevant induction of Cyp c 1-specific IgG3 or IgA responses was observed (Fig. 2B). The analysis of the epitope specificity of this IgE response using seven overlapping Cyp c 1 peptides gave similar results (Fig. 2C, D) as experiments performed with sera from fish allergic patients (Fig. 2E). Cyp c 1-specific mouse IgE antibodies showed no relevant IgE reactivity to any of seven Cyp c 1 peptides but only to the complete Cyp c 1 allergen indicating that the murine IgE antibodies were not directed to linear/sequential epitopes but to the intact conformation of Cyp c 1 (Fig. 2D). Likewise, the IgE epitope mapping of fish allergic patients’ sera (n=10) revealed that also the patients IgE responses were mainly directed to the complete Cyp c 1 allergen molecule and not to the Cyp c 1 peptides (Fig. 2E). Only peptide 7 showed some weak IgE reactivity. The mapping of the Cyp c 1-specific T cell response performed with the overlapping synthetic Cyp c 1 peptides in cultivated splenocytes from sensitized mice identified peptide 5 spanning aa 61-80 of Cyp c 1 as the major T cell epitope in the murine model (Fig. 2F). The ability of the Cyp c 1-specific IgE antibodies to induce allergic reactions was demonstrated by loading RBL cells with serum IgE from sensitized mice which resulted in degranulation and mediator release (Fig. 4).

IgG antibodies induced by immunization with a hypoallergenic Cyp c 1 mutant inhibit the binding of patients’ IgE to Cyp c 1 and cross-react with parvalbumins from many fish species
We have engineered a recombinant hypoallergenic derivative of the major carp allergen Cyp c 1 (mCyp c 1) by mutation of the two active calcium binding sites of the molecule.\textsuperscript{13,14} In order to investigate whether IgG antibodies induced by immunization with mCyp c 1 protect against fish allergy, we produced mCyp c 1-specific rabbit antibodies. Rabbit anti-mCyp c 1 IgG antibodies reacted with wtCyp c 1 even at high dilutions (>1:100.000) (Fig. 3A). Furthermore, rabbit anti-mCyp c 1 antibodies at a dilution of 1:100 inhibited almost completely the binding of fish allergic patients’ IgE to wtCyp c 1 as shown in IgE inhibition ELISA experiments (Fig. 3B). Epitope mapping studies performed with synthetic Cyp c 1 peptides showed that anti-mCyp c 1-specific IgG antibodies reacted also with sequential Cyp c 1 peptide epitopes (peptides 7>1=3>6) (Fig. 3C). Furthermore, mCyp c 1-specific IgG antibodies cross-reacted with natural parvalbumins from six commonly consumed fish species (carp, cod, mackerel, salmon, sardine and tuna) (Fig. 3D). Only parvalbumin from swordfish did not react with mCyp c 1-induced IgG antibodies (Fig. 3D).

\textbf{mCyp c 1-induced IgG antibodies block IgE binding to Cyp c 1, Cyp c 1-induced basophil degranulation and allergic reactions in the murine model of fish allergy}

In a first \textit{ex vivo} experiment we investigated whether mCyp c 1-induced rabbit IgG antibodies can suppress the Cyp c 1-induced degranulation of basophils loaded with IgE from Cyp c 1-sensitized mice (Fig. 4). We found that Cyp c 1 in the presence of mCyp c 1-specific rabbit IgG antibodies did not induce relevant basophil degranulation over background (medium alone) whereas Cyp c 1 without mCyp c 1-specific IgG antibodies induced basophil degranulation (Fig. 4).

Next we injected mCyp c 1-specific IgG antibodies in one group of Cyp c 1 sensitized mice whereas the other Cyp c 1-sensitized mouse group received IgG antibodies specific for the unrelated grass pollen allergen Phl p 1. We then analyzed blood samples collected one day before and one day after treatment for the presence of the injected rabbit IgG antibodies. Cyp c 1-specific rabbit IgG antibodies were only detected in mice who had received anti-mCyp c 1-
specific IgG (Fig. 5A) but not in mice who had been treated with anti-Phl p 1-specific IgG (Fig. 5B). The latter group was found to contain Phl p 1-specific rabbit IgG (data not shown). The binding of mouse IgE to Cyp c 1 was reduced in mice who had been treated with anti-mCyp c 1-specific IgG (Fig. 5C) but not in mice who had been treated with Phl p 1-specific IgG (Fig. 5D) when Cyp c 1-specific IgE reactivity was compared before and after injection of rabbit antibodies.

Next, the two groups of Cyp c 1-sensitized mice which had received mCyp c 1-specific IgG or Phl p 1-specific IgG were challenged by intragastric application of 100µg wtCyp c 1. Upon challenge, mice which had received the Phl p 1-specific IgG antibodies (control group) developed allergic symptoms including scratching and rubbing around nose and mouth, reduced activity and swelling around the eyes and mouth whereas mice which had been treated with mCyp c 1-specific IgG antibodies (therapy group) showed either no or only mild symptoms in response to oral allergen challenge (Fig. 6). Thus a significant reduction of allergic symptoms was found in the group of mice which had been treated with mCyp c 1-specific IgG when compared to the group treated with the Phl p 1-specific IgG antibodies (Fig. 6).
DISCUSSION
Since the original demonstration by Prausnitz & Kuestner that allergic sensitization to fish can be transferred passively with serum,\textsuperscript{21} treatment of fish allergy and other life-threatening food allergies represents a major challenge.\textsuperscript{22,23} Due to the high allergenic activity of fish which can induce severe allergic reactions, SIT is not available as a treatment option for fish allergic patients. In order to establish an \textit{in vivo} model for fish allergy which closely mimics fish allergy in patients and hence would allow studying approaches for SIT of fish allergy, we sensitized mice by the oral route using the recombinant major fish allergen, rCyp c 1. Mice sensitized by oral administration of rCyp c 1 developed IgE antibodies which mainly recognized conformational epitopes of Cyp c 1 similar to those of fish allergic patients. Importantly, IgE antibodies induced by rCyp c 1 also induced basophil activation and allergic reactions when sensitized mice were orally challenged. Epitope mapping studies performed with overlapping peptides of Cyp c 1 identified peptide 5 comprising amino acids 61-80 in the Cyp c 1 sequence as a major T cell epitope which may be useful for studying T cell epitope-targeting strategies in the murine model. Since besides cellular mechanisms, the induction of allergen-specific blocking IgG antibodies has been identified as a major mechanism of SIT for other food allergen sources such as peanut allergy and egg allergy\textsuperscript{9,10} we thought to evaluate the therapeutic potential of mCyp c 1, a recombinant hypoallergenic variant of Cyp c 1 which is currently evaluated as a candidate for subcutaneous immunotherapy of fish allergy in the European Union-funded project FAST.\textsuperscript{7} Within the FAST project, mCyp c 1 has been formulated for subcutaneous injection immunotherapy because it has been shown that immunization of mice and rabbits with mCyp c 1 induces a robust induction of IgG antibodies which recognize the wildtype allergen. In fact, we found that IgG antibodies induced by immunization of rabbits with mCyp c 1 not only recognized the Cyp c 1 wildtype allergen but also inhibited allergic patients’ IgE antibodies to Cyp c 1. Moreover, mCyp c 1-induced rabbit antibodies cross-reacted with parvalbumins from several fish species and inhibited the degranulation of basophils which were loaded with IgE antibodies from Cyp c 1-sensitized mice.
We were therefore interested to study if mCyp c 1-specific antibodies can also suppress symptoms of fish allergy *in vivo* using the fish allergy model. For this purpose we followed the classical experiments performed by Dunbar which basically inspired Noon to conduct the first SIT study for the treatment of grass pollen allergy.\(^{24}\) In fact Dunbar showed that rabbit anti-sera raised against natural allergen preparations were able to suppress allergic inflammation in patients using conjunctival provocation testing.\(^{25}\)

We therefore treated sensitized mice with mCyp c 1-specific rabbit antibodies and could show their presence in the circulation of mice and that they inhibited the binding of IgE from sensitized mice to Cyp c 1 by ELISA. More importantly, we found that mice which had received mCyp c 1-specific antibodies were protected against allergic reactions induced by oral allergen challenge.

We think that the obtained results are important because they indicate that antibodies induced by immunization with mCyp c 1 can suppress allergic reactions caused by the major fish allergen and hence may represent an important mechanism of SIT with mCyp c 1. Since the allergenic activity of mCyp c 1 has been reduced compared to the wildtype allergen it will be possible to vaccinate fish allergic patients with fewer and higher doses to induce protective IgG antibodies directly by active vaccination. Ultimately it may be possible to establish safe and convenient subcutaneous SIT protocols for the treatment of fish allergy which are based on mCyp c 1.
REFERENCES


FIGURE LEGENDS

TABLE I. Sequences, molecular weights and pIs of synthetic peptides spanning the Cyp c 1 sequence.

FIG 1. Sensitization and treatment protocol for a mouse model of fish allergy. Mice were sensitized to wtCyp c 1 five times by intragastric gavage as indicated, treated by the i.p. application of mCyp c 1- or Phl p 1-specific IgG antibodies on day 48 and challenged with wtCyp c 1 on day 51.

FIG 2. Cyp c 1-specific immune responses in sensitized mice. (A) Induction of Cyp c 1-specific IgG1, and (B) IgG2a, IgG3, IgA, IgM as measured by ELISA. (C) Position of synthetic Cyp c 1-derived peptides 1-7 in the Cyp c 1 molecule. (D) Epitope mapping of mouse IgE and (E) allergic patients IgE with Cy p c 1 peptides. Shown are mean antibody levels (y-axes: optical density OD values ± SDs) before sensitization and at day 28. (F) Proliferation of splenocytes from Cyp c 1-sensitized and naïve mice in response to Cyp c 1 peptides, wtCyp c 1 and medium alone. Results are displayed as mean counts per minute ± SDs for the two mouse groups.

FIG 3. Characterization of the IgG antibodies induced by immunization with mCyp c 1. (A) Titration of rabbit IgG antibodies induced by immunization with mCyp c 1 for reactivity to wtCyp c 1. Mean OD values corresponding to bound IgG (y-axis) are displayed for different serum dilutions (x-axis). (B) Inhibition of fish allergic patients’ IgE-binding to wtCyp c 1 by mCyp c 1-induced IgG. Shown is allergic patients’ IgE binding (patients 1-8) (y-axis: mean OD values) to wtCyp c 1 which was pre-incubated with various dilutions of serum from a rabbit obtained before (Pre) or after immunization with mCyp c 1 (x-axis). (C) Epitope mapping of mCyp c 1-induced rabbit IgG antibodies. IgG reactivity (y-axis: mean OD values +/- SDs) of mCyp c 1-specific IgG to wtCyp c 1 and Cyp c 1 peptides 1-7 (x-axis). (D) Cross-reactivity of mCyp c 1-induced rabbit IgG antibodies to nitrocellulose-blotted allergen extracts from other fish species.
Blotted fish extracts were incubated with mCyp c 1-specific rabbit IgG antibodies. Bound IgGs were detected with $^{125}$I-labeled anti-rabbit IgG antibodies and visualized by autoradiography. Molecular weights (kDa) are indicated on the left margin.

**FIG 4.** mCyp c 1-specific IgG antibodies inhibit wtCyp c 1-induced basophil degranulation. RBL cells were loaded with serum from wtCyp c 1-senzitized mice in the presence or absence of mCyp c 1-specific IgG antibodies (x-axis). The mean percentage of total β-hexosaminidase release ± standard deviation is displayed on the y-axis.

**FIG 5.** Reduced allergen-specific IgE reactivity of Cyp c 1-sensitized mice which had been treated with mCyp c 1-specific IgG. Presence of Cyp c 1-specific rabbit IgG in mice before and after injection of (A) mCyp c 1-specific IgG or (B) Phl p 1-specific IgG. Cyp c 1-specific IgE reactivity of mice before and after injection of (C) mCyp c 1-specific IgG or (D) Phl p 1-specific IgG. Antibody reactivities are shown for each mouse (y-axes: OD values) before and after IgG injection (y-axes).

**FIG 6.** Suppression of allergic symptoms in Cyp c 1-sensitized mice after challenge with Cyp c 1 by mCyp c 1-specific IgG. Allergic symptoms (y-axis: mean symptom scores ± standard deviations) in Cyp c 1-sensitized mice having received mCyp c 1-specific or Phl p 1-specific IgG antibodies (x-axis).
Table I. Sequences, molecular weights and pls of synthetic peptides spanning the Cyp c 1 sequence.

<table>
<thead>
<tr>
<th>peptide</th>
<th>aa sequence</th>
<th>MW (Da)</th>
<th>isoelectric point (pI)</th>
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<tr>
<td>P1</td>
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<td>P2</td>
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<td>2182.4</td>
<td>5.95</td>
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<td>P3</td>
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<td>2106.4</td>
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<td>2282.5</td>
<td>4.3</td>
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<td>EDELKLFQNFSAARALTQD</td>
<td>2238.4</td>
<td>4.32</td>
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<td>P6</td>
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<td>2081.2</td>
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<td>P7</td>
<td>GDSDGDKIGVDEFAALVKA</td>
<td>1964.1</td>
<td>4.04</td>
</tr>
</tbody>
</table>
Figure 1

Intragastric gavage
100μg wtCyp c 1 + cholera toxin
Randomization
Antibody treatment
Sacrifice

Blood samples
0 7 14 21 28 43

days

1 8 15 22 38

Intragastric challenge
wtCyp c 1

anti-mCyp c 1

anti-rPh p 1

51

48 49

62
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Summary

Food allergy is a serious problem in developed countries with an increasing prevalence and symptoms ranging from mild reactions to life-threatening anaphylaxis.

Fortunately, molecular cloning technologies have enabled the production of food allergens in a recombinant form, which represents a major step towards component-resolved diagnosis and improved treatment strategies for food allergy.

Allergen-specific approaches for the treatment of food allergy include subcutaneous, oral and sublingual immunotherapy with varying efficacy and safety profiles. However, the development of hypoallergenic food allergen derivatives with reduced IgE reactivity may be an attempt to decrease unwanted side effects of allergen-specific immunotherapy.

The FAST project (food allergy specific therapy), funded by the European Union (http://www.allergome.org/fast/), aims at the development of subcutaneous hypoallergenic immunotherapies for fish and peach allergy as they represent severe and persistent allergies prevalent in the European Union.

Contributing to this project, the allergenic activity of a hypoallergenic mutant (mCyp c 1) of the major fish allergen and recombinant carp parvalbumin (wtCyp c 1) were analyzed with regard to IgE reactivity. This revealed strongly reduced wheal and flare reactions in response to mCyp c 1 in comparison to the wild-type allergen wtCyp c 1 showed by skin prick tests in patients.

The second aim of the master thesis was the development of a mouse model of fish allergy to test mCyp c 1 in the model. Therefore, an oral sensitization scheme with wtCyp c 1 and the mucosal adjuvant cholera toxin was used. As a treatment strategy, mice were passively immunized with mCyp c 1-induced rabbit IgG antibodies. Taken together, the application of mCyp c 1-induced IgG antibodies could protect sensitized mice from allergic symptoms after oral wtCyp c 1 challenge.
Zusammenfassung


Durch molekulare Klonierungstechniken wurde die Produktion von Allergenen in rekombinanter Form ermöglicht. Dies stellte einen großen Schritt in Richtung neuer, auf Allergenkomponenten basierter Diagnostik, sowie verbesserter Behandlungsmöglichkeiten dar. Allergen-spezifische Therapien beinhalten subkutane, orale und sublinguale Immuntherapie, mit unterschiedlicher Effizienz und unterschiedlichem Grad an Nebenwirkungen. Doch die Entwicklung von hypoallergenen Allergen-Derivaten mit reduzierter IgE-Reaktivität ist ein möglicher Ansatz um unerwünschte Nebenwirkungen der Therapien zu senken.

Daher hat das FAST (Food Allergy Specific Therapy) EU-Projekt (http://www.allergome.org/fast/) die Zielsetzung, hypoallergene Immuntherapien mit subkutaner Anwendung gegen Fisch- und Pfirsichallergie zu entwickeln. Diese stellen in der EU weitverbreitete, persistente und schwere Lebensmittelallergien dar.

Als Beitrag zu diesem EU-Projekt war ein Ziel dieser Masterarbeit, rekombinantes Fischallergen (wtCyp c 1) und eine hypoallergene Mutante des Fischallergens (mCyp c 1) bezüglich ihrer IgE Reaktivität zu vergleichen. Eine Hautteststudie mit Patienten zeigte deutlich, dass mCyp c 1 im Vergleich zum Wildtyp-Allergen wtCyp c 1 stark reduzierte Quaddel-Erythem-Reaktionen verursacht.

Das zweite Ziel war die Entwicklung eines murinen Fischallergiemoedells, um mCyp c 1 darin auszutesten. Für die Etablierung des Modells wurde ein orales
Sensibilisierungsschema mit wtCyp c 1 und dem mukosalen Adjuvans Cholera Toxin angewandt. Eine passive Immunisierung mit mCyp c 1-induzierten Kaninchenantikörperrn wurde als Behandlungsstrategie verwendet. Zusammenfassend wurde gezeigt, dass die Injektion von mCyp c 1-induzierten Kaninchenantikörperrn sensibilisierte Mäuse vor allergischen Symptomen nach oraler Provokation mit wtCyp c 1 schützt.
Curriculum vitae

Personal Data

Date of Birth: March 2nd, 1988
Place of Birth: Linz, Austria
Citizenship: Austria

Education

Feb. 2012 – Jan. 2014: Master Thesis at the Department of Pathophysiology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, under supervision of Univ.-Prof. Dr. Rudolf Valenta

January – May 2011: Semester abroad at the University of Eastern Finland, Kuopio, Finland

Since March 2010: Master studies in Molecular Biology with specialization on Molecular Medicine at the University of Vienna

Since Sept. 2009: Master studies in Nutritional Science with specialization on Molecular Nutrition at the University of Vienna

2006-2009: Bachelor studies in Nutritional Science at the University of Vienna

1999-2006: Grammar School, Upper Austria

1994-1999: Primary school, Upper Austria
Awards

Travel Grant of the European Academy of Allergy and Clinical Immunology Food Allergy and Anaphylaxis Meeting (EAACI FAAM) 2013, Nice, France

Erasmus travel grant for the semester abroad at the University of Eastern Finland

Merit grant 2009 and 2010 of foundations and funds of the University of Vienna for study achievements between January 2008 and December 2009

Merit grant 2007, 2008, 2009 and 2010 of the Austrian Federal Ministry of Science and Research for study achievements between October 2006 and September 2010

European Patent

"Hypoallergenic allergen derivatives of Pru p 3 for immunotherapy of IgE-mediated peach allergy" (Application No. 11195132.3)