New Perspectives for Allergy Diagnosis and Therapy

Inauguraldissertation
der Philosophisch-naturwissenschaftlichen Fakultät
der Universität Bern

vorgelegt von

Pamela Marti

von Bern, BE

Leiter der Arbeit:

Prof. Dr. Beda M. Stadler
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Der Dekan:
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A. SUMMARY

Most of the clinically relevant allergens are known and have been molecularly characterised. We have previously used a bioinformatical approach to predict allergenicity of proteins and their cross-reactions with other allergens. The presently known allergens were regrouped according to shared allergenic regions called motifs. More than one thousand allergen sequences can now be attributed to 69 motifs.

The aim of our study was to investigate whether these predicted *in silico* motifs correspond to the epitopes recognised by IgE. Two allergen motifs of clinical relevance were selected. One of them contained manganese superoxide dismutases (MnSODs) of different organisms and the other one comprised tropomyosins of diverse phylogenetic origin.
From the MnSOD motif, the *Aspergillus fumigatus* sequence was chosen as a representative and its 50 amino acid spanning motif region was synthesised as a peptide (MnSOD Mot). We found specific IgE antibodies against MnSOD Mot in sera of patients who showed a high IgE reactivity to the corresponding full length allergen and also in a patient sensitised to the cross-reactive MnSOD of *Malassezia sympodialis*. These results suggest that MnSOD Mot contains an epitope which is involved in the allergenicity of the two cross-reacting fungal MnSODs.
For the tropomyosin allergen, a consensus motif sequence (Trop Mot) was determined and expressed as a recombinant protein in *Escherichia coli*. We detected specific IgE reactivity to Trop Mot in sera of shellfish allergic individuals which correlated in intensity with the one measured for a shrimp full length tropomyosin in the same sera. We concluded that Trop Mot represents an allergenic structure that is comparable to the full length tropomyosin and that may be used as a serological marker for the diagnosis of shellfish allergy in the future. Apart from potentially being useful in diagnostic tests, allergen motifs may also be beneficial for the development of future therapeutic reagents (Dissertation Equivalent I).

Furthermore, for the improvement of atopy therapy, recombinant live lactic acid bacteria were tested as a delivery vehicle for oral anti-IgE vaccination. The presentation of vaccines via the oral route is advantageous to other routes in different aspects. Particularly it is convenient, can be carried out on a large scale and is rather inexpensive.
It has been shown earlier that mimotopes of human IgE are able to induce an anti-IgE response which prevents allergic reactions. To obtain a mucosal anti-IgE vaccine, *Lactobacillus johnsonii* (Lj) bacteria were engineered to express the membrane anchoring domain of proteinase B from *Lactobacillus bulgaricus* fusioned to either the Cε4 IgE mimotope or to an anti-idiotypic single chain fragment variable (α-IdscFv2) which was derived from a Fab mimicking the same region of IgE as the mimotope but covering a larger area. When used for subcutaneous and intranasal immunisation of mice, these recombinant Lj induced a systemic IgG response against human IgE, indicating that recombinant Lactobacilli expressing IgE epitopes might be used to induce *in vivo* a beneficial anti-IgE response by parenteral as well as oral vaccination (Dissertation Equivalent II).
Thus it may be envisaged to use consensus sequences derived from motifs expressed on such oral vaccines for immunotherapy of allergic disease.

B. ABBREVIATIONS

MnSOD = Manganese Superoxide Dismutase
ABPA = Allergic Bronchopulmonary Aspergillosis
AD = Atopic Dermatitis
IL = Interleukin
PBMC = Peripheral Blood Mononuclear Cells
CF = Cystic Fibrosis

A. fumigatus = Aspergillus fumigatus
M.sympodialis = Malassezia sympodialis
E.coli = Escherichia coli
D.melanogaster = Drosophila melanogaster
S.cerevisiae = Saccharomyces cerevisiae
P.aztecus = Penaeus aztecus
P.americana = Periplaneta americana
D.pteronyssinus = Dermatophagoides pteronyssinus
T.cornutus = Turbo cornutus

FAO = Food and Agriculture Organisation
WHO = World Health Organisation

C. SCIENTIFIC OVERVIEW

Atopic diseases

Epidemiology

The prevalence of atopic diseases such as allergic rhinitis, asthma, eczema and food allergy has substantially increased during the past 30 years and it is estimated that more than 20% of the population worldwide is suffering from an atopic disease [1]. Studies of the population prevalence of these diseases show a considerable worldwide variation with industrialized and Western countries being more affected than developing countries. A study conducted with Swedish children shows a double increase of asthma, allergic rhinitis and eczema symptoms between 1979 and 1991 [2]. The most striking differences can be found in asthma symptoms. A study conducted by the ECRHS (European Community Respiratory Health Survey) reports that English-speaking countries have the highest asthma symptom prevalence rates, with centres in the UK, Australia, New Zealand and Republic of Ireland reporting the highest incidences. The lowest prevalences are reported from centres in several Eastern European countries [3, 4].

The reasons for this striking increase of atopic disorders are still a matter of debate. It can certainly not be simply attributed to the improvement of diagnostic techniques or to the greater general awareness in the public. These diseases result from complex interactions between various environmental influences and genetic predisposition, but considering the time period over which the increase in prevalence has occurred, it is unlikely to be primarily the consequence of genetic changes. Of much greater importance to the process of allergic sensitisation seems to be the contribution of environmental factors, mainly in early life [1]. The so called “hygiene hypothesis” states that a reduced exposure to infectious agents during childhood, as it occurs in Western societies as part of a changed lifestyle, is associated with a higher risk of developing allergic diseases later in life [5]. This assumption is based on experimental evidence suggesting that infectious agents such as bacteria and viruses guide the immune system to a Th1-phenotype dominated response, and therefore the lack of experienced infections may deviate the immune system to a Th2-cytokine profile, which triggers IgE antibody production. Studies on infections with *Helicobacter pylori*, *Toxoplasma gondii* and *Mycobacterium tuberculosis* support the idea of an inverse relationship between atopy and repeated infections [6-8]. However, the interactions between infections and development of allergy are complex and in part still contradictory as also allergy enhancing effects of microbes have been observed. *Bordetella pertussis* for example, is a strong enhancer of IgE formation [9].
Nevertheless, it is widely accepted that factors such as exposure to farm animals or domestic pets, day care attendance, large family size and high household endotoxin levels can protect against allergic sensitisation [5]. Another protective factor is breast feeding, which has been shown to significantly reduce the risk of asthma and atopy at 6 years of age if exclusively continued till at least 4 months after birth [10]. The multifactorial determinants influencing the development of atopic disease are schematically summarized in Figure 1.

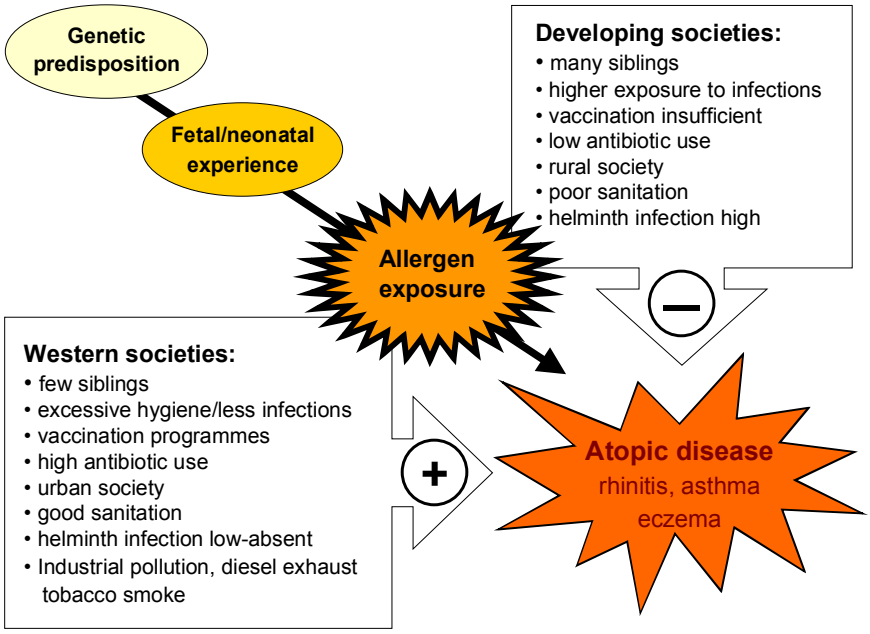

Figure 1: Multifactorial determinants influencing the development of atopic disease (adapted from 10)

After the increase in prevalence of asthma that has continued for decades, recent reports provide evidence that the burden of asthma may have reached a plateau in some areas. A study from Canada for instance, reports that physician-diagnosed asthma prevalence increased from 1991 to 1996, whereas in 1997 and 1998 the rate was either stable or decreased, depending on the age group [11]. The reasons for these signs of a reversing trend are thought to lie in the earlier detection and improved treatment of asthmatics. Furthermore, environmental influences associated with modern lifestyle may have reached the maximum possible impact on inducing symptoms [12].

Initiation of Immediate Hypersensitivity

The principal immunologic mechanism leading to an allergic reaction is termed immediate hypersensitivity (type I hypersensitivity) because it begins rapidly, that is within minutes of initial antigen exposure. The sequence of events in immediate hypersensitivity is initiated by the production of IgE antibodies to an environmental protein antigen, commonly called an allergen. In atopic individuals, $CD4^+$ helper T cells differentiate predominantly into the T_H2 subtype of effector cells upon encounter with allergens [13]. T_H2 cells produce cytokines like IL-4 and IL-13 to stimulate B lymphocytes specific for the allergens to switch to IgE producing plasma cells [14, 15]. In addition to these cytokines, B cells require contact-mediated signals to undergo isotype switching , which are also provided by the T_H2 cells (Figure 2). These contact-mediated signals include the interaction of the antigen-specific T cell Receptor (TCR) to the MHC II associated and processed allergenic peptide on the B cell, the subsequent interaction between induced CD40L on the T cell and constitutively presented CD40 on the B cell and the binding of induced CD80 (B7-1) and CD86 (B7-2) to CD28 [16]. It is the interaction of CD40 with its ligand together with the cytokines IL-4 and IL-13 that promotes the strong B cell growth and the isotype switch to IgE [17].

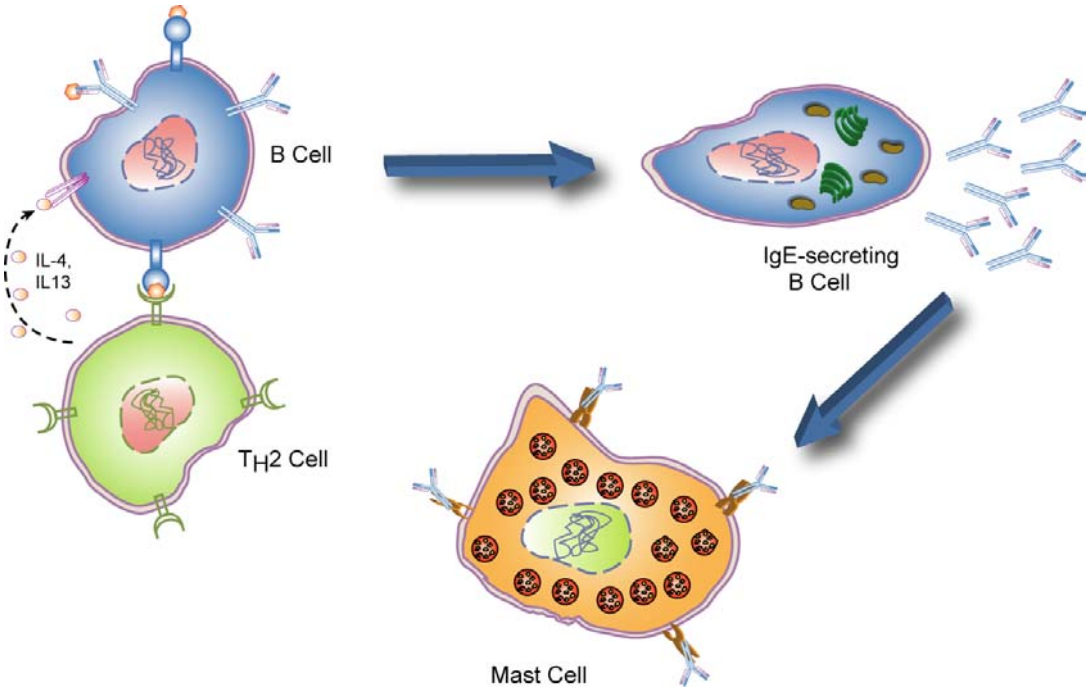

Figure 2: Initiation of immediate hypersensitivy reactions: IgE production and binding to mast cells

Specific Fc receptors present on the surfaces of mast cells and basophils bind the circulating IgE and re-introduced antigen induces cross-linking of the bound IgE which in turn leads to activation of the cells and to release of pre-formed mediators (Figure 3). These mediators are stored in cytoplasmic granules and include biogenic amines, the prototype of which is histamine, several enzymes and also proteoglycans, which collectively cause increased vascular permeability, vasodilation and bronchial and intestinal smooth muscle contraction resulting in a wheal and flare reaction on the skin [18].

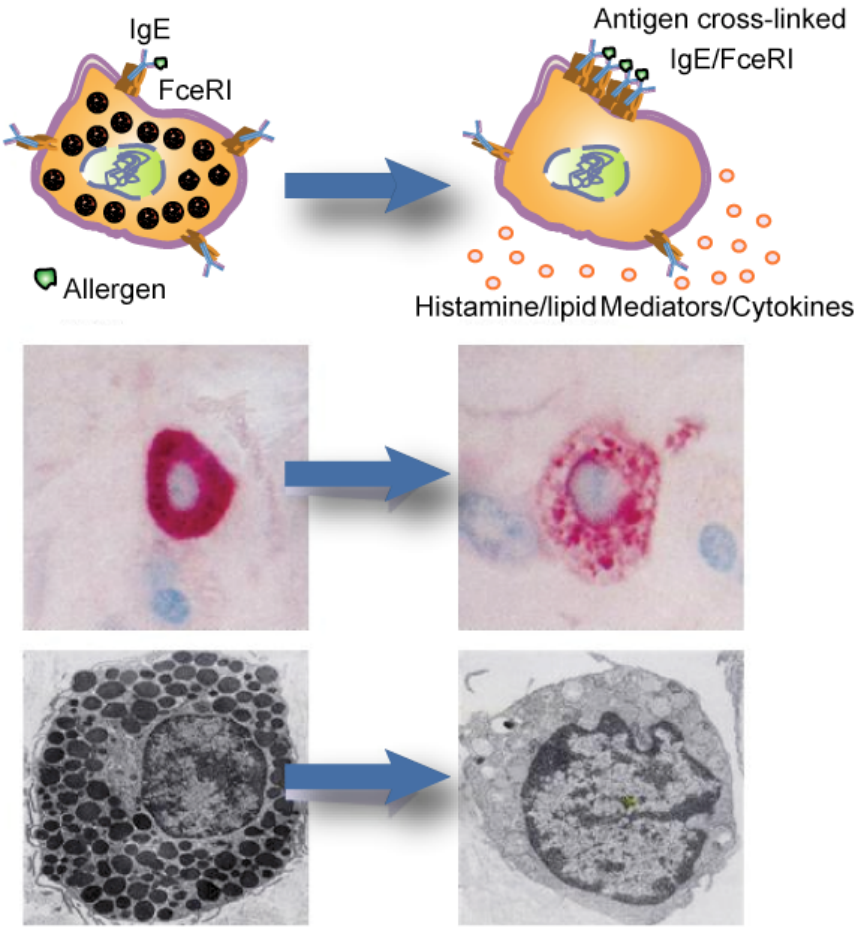

Figure 3: Mast cell activation (adapted from [18])

Mast cells also release newly synthesized mediators including lipid mediators like prostaglandin D_2, leukotrienes and platelet-activating factor, and cytokines like tumor necrosis factor (TNF), IL-1, IL-4, IL-5, IL-6 and various colony-stimulating factors. These inflammatory cytokines recruit eosinophils and neutrophils, which liberate proteases causing tissue damage, and T_H2 cells that may exacerbate the reaction by producing more cytokines. These components of immediate hypersensitivity are responsible for the initiation of the late phase reaction which follows 2 to 4h after the rapid wheal and flare reaction [19-21].

The FcεRI-IgE Interaction

The biologic effects of IgE in immediate hypersensitivity are mediated by a high-affinity Fc receptor specific for ε heavy chains, called FcεRI. It is constitutively expressed on mast cells and basophils and on a number of other immune cells such as eosinophils, monocytes, macrophages, Langerhans cells, platelets, megakaryocytes and neutrophils. By binding to the FcεRI through their heavy chain, IgE antibodies can function as an antigen receptor on the surface of these cells. The binding of IgE to FcεRI is much stronger than that of any other Fc receptor to its ligand, the dissociation constant (K_d) of FcεRI for IgE being about $1x10^{-10}$ M [21]. Each FcεRI molecule is composed of three separate transmembrane subunits (Figure 4), one α chain that mediates ligand binding, and three chains that contribute to signalling, including one β chain and a dimer of identical γ chains [22]. For cell surface expression, both the α and γ chains must be present [23]. The $\alpha\beta\gamma_2$ form of the receptor is expressed on IgE-dependent effector cells

like mast cells and basophils, but in antigen presenting cells such as macrophages, monocytes, Langerhans cells and dendritic cells, the β chain is lacking ($\alpha\gamma_2$). The binding site for IgE is formed by two Ig-like domains which are included in the amino terminal extracellular portion of the α chain.

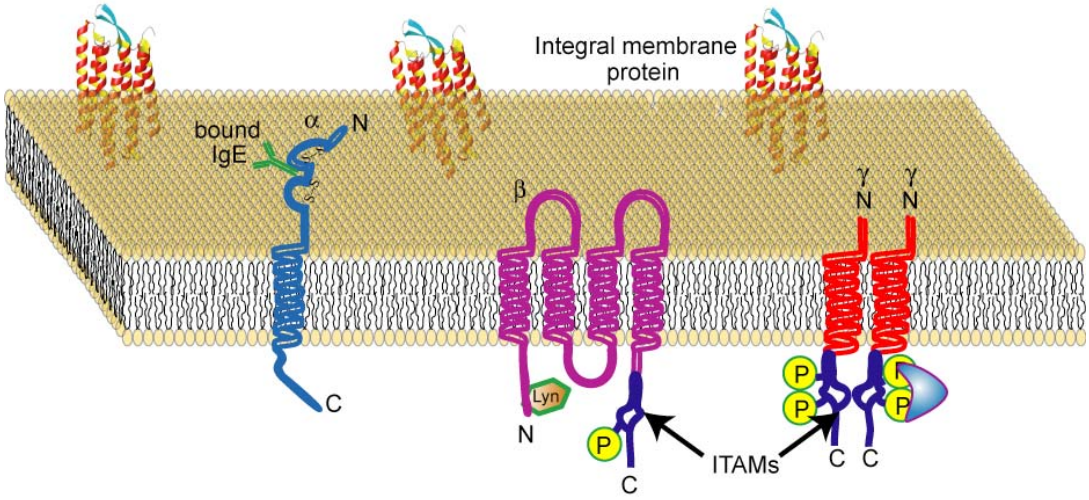

Figure 4: Polypeptide chain structure of the high-affinity IgE Fc receptor FcεRI

The β chain of FcεRI crosses the membrane four times and contains a single immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic carboxy terminus. The cytoplasmic parts of the γ chains each contain one ITAM, and together with the ITAMs of the α chain, they account for the signalling functions of FcεRI [18].

IgE antibodies consist of two identical, disulfide-linked heavy chains, each of which contains 1 variable and 4 constant domains (Cε1-4). The heavy chains associate via disulfide bonds with two identical light chains either of the κ or the λ subtype. The half-life of IgE in solution is approximately 2.5 days and increases when IgE makes small immune complexes with IgG.

When complexed to FcεRI, IgE adopts a bent structure and the binding occurs via the convex side of the molecule through its Cε3 domains [24]. Due to steric hindrance only one FcεRI can bind to an IgE molecule. The interaction between the receptor and IgE stabilises the complex so that the half-life of the IgE- FcεRI complex is around 21 days.

Effector Functions of Mast Cells and Basophils

Mast cells and basophils exert their effector functions through the release of soluble molecules upon activation (Table 1).

Mediator category	Mediator	Function/ Pathologic Effects
Mast cells		
Preformed, stored in cytoplasmic granules	Histamine	Increases vascular permeability; stimulates smooth muscle cell contraction
	Enzymes: neutral proteases	Degrade microbial structures; tissue damage/remodelling
Major lipid mediators produced on activation	Prostaglandin D_2	Vasodilation, bronchoconstriction, neutrophil chemotaxis
	Leukotriene C_4, D_4, E_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
	Platelet-activating factor	Chemotaxis and activation of leukocytes, bronchoconstriction, increased vascular permeability
Cytokines produced on activation	IL-3	Promotes mast cell proliferation
	TNF-α, MIP-1α	Promotes inflammation/late phase reaction
	IL-4, IL-13	Promote T_H2 differentiation
	IL-5	Promotes eosinophil production and activation
Basophils		
Preformed, stored in cytoplasmic granules	Histamine	Increases vascular permeability; stimulates smooth muscle cell contraction
	Enzymes: neutral proteases	Degrade microbial structures; tissue damage/remodelling
Major lipid mediators produced on activation	Leukotriene C_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
Cytokines produced on activation	IL-4, IL-13	Promote T_H2 differentiation
Eosinophils		
Preformed, stored in cytoplasmic granules	Major basic protein Eosinophil cationic protein	Toxic to helminths, bacteria, host cells
	Eosinophil peroxidase, Lysosomal hydrolases, Lysophospholipase	Degrades helminthic and protozoan cell walls; tissue damage/remodelling
Major lipid mediators produced on activation	Leukotriene C_4, D_4, E_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
	Lipoxins	Promote inflammation
Cytokines produced on activation	IL-3, IL-5, GM-CSF	Promote eosinophil production and activation
	IL-8, IL-10, RANTES, MIP-1α, eotaxin	Chemotaxis of leukocytes

Table 1: Mediators produced by mast cells, basophils and eosinophils (adapted from [25]).

These mediators have classically been divided into three categories: preformed mediators, newly sythesised mediators and cytokines [26]. As mentioned earlier, preformed mediators are packaged within secretory granules and are released into the extracellular environment within minutes after activation of the cells. Many of the biologic effects of mast cell activation are mediated by biogenic amines, sometimes also called vasoactive amines, nonlipid low molecular weight compounds that share the structural feature of an amine group [18]. The principal mediator of this class in human mast cells is histamine. Each cell contains approximately 2 to 5 pg of histamine. Histamine acts by binding to target cell receptors and has effects on smooth muscle (contraction), endothelial cells, nerve endings and mucous secretion. Binding of histamine to endothelium for instance, causes cell contraction leading to leakage of plasma into the surrounding tissue (Figure 5). It also stimulates endothelial cells to synthesise vascular smooth muscle relaxants, such as prostacyclin (PGI_2) and nitric oxide, which cause vasodilation. Collectively, these actions of histamine produce the wheal and flare response of immediate hypersensitivity. Histamine may also contribute to the increased peristalsis and bronchospasm associated with ingested allergens and asthma by inducing construction of intestinal and bronchial smooth muscles. The actions of histamine are short lived though, because histamine is rapidly removed from the extracellular milieu by amine-specific transport systems.

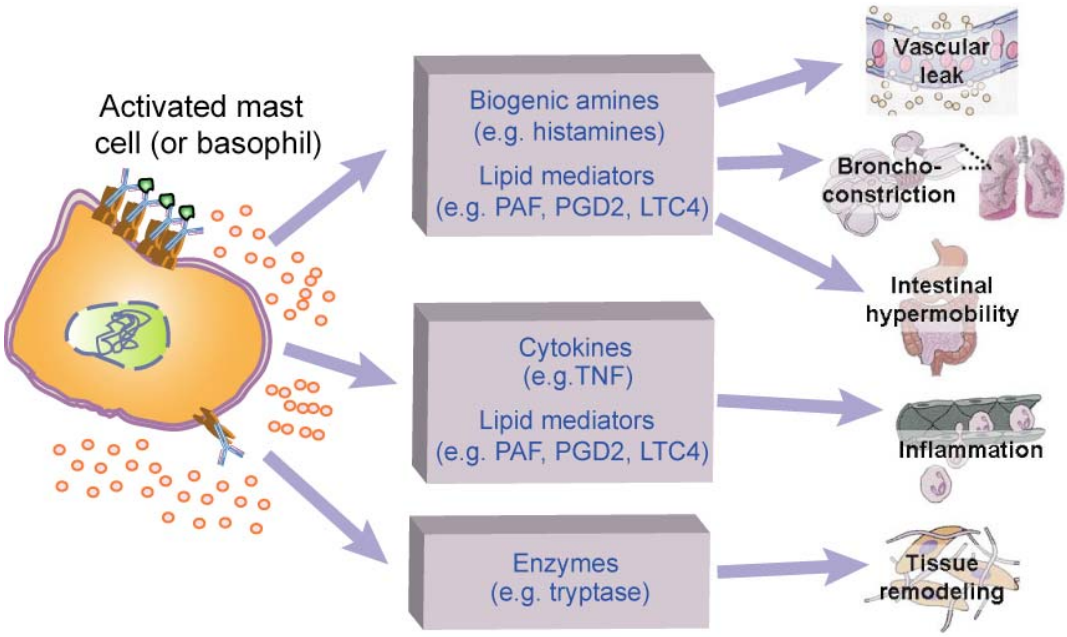

Figure 5: Biologic effects of mediators in immediate hypersensitivity (adapted from [18])

Neutral serine proteases, including tryptase, chymase and carboxypeptidase, constitute most of the protein content in mast cell granules. The presence of tryptase in human biologic fluids is thought to be a marker of mast cell activation. Tryptase can activate fibroblasts and promote accumulation of inflammatory cells *in vitro*, but its function *in vivo* is still unknown [26].

Activated mast cells and basophils also synthesise and release lipid-derived mediators that have a variety of effects on blood vessels, bronchial smooth muscle and leukocytes. The most important of these mediators in mast cells include prostaglandin D_2 (PGD_2), the major cyclooxygenase metabolite of arachidonic acid, and the lipoxygenase product leukotriene C_4 (LTC_4). Basophils do not make significant quantities of PGD_2. The actions of PGD_2 mainly involve smooth muscle cells, vasodilation and bronchoconstriction.

PGD_2 also promotes neutrophil chemotaxis and accumulation at inflammatory sites. Extracellular peptidolytic processing of LTC_4, which is equally important both in mast cells and basophils, yields the active metabolites LTD_4 and LTE_4 and all three of them cause prolonged bronchoconstriction and enhance vascular permeability. Another type of lipid mediator produced by mast cells is called platelet-activating factor (PAF). PAF is also a bronchoconstrictor.

A variety of cytokines that may contribute to allergic inflammation are produced by mast cells and basophils. These cytokines include TNF-α, IL-1, IL-4, IL-5, IL-6, IL-13, MIP-1α, MIP-1β and various colony-stimulating factors such as IL-3 or GM-CSF. They are released upon IgE-mediated mast cell or basophil activation and likely seem to be predominantly responsible for the late phase reaction.

Allergic Diseases in Humans

The release of mediators by mast cells is central to all allergic diseases in humans, and the pathologic manifestations of the diseases depend on the tissues in which the mediators act, as well as the chronicity of the resulting inflammatory process. The most common forms of atopic disease are allergic rhinitis (hay fever), bronchial asthma, atopic dermatitis (eczema), and food allergies. The clinical and pathologic outcome of allergic reactions depends on the organs or tissues which are involved and these are determined by the point of contact with the allergen. For example, inhaled antigens cause rhinitis or asthma, ingested antigens often cause vomiting and diarrhoea , and injected antigens cause systemic effects on the circulation. The severity of the response is influenced by the concentration of mast cells in the affected tissues. They are particularly abundant in the skin and the mucosa of the respiratory and gastrointestinal tracts, so that these tissues often suffer the most injury in allergic reactions.

Allergic rhinitis, commonly called hay fever, is perhaps the most common allergic disease and is the clinical manifestation of immediate hypersensitivity reactions to common allergens such as plant pollen or house dust mites localised to the upper respiratory tract by inhalation. The pathology of allergic rhinitis is characterised by mucosal edema, leukocyte infiltration with abundant eosinophils, mucus secretion, coughing, sneezing, and difficulty breathing. Allergic conjunctivits with itchy eyes is commonly associated with allergic rhinitis. Food allergies are immediate hypersensitivity reactions to ingested foods that lead to the release of mediators from intestinal mucosal and submucosal mast cells. Clinically, these events result in enhanced peristalsis, increased fluid secretion from intestinal lining cells, and associated vomiting and diarrhoea. Allergic reactions in the skin initially manifestate as wheal and flare reactions as a result of the acute effects of mast cell mediators. It is then followed by swelling and induration , a reflection of the inflammatory events of the late phase reaction. These skin manifestations can occur in response to direct contact with the allergen or after an allergen enters the circulation via the intestinal tract or by injection. Skin reactions are largely mediated by histamine. In systemic immediate hypersensitivity, mast cell and basophil mediators gain access to vascular beds throughout the body and cause vasodilation and exudation of plasma. Usually these effects result from the systemic presence of antigen introduced by an insect sting, injection, or absorption across an epithelial surface such as the skin or gut mucosa. Released mediators cause a decrease in vascular tone and leakage of plasma, which can lead to a fall in blood pressure or shock, called anaphylactic shock, which is often fatal. The inflammatory disease bronchial asthma is caused by repeated immediate hypersensitivity reactions in the lung leading to the clinicopathologic triad of intermittent and reversible airway obstruction, chronic bronchial inflammation with eosinophils, and bronchial smooth muscle cell hypertrophy and hyperreactivity to bronchoconstrictors. Although most cases of asthma are due to immediate hypersensitivity, in about 30% of the patients asthma may not be associated with atopy, although all are caused by mast cell activation. In some individuals, asthma may be triggered by nonimmune stimuli such as drugs, cold and exercise; how these

cause mast cell activation is unknown. The major mediators released by mast cells in acute bronchoconstriction are leukotrienes and platelet-activating factor (PAF), whereas histamine is of minor importance in airway constriction [18].

Therapy of Allergic Disorders

Current Clinical Concepts

Current therapeutic approaches aimed at atopic diseases include avoidance of allergens and environmental triggering factors, anti-allergic pharmacotherapy and immunotherapy for specific allergens, also known as hyposensitisation or desensitisation [9, 27]. The most frequently used drugs currently used are histamine antagonists and anti-cholinergic agents for the relief of symptoms and corticosteroids for the suppression of allergic inflammation. Recent advances in the understanding of the inflammatory and immunological processes of atopy have led to the development of new therapeutic strategies for allergic diseases [28, 29]. Promising approaches such as anti-IgE immunotherapy and, DNA vaccination, CpG oligonucleotides and mycobacterial vaccination are of considerable interest to prevent or cure atopic diseases in the future (reviewed in [9]).

Immunotherapy

Conventional allergy immunotherapy involves administering small but increasing doses of allergens with the goal of altering the immune systems responses, that is, desensitising the patient to those allergens and reducing symptoms triggered by subsequent exposures. Immunotherapy has been successfully used for the treatment of allergic rhinitis [30], allergic asthma and insect venom anaphylaxis [31], and can induce prolonged remission. It is thought to shift the immune response from the production of IgE to the production of so-called blocking IgG antibodies which may participate in the desensitisation process by competing with IgE for binding of the allergen [27]. A number of studies have shown that immunotherapy inhibits the release of mediators by mast cells and basophils [32], prevents the recruitment of eosinophils into the skin and nose after allergen provocation [30] and decreases the number of mast cells in subcutaneous tissues [33]. It has also been suggested that immunotherapy may shift the balance from T_H2 lymphocytes, which predominate in allergic individuals, to T_H1 lymphocytes [34, 35]. The resulting changes in cytokine production may explain the allergen-specific IgG response and the inhibition of the cytokine-mediated eosinophil recruitment.

Immunotherapy is successful in reducing the clinical symptoms in 60-90% of the cases, however, few patients are actually cured [36]. The occurrence of anaphylactic reactions and even death in a few patients have led to the development of different strategies to improve efficacy of immunotherapy and minimise side effects. Recombinant allergens with reduced allergenic and anaphylactic activity have been produced and offer various advantages in the diagnosis and the therapy of allergic diseases.

Instead of whole allergens, peptide fragments of T-cell allergens have been used as immunotherapeutic agents. These T-cell epitopes may disrupt allergen-induced CD4 T-cell responses and thereby B-cell activation and subsequently the production of IgE [37]. Thus the danger associated with IgE-mediated anaphylaxis following cross-linking of allergen-specific IgE on the surface of mast cells and basophils can be minimised. In mouse models, pre-treatment with peptide obtained from the major cat allergen Fel d 1, can prevent immediate hypersensitivity and airway hyperresponsiveness [38]. However, side effects associated with peptide immunotherapy have been reported.

As mentioned earlier, a lack of microbial stimulations in childhood is thought to play a role in the development of atopy in genetically pre-disposed individuals. Early virus or

bacteria infections normally trigger a T_H1 immune response which may switch a T_H2 biased allergic immune response to T_H1 reactions that can protect against both infections and allergic reactions [39]. This observation led to the idea of inducing protective T_H1 responses by vaccination and thereby preventing sensitisation and the development of atopic disease [40]. Vaccination studies in humans using Bacille Calmette-Guérin (BCG) as the immuno-stimulatory agent however, have shown no clear conlusion as to whether it can inhibit atopy, and the efficacy of the concept has to be further evaluated. The same is true for other approaches using DNA vaccination strategies. Intradermal injection of rats with a plasmid encoding the house dust mite allergen Der p 5 [41] , and immunisation of mice with a plasmid encoding ovalbumin [42] have shown promising results. Analysis of the mechanism of inducing T_H1 responses by plasmid vaccination in rodents has also revealed that non-coding sequences in the plasmid DNA such as unmethylated CpG (cytosine-phosphate-guanosine) oligodeoxynucleotides (CpG-ODN) are responsible for inducing the T_H1 response. In mice experiments, administration of CpG-ODNs has effected a redirection of the immune system towards a T_H1 response and a suppression of the development of allergen-induced asthma [42]. The potency of DNA vaccines in patients with atopic diseases has yet to be assessed.

Passive anti-IgE Therapy

The neutralisation of IgE by antibodies that are directed against the region of IgE which is involved in the binding of IgE to the receptors is one of the most promising therapeutic approaches. Murine monoclonal anti-IgE antibodies to human IgE have been generated that bound to IgE but were non-anaphylactogenic and inhibited the binding of IgE to FcεRI making them attractive candidates for therapeutic application [43-45]. The target structure of these non-anaphylactogenic antibodies has been located within the third domain of the IgE molecule which corresponds to the site recognised by the IgE receptors [46]. Characteristically, these antibodies prevent IgE from binding to its cell-bound receptors and thereby block effector functions. One such antibody, named BSW17, has been shown to recognise receptor-bound IgE without triggering human basophils [47]. Upon binding of the antibody, a conformational change of the IgE molecule was induced, resulting in the dissociation from the IgE receptor. BSW17 was also shown to inhibit IgE synthesis through divalent recognition of surface-bound IgE (Figure 6) [48].

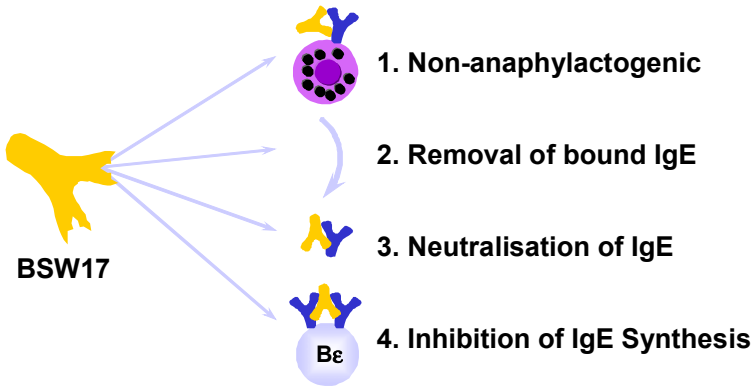

Figure 6: Properties of the murine anti-human IgE mAb BSW17

To circumvent the problem of antigenicity in humans, murine anti-human IgE antibodies have been humanised using a method established by Riechmann et al. [49]. Toxicity studies with the antibody rhuMAb-E25 have produced promising results so far. Administration of rhuMAb-E25 induces a dose-dependent decrease of serum free IgE

level to 1% of pre-treatment level associated with a marked down-regulation in basophil FcεRI expression and histamine release [50]. In cases of moderate and severe asthma [51, 52] and also allergic rhinitis [53], a clear improvement in symptoms and reduced use of medication was achieved. At this time, rhuMAb-E25 is approved for the additional treatment of severe asthma, but more results are required to evaluate its benefits on other allergic diseases such as mild asthma and allergic rhinitis.

Active anti-IgE Treatment

Alternatively, the neutralisation of IgE can be achieved by active immunisation using IgE epitopes. Thereby, a natural autoimmune response is induced which results in the formation of autoantibodies that have the same effect as passively administered non-anaphylactogenic anti-IgE antibodies. To isolate such IgE epitopes, the monoclonal anti-IgE antibody BSW17 was used as the selecting antigen [54, 55]. By screening random peptide phage display libraries, two peptides were isolated which mimic the epitope recognised by BSW17 on the IgE molecule. Hence, these peptides were called mimotopes [56]. Both mimotopes were recognised exclusively by BSW17 and fully inhibited the binding of BSW17 to IgE. This suggests that BSW17 has two binding sites on the IgE molecule. Coupled to keyhole limpet hemocyanin (KLH) as a carrier protein, the mimotopes were used for immunisation of rhesus monkeys [57]. In an in vivo passive cutaneous anaphylaxis (PCA) test, both mimotopes were shown to completely abolish PCA reactivity in the event of allergen challenge. Furthermore, one of the mimotopes, called Cε4, was able to significantly reduce PCA reactivity for minimally 10 months without repeated administration. Boosting the monkeys after 10 months evoked a high titer of antibodies against the Cε4 mimotope, indicating that active immunisation was able to induce memory B cells. The applicability of such carrier protein coupled mimotopes as anti-allergy vaccines in humans still needs to be investigated.

Active anti-IgE treatment via mucosal immunisation might offer an advantageous alternative to parenteral immunisation routes, as it is more convenient for the patient and rather inexpensive. As a carrier to deliver the antigen via the mucosal and in particular via the oral route, lactic acid bacteria seem to be suitable. They are non-pathogenic constituents of the normal human gut flora and are thus adapted to the acidic conditions found in the gastrointestinal tract. The IgE mimotope Cε4 described above was expressed on the surface of *Lactobacillus johnsonii* (Lj), fusioned to a cell wall anchored proteinase from another *Lactobacillus* species [58] (Dissertation Equivalent II). In addition, an anti-idiotypic single chain fragment variable, which was derived from an Fab mimicking the same region of IgE as the mimotope but covering a larger area, was expressed on the surface of these lactic acid bacteria in the same way. The recombinant Lj strains were used to immunise mice either subcutaneously or intranasally. A systemic IgG response against human IgE was induced both by the parenteral and oral route of immunisation, suggesting that recombinant Lactobacilli expressing IgE epitopes might serve as the basis of future immunotherapeutic approaches aimed at the induction of a beneficial anti-IgE response.

Allergens

A great number of allergenic proteins have already been identified and characterised, but it is still not known why some antigens cause allergic reactions and others do not. The allergenicity may reside in the biochemical properties of the antigens themselves. Properties such as low molecular weight, glycosilation and high solubility in body fluids are believed to be characteristic for allergens [9]. The allergens that most commonly produce reactions in susceptible individuals are known as major allergens. According to their place of entrance into the body, allergenic proteins can be divided into invasive, skin, airborne or food allergens, the latter two are described in the sections below.

Airborne Allergens

Inhaled bioparticles from different outdoor and indoor biological sources such as pollen grains, fungal spores or insects are the main cause of respiratory allergy. Particular allergens are associated with different types of allergic diseases in the respiratory tract. Grass and tree pollens cause hay fever whereas allergens derived from house dust mites, cats and cockroaches induce asthma [59]. The reason for this difference is probably the size of the particles, as pollen particles are too large to penetrate low into the respiratory tract.

The major inhalant allergens present in Western developed countries include Der p1 and Der p 2 from the house-dust mite *Dermatophagoides pteronyssinus*; Fel d 1 from the cat (*Felis domesticus*); several tree allergens including Bet v 1 from the birch tree (*Betula verrucosa*); and many grasses, such as Phl p 1 and Phl p 5 from timothy (*Phleum pratense*) [60]. The allergens Amb a 1, 2, 3, 4, 5, and 6 from the short ragweed (*Ambrosia artemisiifoglia*) are dominant respiratory allergens in North America and are of increasing importance also in Europe. The prevalence of outdoor allergens such as tree and grass pollens is subject to seasonal variation, whereas indoor allergens from house dust mites and domestic animals are present throughout the year. Indoor air exposures are more strongly linked to the increase in asthma prevalence in industrialised countries than outdoor air exposures [61].
The treatment of atopic diseases caused by airborne allergens consists of allergen avoidance, antiallergic medication and specific immunotherapy. The drugs currently used are antihistamines and anticholinergic agents for the relief of symptoms, and topical corticosteroids to suppress allergic inflammation. Specific immunotherapy is very effective in patients with seasonal allergic rhinitis and, to a lesser extent, in those with perennial rhinitis. Its effects can be long lasting, especially when treatment is continued for several years [62].

Allergy to Fungi

Fungi are eukaryotic, nonchlorophyllous, unicellular to multicellular organisms that can be found in all kinds of environments [63]. At least 60 species of fungi have spores which are thought to be allergenic, with the most predominant fungi implicated in allergy belonging to the genera *Aspergillus, Alternaria, Cladosporium* and *Penicillium* [61]. Fungal spores are present outdoors as well as indoors. Concentrations in the environment depend on factors such as climate and vegetation and vary according to the season. The prevalence of fungi present indoors depends on moisture, ventilation, presence or absence of carpets, pets, and houseplants. Fungi found indoors usually consist of a mixture of spores which have entered from outdoors and those that grow and multiply indoors. *Aspergillus* and *Penicillium* are considered classical indoor fungi, whereas *Alternaria* is a major outdoor mold [64].

The major allergic diseases induced by fungi are asthma, rhinitis, allergic bronchopulmonary mycoses and hypersensitivity pneumonitis. Up to 30% of all allergic asthma cases are caused by mold allergy, children being the most sensitive population. Fungi impact human health not only as allergens, but also as infectious or toxigenic agents. The airborne spores are inhaled down to the bronchia and alveoli where they are lysed and the human body thereby exposed to primary and secondary metabolites, including mycotoxins [65].

The importance of fungi as allergens has long been underestimated because of inadequate, non-standardised diagnostic antigen preparations. Recently, the use of molecular biology to clone and express relevant standardisable fungal allergens has gained significant progress. Many allergens have already been cloned and the proteins expressed.

Aspergillus Fumigatus

Over 90% of human disease attributable to *Aspergillus* species is caused by *A.fumigatus*. The spores of this saprophytic, ubiquitous fungus are remarkably thermotolerant; they grow at temperatures from 15°C to 53°C. The filamentous hyphae are 7 to 10 µm long and 3 to 4 µm wide; they septate and branch at 45° angles. The spores are 2 to 3.5 mm in diameter, typically coloured green, but sometimes they can also be white [66].

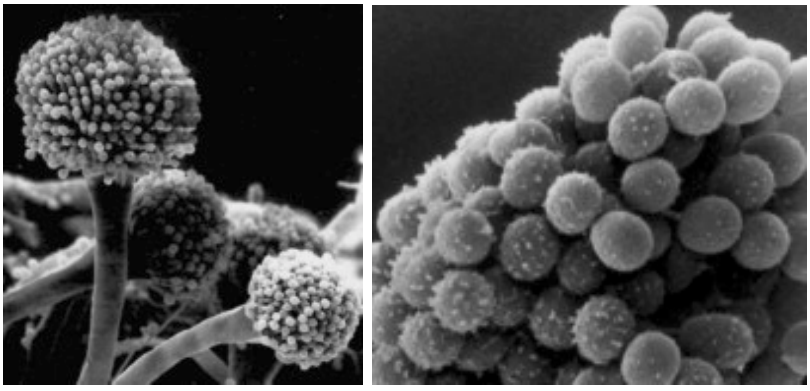

Figure 7: Conidia and spores of *Aspergillus fumigatus* (adopted from www.pasteur.fr/recherche/unites/aspergillus/spores_af.jpg)

Up to date, 29 different *A.fumigatus* allergens have been identified, several of which have been cloned and expressed as recombinant proteins. Their molecular structures can be attributed to a wide range of functional proteins, including toxins, enzymes, heat shock proteins and several unique proteins with no homology to any of the known proteins. Asp f 1 is a ribotoxin that inhibits protein translation and shows positive skin test reactivity in 50% of asthmatic patients [67]. It has been shown to be suitable for in vivo and in vitro diagnosis of sensitisation to *A.fumigatus* [68]. Another major allergen is Asp f 2, a 310 amino acid protein which can bind to fibrinogen [69]. Asp f 3 is a 18.5 kD protein with homology to a peroxisomal protein from *Candida boidinii* and Asp f 4 is an intracellular protein with unknown biochemical function. The metalloproteinase Asp f 5 exhibits skin test reactivity in more than 70% of allergic asthmatics and appears to be useful as an indicator for sensitisation to *A.fumigatus*. The manganese superoxide dismutase Asp f 6 will be described in more detail later. Asp f 8 is an acidic P2 ribosomal protein which is homologous to allergens from *Alternaria* and *Cladosporium*, and the cyclophilin Asp f 11 shows homology to a protein from *Malassezia furfur*. The heat shock protein (HSP) Asp f 12 has homology with proteins from phylogenetically distant organisms such as *Candida albicans, Saccharomyces, Trypanosoma*, housefly, mouse and humans because of the extremely conserved *HSP* gene [70].

Manganese Superoxide Dismutase

Superoxide dismutases are members of the metalloprotein superfamily and catalyse the dismutation of superoxide radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) [71]. They are of great importance in the primary defense against the toxicity of oxygen-related radicals and are thus essential for all organisms depending on oxygen.

The allergen Manganese superoxide dismutase of *A.fumigatus*, termed Asp f 6, was first isolated from a cDNA library displayed on the surface of filamentous phage using IgE antibodies of *A.fumigatus*-sensitised subjects as ligands [72]. The sequence encoding the mature enzyme was then cloned and expressed as a recombinant protein in *Escherichia coli* cells [73]. Asp f 6 has been demonstrated to be an allergen showing a high degree of homology with MnSODs of phylogenetically distant origin. The MnSODs of man, yeast, *Drosophila melanogaster, Saccharomyces cerevisiae* and *E.coli* have about 50% identity and 70% similarity in their amino acid sequences when compared with their counterpart in *A.fumigatus*. Sera from individuals sensitised to *A.fumigatus* MnSOD showed IgE reactivity to the recombinant proteins of all these organisms in Western blot analysis. IgE binding to all MnSODs could be inhibited by pre-incubation of the sera with *A.fumigatus* MnSOD indicating that these homologous enzymes from different organisms share IgE-binding epitopes. These results suggest that MnSODs from phylogenetically distant species represent a family of cross-reactive structures involved in allergic reactions. Interestingly, both *A.fumigatus* and human MnSOD were recognised by IgE antibodies from patients allergic to *A.fumigatus* MnSOD and elicited specific hypersensitivity skin reactions in these patients. Furthermore, both human and *A.fumigatus* MnSOD induced a proliferative response in PBMC of individuals sensitised to *A.fumigatus* who showed specific IgE responses and reacted in skin tests to *A.fumigatus* MnSOD [72]. In contrast, an *A.fumigatus*-sensitised person with no IgE antibodies against the fungal MnSOD only responded to the fungal extract. Taken together, these data strongly suggest the occurrence of in vitro and in vivo humoral and cell-mediated autoreactivity to human MnSOD in *A.fumigatus* allergic individuals caused by molecular mimicry between conserved T and B cell epitopes present on the fungal and the structurally related human enzyme [74].

The human MnSOD has been crystallised and its structure has been solved at 2.2 Å resolution [75]. It forms a homotetramer with the manganese ions coordinated by three histidines, one aspartic acid and one water molecule. Each of the four chains contains 7 α-helices and 5 strands of β–sheet (Figure 8).

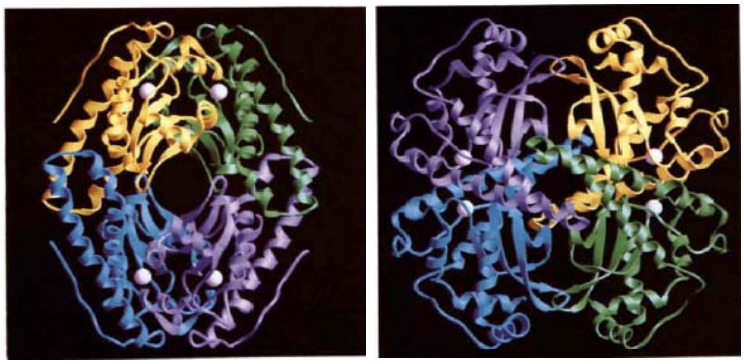

Figure 8: The human MnSOD tetramer as a ribbon diagram. The 2 views are related by a 90° rotation about a vertical axis. The 4 subunits are indicated by different colours. Manganese ions are shown as white spheres (adopted from 73).

Also the MnSOD of *A.fumigatus* has been crystallised and its structure has been solved at 2-Å resolution by molecular replacement using the structure of the human MnSOD as a

search model [76]. The obtained results confirmed the high homology of human and *A.fumigatus* MnSOD at the level of tertiary structure. Eukaryotic MnSODs are usually tetrameric, whereas the prokaryotic enzymes are mostly dimeric.

Since only the residues being at least partly solvent exposed can contribute to IgE binding, Flückiger et al. used a suitable program to calculate the solvent-accessible surface area of the two structures and the relative residue-accessible area. Of the totally 101 identical amino acids, only 10 are at least 50% solvent exposed in both structures. Sequence alignment of *A.fumigatus* and human MnSOD with the homologous enzymes of *S.cerevisiae*, *D.melanogaster* and *E.coli* revealed 48 amino acids that are identical in all the five sequences. Of those, only four (Pro^{8}, Pro^{19}, Lys^{32}, and Asn^{39}) are at least 30% and only two (Pro^{19} and Asn^{39}) are at least 50% solvent exposed in the fungal and the human crystal structure [74]. It seems thus very likely that Pro^{8}, Pro^{19}, Lys^{32}, and Asn^{39} re involved in the IgE-mediated cross-reactivity between the homologous proteins (Figure 9).

Figure 9: Alignment of the homologous MnSOD sequences of *A.fumigatus, H.sapiens, S.cerevisiae, D.melanogaster and E.coli*. Asterisks designate identical and colons designate similar amino acids in the five sequences. The two identical amino acids which are at least 30% solvent exposed are shown in red and the two identical amino acids which are at least 50% solvent exposed are shown in bold red.

Allergic Bronchopulmonary Aspergillosis (ABPA)

A.fumigatus is also involved in severe complications of the lung such as the potentially life-threatening hypersensitivity disease Allergic Bronchopulmonary Aspergillosis, which occurs primarily in patients with asthma or cystic fibrosis (CF). The *A.fumigatus* conidia inhaled from the environment colonise the airways and the lung, where the defence mechanisms of these patients are particularly impaired [77]. The presence of large numbers of eosinophils and lymphocytes in the airway walls, increased serum IgE levels and serum precipitins to *A.fumigatus* allergens leading to pulmonary infiltrates, bronchiectasis and fibrosis are characteristic for the disease [78]. As a result to the inflammatory immune response the function of the lung is impaired [79].

The prevalence of ABPA in CF patients has been reported to range from 0.9% to 23% and from 7% to 14% in asthmatic subjects sensitised to *A.fumigatus.* The wide ranges of

prevalence most likely result from variability in the criteria being used to define ABPA. The diagnosis of ABPA in CF and asthma patients is hampered by the fact that a number of characteristis are shared by ABPA and the two underlying diseases. These include sensitisation to *A.fumigatus,* positive precipitins and elevated specific serum IgE and IgG to *A.fumigatus* extracts, elevated total serum IgE and pulmonary infiltrates [80]. Therefore, serologic identification of ABPA has a great diagnostic potential. However, the serological discrimination between ABPA and *A.fumigatus* sensitisation or CF by the use of skin tests or serologic investigations has long been difficult. The reliability of these diagnostic procedures mainly depends on the quality of the antigen preparations used which is often not satisfactory due to large batch to batch variations of the allergenic components related to standardisation problems [81, 82].

Recently, the application of molecular DNA/RNA technologies and biotechnical procedures has led to cloning, characterisation and production of various highly pure recombinant allergens. The isolation of candidate cDNAs encoding allergens has been facilitated by the development of phage surface display technologies for cloning cDNAs [83]. Screening of an *A.fumigatus* cDNA library displayed on the surface of phage M13 resulted in characterisation and production of a panel of *A.fumigatus* allergens. Large-scale serological investigations have shown that IgE reactivity to recombinant Asp f 4 (rAsp f 4) and recombinant Asp f 6 (rAsp f 6) is specifically observed with sera of patients suffering from ABPA. Specific IgE against these allergens was not detectable in the tested sera from asthmatic individuals sensitised to *A.fumigatus*. In contrast, 78% and 56% of the sera from ABPA patients recognised rAsp f 4 and rAsp f 6, respectively. Approximately 90% of the sera recognised at least one of the two allergens. Hence, serological diagnosis with rAsp f 4 and rAsp f 6 has a specificity of 100% and a sensitivity of around 90% [84]. In addition, the allergens rAsp f 2 and rAsp f 8 have proven useful as specific markers for the serological diagnosis of ABPA. Patients with ABPA showed high levels of serum IgE to Asp f 2, whereas patients with asthma and *A.fumigatus* skin test reactivity but no evidence of ABPA showed only low IgE reactivity to Asp f 2 [85]. Sensitisation to the P_2 protein Asp f 8 was demonstrated in 15% of investigated patients suffering from ABPA, but no sensitisation to this allergen was observed in *A.fumigatus*-sensitised individuals without ABPA.

The MnSOD rAsp f 6 (see preceding chapter) and the P_2 protein Asp f 8 are both enzymes which strictly remain intracellular. The biochemical function of Asp f 4 is not known, yet preliminary experiments aiming at localising the protein with monoclonal antibodies raised against Asp f 4 suggest that the protein is not secreted by *A.fumigatus*. Thus, these proteins are supposably not present in free form as aeroallergens and therefore no specific IgE is raised against them in *A.fumigatus* allergic asthmatics. In contrast, patients suffering from ABPA also become exposed to nonsecreted proteins because of host defense mechanisms leading to damaged fungal cells [86, 87].

Medical treatment for patients suffering from ABPA mainly consists of oral corticosteroids and antifungal agents as an adjunctive therapy [66].

Atopic Dermatitis (AD)

Atopic dermatitis is a common chronic relapsing inflammatory skin disorder with a multifactorial aetiology involving genetic, immunological and environmental factors. 10-20% of children and 1-3% of adults are affected worldwide, the prevalence being on the rise in highly industrialised countries [88]. The pathophysiology of AD is still not well understood, but in recent years, the skin colonisation with various microbial organisms has been recognised as an important factor [89]. Among others, particular attention has been paid to yeasts belonging to the *Malassezia* genus. These lipophilic fungi are a permanent component of the microflora of normal human skin. The highest concentrations of the organism are found on the skin of the face, neck, scalp, upper chest and back, where the presence of high amounts of skin lipids probably offers favourable growth conditions. Different *Malassezia* species have been shown to be capable of

inducing IgE-mediated and T-cell mediated immune responses postulated to contribute to chronic inflammation in the skin. Johansson et al. [90] have found that 67% of 132 examined AD patients and none of 33 healthy controls were sensitised to *Malassezia sympodialis*, demonstrated by either a positive skin prick test, positive atopy patch test or specific IgE antibodies to *M.sympodialis*. These results suggest that anti-*M.sympodialis* IgE production is AD-specific, as it only appears in AD patients and is missing in the healthy control group.

11 different *M.sympodialis* allergens have been identified so far, and one of them, Mala s 11, is a Manganese superoxide dismutase. As mentioned earlier, MnSODs are highly conserved among phylogenetically distant organisms. Also the MnSOD from *M.sympodialis* and the human MnSOD show a high degree of sequence similarity, which leads to IgE-mediated cross-reactivity between the two homologues. It has been postulated recently, that IgE-mediated autoreactivity against human proteins could be a possible pathogenic factor leading to aggravation of symptoms in AD patients [91-93]. Schmid-Grendelmeier et al. have found specific IgE antibodies against human MnSOD (hMnSOD) in 29 out of 67 patients with AD [94]. All patients with AD showing serum IgE against hMnSOD were co-sensitised to *M.sympodialis*, and IgE raised against hMnSOD and yeast extract correlated nicely. Thus, primary sensitisation most likely occurs through sensitisation to the environmental MnSOD of *M.sympodialis*. Interestingly, the amount of specific IgE raised against hMnSOD strongly correlated with disease severity, an observation which supports the idea of autoreactivity as a mechanism responsible for the exacerbation of AD.

Food Allergens

Foreign antigens, including food antigens and commensal bacteria encountered in the gastrointestinal tract, are usually well tolerated. However, some food proteins induce adverse immune-mediated responses in susceptible individuals, commonly called food allergy. Considering the large number of food proteins comprising the human diet, the number of foods inducing IgE-dependent allergic reactions is small [95]. Food allergies are common in infants and young children: as many as 8% of children under the age of 3 years suffer from allergy to one or more foods [96]. Adult food allergy is estimated at 3% worldwide [97]. Which foods are likely to cause hypersensitivity reactions is dependent on the local eating habits and on age. Cow's milk, eggs and peanuts account for 80% of allergic reactions in childhood, but allergy to soybeans and wheat is also common in infants [98]. Seafood, peanuts and tree nuts are the more common causes of food allergy in adult life. Childhood food allergy usually does not persist after 30 years of age, peanut allergies building the exception. More than 80% of peanut allergies persist into adulthood [99]. When food allergy is once established in adults, it is rarely cured. Children often suffer from the multiple food allergy syndrome, including allergy to eggs, milk, meat and fish, whereas hypersensitivity reactions to animal allergens in adults are mostly mono-allergies.

In contrast, allergies to fruit and vegetables are often multiple, reflecting the fact that many of the proteins involved are highly conserved across species. Fruit and vegetable allergy is often a result from cross-sensitisation to pollens, e.g. from *Betulaceae* and *Artemisia* species. Individuals allergic to birch tree pollen for example, often display allergic symptoms when eating fruit such as apple, peach or pear, or vegetables like carrot or celery. This is mainly due to cross-reactivity of the major birch pollen allergen, Bet v 1, and its homologues in fruit and vegetables [100].

Another factor favouring the acquisition of food allergy by adults is the occupational sensitisation by inhalation of food proteins in food industries. After absorption of the food allergens by the gastrointestinal route through ingestion, the IgE-mediated symptoms classically develop within minutes to at maximum a few hours. Clinical manifestations include the so-called oral allergy syndrome, characterised by localised mouth tingling, itching and swelling, and also more severe symptoms such as nausea, vomiting, cramping abdominal pain and diarrhoea. Extragastrointestinal reactions include flushing, urticaria, angioedema and even life-threatening anaphylaxis, which is more common in adults than in children. Anaphylaxis may include cardiovascular collapse, systemic reactions affecting several organs, laryngeal angioedema and serious acute asthma. In industrialised nations, food allergy is the major cause of anaphylaxis, involving 17% to 37% of patients admitted to hospital emergency units [101, 102]. The fatality risk is estimated at 1% in severe anaphylaxis [103, 104]. In the United States, 90% of fatal cases are caused by peanuts and hard-shelled fruit [105].

The only reasonable strategy available to date to treat or prevent food allergy, is the strict avoidance of the offending allergen. This is of particular importance in cases of food allergies such as peanut allergy in which trace amounts of allergen can cause significant reactions. There is no clear evidence so far, that oral desensitisation , injection immunotherapy or prophylactic medication are beneficial in the prevention or modulation of food allergy [95].

Allergy to Fish and Shellfish

Fish and Shellfish are considerably important in human nutrition and in world economy. Unfortunately, they also can be important causes of severe acute hypersensitivity reactions, including fatal anaphylaxis. In the United States, seafood allergy is reported by 3.3% of the general population [106]. Edible seafood includes sea organisms from 3 major divisions, each of them belonging to a different phylum : the *Chordata*, the *Mollusca* and the *Arthropoda*. The major component of the *Chordata* are the ray-fin fishes. The *Mollusca* include snails and abalone, mussels, oysters, scallops, clams, squid and octopus. The *Arthropoda* phylum contains the major class *crustacea*, which includes shrimp, crabs, lobster and crawfish. The seafood species most frequently consumed in the United States are shown in Table 2 (adapted from [107]).

		Pounds per capita
1.	Shrimp	3.4
2.	Tuna	2.9
3.	Salmon	2.02
4.	Alaska Pollock	1.2
5.	Catfish	1.15
6.	Cod	0.56
7.	Clams	0.46
8.	Crab	0.44
9.	Flatfish	0.39
10.	Tilapia	0.35

Table 2: Top 10 most frequently eaten seafoods in the United States, per the National Fisheries Institute in 2001 (adapted from [107]).

Improved transportation and preservation have led to an increased availability and consumption in areas that are far removed from where the fish is grown and harvested. In addition to allergy in the consumer, fish and shellfish have also been recognised as important allergens in occupational environments. Seafood workers suffer from allergic reactions most commonly associated with direct contact or inhalational exposure rather than with ingestion [108, 109].

A number of major allergens and important potential cross-reacting allergens have been identified within the fish family and between shellfish. Homologous inhalant allergens have also been identified in insects such as the cockroach, and arachnids such as house dust mites. Codfish and shrimp have been the models used to study allergy to fish and shellfish, respectively. The major allergens responsible for cross-reactivity among distinct species of fish are parvalbumins [110]. Their function is to control the flow of calcium in and out of cells. They can be found only in the muscles of amphibians and fish and have a molecular weight of approximately 12 kD. It has been demonstrated that more than 95% of fish-allergic patients have specific IgE to this protein. It is estimated that 50% of individuals allergic to some type of fish are at risk for reacting to a second species, due to the cross-reactivity among parvalbumins [111]. The major allergen of shellfish has been identified as the muscle protein tropomyosin (s. next chapter).

Tropomyosin

Tropomyosin is an essential protein in muscle contraction, both in invertebrates and in vertebrates. This molecule seems to be highly conserved based on the significant primary structure identity of tropomyosins from unrelated species. In invertebrates, they have a molecular weight of between 34 and 41 kD and show great homology in their amino acid sequence. They are the panallergens responsible for cross-reactivity between crustaceans (shrimp, crab, crawfish, lobster), mollusks (squid, oyster, snail, mussels, clams), insects (cockroaches) and arachnids (house dust mites) [112].

Shrimp is the best studied among these homologous allergens. The tropomyosin of *Penaeus aztecus* has been isolated as a 36 kD protein designated Pen a 1. Approximately 85% of the shrimp-specific IgE from shrimp-allergic individuals binds to Pen a 1, whereas all other shrimp allergens bind IgE from less than 25% of the shrimp-allergic subjects [113, 114]. Not only the tropomyosins of several different crustaceans and mollusks, but also the ones of the cockroaches *Blattella germanica* [115] and *Periplaneta Americana* [116], and of the house dust mites *Blomia tropicalis* and *Dermatophagoides pteronyssinus* [117] have been cloned and expressed as recombinant proteins. Although cockroach and house dust mite tropomyosins represent minor allergens, cross-reactivity to the homologous shellfish allergens has been clearly demonstrated.

Tropomyosin is present in all eukaryotic cells, which contain different isoforms of tropomyosin in muscle (skeletal, cardiac and smooth), brain, fibroblast, platelets and many other nonmuscle cells. The tropomyosins from different tissues are highly homologous, nevertheless, structural differences do exist among these isoforms [118]. In muscle, tropomyosin is associated with the thin filament and with microfilaments in many nonmuscle cells. The rod-shaped dimeric molecule consists of two α-helical chains arranged in a parallel coiled-coil configuration. The single molecules are bonded head to tail (Figure 10).

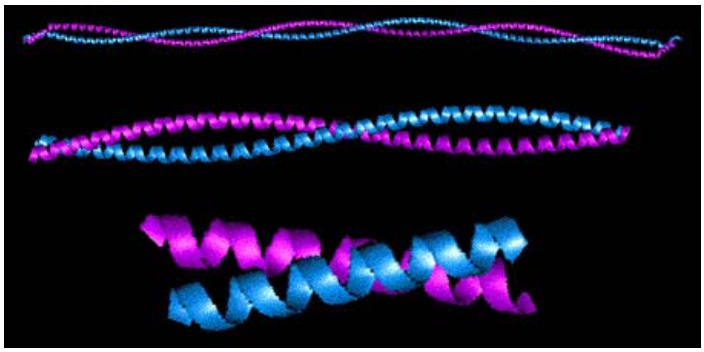

Figure 10: The coiled-coil structure of a tropomyosin dimer. (adopted from http://membranes.nbi.dk/article_coiled-coil/coiled-coil_proteins.html).

The amino acid sequence shows a repeating pattern of nonpolar and polar amino acids , which is characteristic for a coiled-coil structure. The two α-helices interact along their length with the nonpolar residues forming a hydrophobic core. Polar and ionic sidechains are directed toward the exterior of the ropelike molecule, where they can interact with solvent and/or other proteins.

Dimeric tropomyosin binds to the sides of actin filaments and, together with troponins, regulates the interaction of the filaments with myosin in response to Ca^{2+} (Figure 11) . This has an impact on the contractile activities of the cells , as well as on the regulation of cell morphology and motility [119].

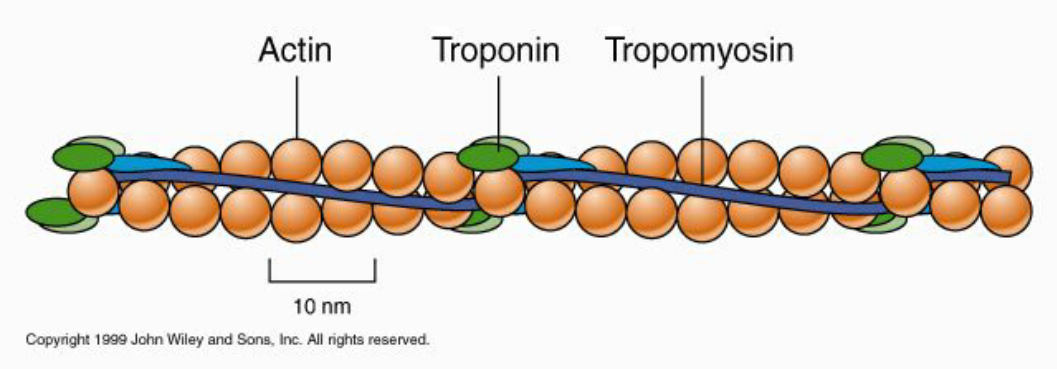

Figure 11: Actin, Troponin and Tropomyosin together constitute a thin filament.

In skeletal muscle, Tropomyosin accounts to about 3% of the total muscle protein.

To elucidate the basis of cross-reactivity between tropomyosins from different organisms, several groups have attempted to identify IgE-binding regions of the homologous allergens. Initially, Shanti et al. identified two IgE-binding regions of Pen i 1 by analysing IgE antibody reactivities of patients allergic to shrimp and healthy controls to nine tryptic fragments of Pen i 1 [113]. When peptides 6 and 9, consisting of amino acid residues 163-161 and 50-66 respectively, were used as inhibitors, inhibition of binding of Pen i 1-specific IgE antibodies was maximal (50%). Reese et al. identified four peptides of Pen a 1, between 13 and 21 amino acids long, that were reactive to IgE antibodies of shrimp-allergic subjects [120]. All four peptides were located in the second half of the molecule including the C terminus. In the first systematic analysis of IgE-binding epitopes of Pen a 1, Ayuso et al. synthesised 46 overlapping peptides, 15 amino acids long, spanning the whole length of the Pen a 1 tropomyosin [121]. The peptides were tested for IgE binding with sera of shrimp-allergic patients. A wide range of individual reactivity patterns was observed, but based on frequency and intensity of IgE binding, 5 major allergenic regions could be identified: region 1 (43-57), region 2 (85-105), region 3 (133-153), region 4 (187-201) and region 5 (247-284) (Figure 12). The IgE binding sequences occur at regular intervals in the molecule, suggesting some relation with the coiled-coil structure of tropomyosin. Some of these allergenic regions have also been recognised as allergenic in tropomyosins from different organisms such as those from the mollusks *Turbo cornutus* [122] and *Crassostrea gigas* [123] or in the shrimp *Penaeus indicus* [113]. Further analysis of two allergenic regions of Pen a 1, regions 3 and 5, has led to the identification of tropomyosin epitopes (shortest peptide with maximal IgE-binding capacity) [118]. The core epitope found in region 3 was at the position 137-141 (DEERM). This sequence was found to be identical to the homologous sequences of American cockroach (*Periplaneta Americana*) and house dust mite (*Dermatophagoides pteronyssinus*) tropomyosins, Per a 7 and Der p 10, respectively. In region 5 two relevant epitopes have been identified. The first one, found to be at position 266-273 (KYKSITDE), differs from homologous regions of other invertebrate tropomyosins. The second one, which was found at position 251-259 (KEVDRLEDE), is identical to the homologous sequences of Per a 7 and Tur c 1 (Figure 12). The identity of some of these epitopes with homologous, allergenic tropomyosins of other invertebrates such as American cockroach and house dust mite may explain the reported cross-reactivites between shrimp and the latter organisms.

Figure 12: Alignment of the homologous Tropomyosin sequences of *P.aztecus, P.americana, D.pteronyssinus and T.cornutus*. Asterisks designate identical and colons designate similar amino acids in the four sequences. The five major allergenic regions of Pen a 1 identified by Ayuso et al. are shown in red and the three epitopes (shortest peptide with maximal IgE-binding capacity) along with the identical sequence segment in the homologous sequences are shown in bold red.

Tropomyosin has also been shown to act as an autoantigen in vertebrates. It is associated with various heart and autoimmune diseases such as acute rheumatic fever, myasthenia gravis and ulcerative colitis [124-128]. Amino acid sequence alignments between invertebrate and vertebrate tropomyosin allowed the identification of protein regions sharing 50 to 60% identity. In one of these regions, both an allergenic IgE binding [113, 129] and an autoantigenic epitope have been identified [130].

Autoimmunity and Allergy

Several environmental allergens have been shown to share structural and functional properties with human proteins, which can lead to humoral and cell-mediated autoreactivity in susceptible individuals. IgE antibodies from patients allergic to profilin, e.g. from birch pollen, were able to bind to human profilin and in addition, human profilin induced histamine release from blood basophils of profilin-allergic individuals [131]. Also the human proteins manganese superoxide dismutase (MnSOD) [72], ribosomal P_2 protein [132] and cyclophilin [133] were shown to bind IgE antibodies from sera of patients sensitised to the corresponding allergens of *A.fumigatus*. Human MnSOD and ribosomal P_2 protein induced proliferation of PBMC in *A.fumigatus*-allergic individuals and furthermore, all the three allergens elicited immediate type skin reactions in patients sensitised to the homologous fungus proteins (also see previous chapters). Moreover, it has been shown that the levels of IgE autoantibodies against MnSOD in atopic dermatitis patients correlate with severity of disease [94]. A considerable reduction of IgE autoreactivity was observed in an AD patient during systemic treatment with cyclosporin A. The improvement of skin lesions was associated with a lower level of IgE autoantibodies. After exogenous allergen contact, skin symptoms were aggravated and IgE autoreactivity increased [134]. In summary, these findings suggest that certain allergens can also be regarded as autoantigens and that IgE autoreactivity can serve as a diagnostic parameter for chronic atopic inflammation [135].

Allergen Characterisation by Bioinformatics

Until recently, public allergen databases have been characterised by a low coverage of allergens and a high level of entry redundancy [136]. The number of characterised allergens and related information is increasing, leading to a need for advanced information storage, retrieval and analysis [137]. Therefore, substantial effort has been made to ameliorate the quality of allergen databases. Up-to-date publicly available databases include The Allergen Database (http://www.csl. gov.uk/allergen) and the Allergome Database (http://www.allergome.org/) [138], the latter being the most carefully updated allergen database today [139]. In order to manage and analyse the rapidly increasing amounts of data in the field of allergy, bioinformatic and computational tools are continually being developed. One of the key issues to be solved is the molecular basis of allergenicity of proteins, which can be studied through the analysis of its sequence, structure, B- or T-cell epitopes.

In recent years, much attention has been paid to safety issues such as the potential risk associated with the introduction of genetically modified proteins into the food chain. In addition to laboratory and clinical testings, current procedures for allergenicity assessment involve the comparison of the novel protein's amino acid sequence with those of known allergens [140-142]. According to the FAO/WHO allergenicity evaluation scheme for foods derived from biotechnology, the protein in question is potentially allergenic if it either shows a match of six consecutive amino acids, or 35% homology over a window of 80 amino acids when compared with known allergens. The first criterion has been criticised by several reports as being fairly non-specific, since it produces a large number of false positives [143-145]. On the other hand, the second criterion is too stringent to find most true allergens [146]. Therefore, a number of alternative strategies for allergenicity evaluation of query proteins have been proposed. One of them involves an initial search for matches of identical amino acids, followed by examination using either a biochemical or an evolutionary substitution matrix [147, 148]. Other reports have described the comparisons of primary sequences or structural properties with IgE epitope data from literature, a method which can also be used in combination with searches for short identical stretches [145, 149, 150].

An allergenicity prediction method gaining importance is the analysis of allergens by the use of protein motifs, which is described in more detail in the next chapter.

The Motif Approach

The identification of regions typical for allergenic proteins by using an iterative motif-finding approach has first been described by Stadler et al. [151]. First, a reference allergen database was generated by extracting accession numbers in published allergen lists and downloading the corresponding sequences from the public general databases (Swiss-Prot, PIR and GenBank), resulting in a total of 779 nonredundant sequences. Then the motif identification tool MEME [152] was used to identify relevant motifs in the allergen sequences in an iterative procedure, meaning that sequences matching an identified motif were removed from the database and the remaining sequences were submitted to the next iteration of motif discovery. A total of 52 statistically relevant allergen motifs was found, with 135 of 779 sequences not matching any motif. The motifs were then used to evaluate the potential allergenicity of a query protein sequence, resulting in a recall (sensitivity), which was comparable to the one that is obtained applying the FAO/WHO guidelines for allergenicity prediction of genetically modified foods. The precision however, defined as the percentage of relevant hits in relation to the total number of hits, reached 95.5% and was thus superior to the FAO/WHO method with 36.6%.

Recently, the described motif analysis was repeated using the more complete and continuously updated allergome database (www.allergome.org) [153] as the basis. The latest analysis included 1102 allergenic sequences and resulted in the assignment of 912 sequences to 69 motifs. Still there were 190 sequences that did not match any motif [Truffer et al., manuscript in preparation]. Therefore, it seems to be likely that future motif searches will not yield many more new motifs and that the total number of actual allergenic epitopes could be below 100.

The Biological Relevance of Allergen Motifs

The bioinformatical analysis described above has shown that similar allergens can be characterised by common motifs found in their primary structure. As much as 83% of all known allergens were regrouped into a limited set of only 69 statisitically relevant motifs. Cross-reactivities of clinical relevance, e.g. between the major birch pollen allergen Bet v 1 and the homologous proteins of celery, carrot, apple, hazelnut, apricot and other fruits, were confirmed by the motif analysis.

The aim of our work was to investigate the clinical significance of allergen motifs in more detail, namely to assess the value of allergen motifs as determinants of allergenicity and as a means of predicting cross-reactivity between allergens from different organisms. We focused our studies on the motif 22, which contains Manganese superoxide dismutases from different phylogenetic origin, and on the motif 6, which includes tropomyosins from various organisms. From motif 22, we selected the MnSOD of the mold *A.fumigatus,* Asp f 6, as a representative. As we intended to compare the immunological reactivities of this allergen motif and its full length allergen, we synthesised the fifty amino acid spanning motif region as a peptide and expressed the complete allergen sequence as a recombinant protein. Subsequently, we tested the sera of patients suffering from ABPA, who are sensitised to Asp f 6, for IgE reactivity to the motif peptide and to the full length allergen. The same was done with sera of AD patients, who have IgE antibodies against the MnSOD of the fungus *M.sympodialis*, which are predicted to be cross-reactive with *A.fumigatus* MnSOD.

With the tropomyosin motif we strived for the same goal, but applied a slightly different strategy. Here we aligned the motif regions of the different tropomyosins, determined a consensus sequence which is representative for all the included sequences, and expressed it as a recombinant protein. To determine whether the consensus motif represents a common IgE epitope of the included tropomyosins, we tested its binding to sera of individuals sensitised to either crab or shrimp. In both these organisms tropomyosin is the major allergen. For comparison, the same sera were tested for binding to the full length shrimp tropomyosin Pen a 1 (Dissertation Equivalent I).

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D. DISSERTATION EQUIVALENTS

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Are Allergen Motifs Predicting Epitopes?

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Abbreviations:

MnSOD: Manganese superoxide dismutase

Trop : Tropomyosin

Mot : Motif

ABPA : Allergic bronchopulmonary aspergillosis

AD: Atopic dermatitis

Abstract

Background: We have recently shown that the majority of known allergens can be regrouped according to shared allergen motifs found in their primary structures.
Objective: The aim of this study was to investigate the predicted *in silico* motifs with regard to IgE binding and potential cross-reactions with other allergens.
Methods: Two example motifs were selected to conduct *in vitro* studies. First, a 50 amino acid motif stretch of *A.fumigatus* MnSOD was synthesised as a peptide (MnSOD Mot) and tested for IgE reactivity with sera of patients sensitised to the MnSOD of *A.fumigatus* or *M.sympodialis*. For comparison the IgE binding activity of the same sera was tested against the full length MnSOD. Second, a consensus motif region of invertebrate tropomyosins was expressed as a recombinant protein (Trop Mot) and tested for binding to IgE antibodies of individuals allergic to shellfish. Analysis of Trop Mot IgE reactivity was performed in ELISA and was compared to the one of recombinant shrimp *Penaeus aztecus* tropomyosin (rPen a 1) in ImmunoCAP.
Results: Specific IgE antibodies against MnSOD Mot were detected in sera of patients highly sensitised to *A.fumigatus* MnSOD as well as in a patient sensitised to the cross-reactive *M.sympodialis* MnSOD. We also found specific IgE reactivity to Trop Mot that was correlating in intensity with the one observed for shrimp full length tropomyosin in the same sera.
Conclusion: Our results suggest that allergen motif analysis can serve to identify IgE epitopes in allergens that may be used as possible candidates for diagnostic and therapeutic approaches.

1. Introduction

Atopic diseases are an issue especially in Western countries where their prevalence has steadily been increasing during the past decades. It is assumed that today most allergens of clinical relevance are known and the characterisation of these allergens is constantly making progress.[1] Nonetheless, the molecular basis of allergenicity of proteins remains unknown.

A method to reliably predict the allergenicity of a given protein and its possible cross-reactions with other allergens is of general need for various reasons. In the search of making allergen-specific immunotherapy more safe and effective, a lot of interest has recently been paid to the production of hypo-allergenic protein variants by site-directed mutagenesis of amino acid residues known to be involved in IgE binding.[2] Since this technique requires prior investigation of IgE epitopes for every single allergen intended to be used for desensitisation, its practicability is rather limited. A more general approach to identify allergenic regions of proteins would be considerably useful for the development of future diagnostic procedures. Current diagnostics either use whole extracts from one organism or recombinant allergens classified according to their organism of origin to assess the sensitisation pattern of atopic patients. A classification of allergens with regard to common allergen motifs could potentially simplify the diagnosis of atopy and in addition it would allow the prediction of possible cross-reactions. Furthermore, the advancing technology of genetically modifying agricultural crops and the safety issues linked to the potential risk of novel allergenic proteins ask for a reliable, standardised approach to predict allergenicity.

We have recently presented a bioinformatical approach to assess the allergenicity of proteins and to predict cross-reactions with other allergens by the identification of shared motifs in the amino acid sequences of allergenic proteins.[3] For our initial allergen motif analysis we used an allergen database which was generated at our institute by downloading all sequences of publicly available allergen lists and subsequent removal of redundancies. In the meantime, the continuously updated Allergome database (www.allergome.org) has evolved into one of the most complete allergen databases currently available.[4] The motif search using the 1102 allergen sequences contained in the Allergome database at the time, resulted in the assignment of 912 sequences to 69 statistically relevant allergen motifs.[5] (Truffer R, et al. Manuscript in preparation) In the current study, we aimed at evaluating the significance and the usefulness of such allergen motifs as a means of defining allergenicity and predicting cross-reactivity between highly homologous allergens. We have focused our work on two different motifs of clinical

relevance, one of them contains manganese superoxide dismutases (MnSODs) of different organisms and the other one tropomyosins of variable phylogenetic origin. The enzyme manganese superoxide dismutase catalyses the conversion of toxic superoxide radicals to oxygen and hydrogen peroxide.[6] It has been identified as an allergen of the pathogenic fungus *Aspergillus fumigatus (A.fumigatus)* and is of clinical relevance especially in individuals who are allergic to *Aspergillus fumigatus* and who additionally suffer from allergic bronchopulmonary aspergillosis (ABPA), an intense inflammatory response to *A.fumigatus* in the lung. Furthermore it has been shown that MnSODs of phylogenetically distant organisms are recognised by IgE antibodies from individuals sensitised to *A.fumigatus* MnSOD.

Tropomyosin is an ubiquitous protein, existing in both vertebrates and invertebrates, with essential functions in muscle contraction.[7] Previous studies have identified tropomyosin as the major crustacean allergen including shrimp, crawfish, lobster and crab.[8-11] Shellfish allergy is of great clinical concern, since it can be the cause of severe immediate hypersensitivity reactions and eventually even fatal anaphylaxis. In addition tropomyosin has been proven to be allergenic in other invertebrates such as mollusks (squid, oyster, snail, mussels, clam), insects (cockroaches) and arachnids (house dust mites).[12-15] Further studies have shown that tropomyosin is responsible for cross-reactivity found among these organisms and can therefore be regarded as an invertebrate panallergen.[16-18]

In the present study, we report the first analysis of the *in vitro* immunogenicity of MnSOD and tropoyosin motifs by comparing their IgE reactivities with the ones of their corresponding full length allergens.

2. Materials and Methods

2.1 Antigens, allergens and allergen extracts

MnSOD Mot was purchased from Thermo Electron GmbH (Ulm, Germany) and was 36% pure and characterised by mass spectrometry and analytical HPLC. The first two amino acids of the motif (QQ) were omitted because of stability problems linked to the presence of QQ.

Tropomyosin isolated from chicken gizzard was bought from Sigma (Fluka Chemie GmbH, Buchs, Switzerland).

ImmunoCAPs carrying *A.fumigatus* (m3), crab (*C.pagurus*; f23), shrimp (*P.borealis*; f24) or grass pollen (*P.pratense*; g6) extract, recombinant *A.fumigatus* MnSOD (rAsp f 6; m222) or recombinant shrimp tropomyosin (rPen a 1; Rf351) were purchased from Pharmacia (Uppsala, Sweden) and specific serum IgE levels were determined as recommended by the manufacturer.

2.2 Bacteria and reagents

Bacterial strain BL21(DE3)pLysS was purchased from Novagen (Madison, WI, USA). Clones encoding the *A.fumigatus* MnSOD or the tropomyosin consensus motif (Trop Mot) were grown in super broth (SB) medium containing 30 g/l tryptone and 20 g/l yeast extract, both purchased from Becton Dickinson (Sparks, MD, USA), and 10 g/l MOPS (Applichem, Darmstadt, Germany). All antibiotics (ampicilline, kanamycine, chloramphenicol and tetracycline) were purchased from Fluka Chemie AG (Buchs, Switzerland). Protein expression was induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) from Promega (Madison, WI, USA).

The monoclonal anti-human IgE antibody Le27 was produced in our laboratory as described elsewhere.[18]

2.3 Patients sera

For the studies on the *A.fumigatus* MnSOD, sera from four groups of donors were analysed: patients suffering from allergic bronchopulmonary aspergillosis (ABPA) or atopic dermatitis (AD), patients sensitised to *A.fumigatus* extract and control subjects. Patients suffering from ABPA fulfilled the diagnostic criteria given by Greenberger and Patterson.[19] Individuals with AD were diagnosed according to the criteria of Hanifin and Rajka.[20] ABPA and AD patients sera were kindly provided by Prof. Dr. R. Crameri

(Swiss Institute of Allergy and Asthma Research SIAF, Davos, Switzerland) and by PD Dr. med. P. Schmid-Grendelmeier (Allergy Unit, Dept. of Dermatology, Zürich, Switzerland), respectively. Sensitisation to *A.fumigatus* extract was determined using the Pharmacia CAP system. For control purposes, sera from donors who had no specific serum IgE to rAsp f 6, as determined by ImmunoCAP, were included.

The sera analysed for the studies on the tropomyosin consensus motif included individuals having strong IgE reactivity to crab or shrimp extract, as determined by ImmunoCAP.

2.4 Allergen motif search and generation of consensus sequence

Allergen sequences were retrieved from the continuously updated Allergome database (http://www.allergome.org), which contained 1102 non-redundant sequences at the time of our analysis. Allergen motifs of 50 amino acid residues were identified using the motif discovery tool MEME[21] in an iterative procedure, where after each iterative round matching allergen sequences were removed from the allergen database and remaining sequences were submitted to the next iteration round of motif discovery. Of 1102 allergen sequences, 912 were grouped into a limited set of only 69 statistically relevant motifs.

To investigate the biological relevance of these motifs, two motifs were chosen for further analysis. From the motif 22, which contained amino acid sequences from MnSODs of different organisms, a stretch of 50 amino acids corresponding to the motif region of Asp f 6, was synthesised as a peptide, named MnSOD Mot.

The second motif regrouped tropomyosin sequences that were used to generate a consensus sequence corresponding to the motif region. The consensus sequence was obtained by choosing at each position in the motif region either the most representative amino acid or the one which corresponds to the best to the coiled-coil structure of the tropomyosin proteins.

2.5 Expression and purification of *A.fumigatus* MnSOD

The *Escherichia coli* M15 clone encoding the full length *A.fumigatus* MnSOD was obtained from Prof. Dr. R. Crameri (SIAF).[22] Transformed bacteria were cultivated in 1500ml super broth (SB) medium containing 50µg/ml ampicilline and 25µg/ml kanamycine at 37°C until an OD at 600nm of 0.6 was reached. Protein expression was induced with 1mM IPTG for 4 h at 37°C and the hexahistidine-tagged MnSOD was purified from the cytoplasm using a BD TALON™ Metal Affinity Resin (BD Biosciences, San Jose, CA, USA) according to the manufacturers instructions. Further

purification was achieved by size exclusion chromatography using a HiLoad 26/60 Superdex 75 column (Amersham Pharmacia, Piscataway, NJ, USA). Purity and molecular size of the recombinant MnSOD were analysed by SDS-polyacrylamide gel electrophoresis (15% acrylamide) and Western blotting under denaturing conditions as described elsewhere.[23] Western blots were incubated with mouse anti-histidine antibody (Amersham Biosciences Europe, Otelfingen, Switzerland) at 1:1000 overnight at 4°C followed by incubation with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (DakoCytomation, Baar, Switzerland) at 1:1000 for 4 h at RT (data not shown).

2.6 Cloning, Expression and Purification of Trop Mot

The synthesis of the DNA fragment encoding the consensus sequence of the tropomyosin motif (Trop Mot) was performed using the following 3 overlapping oligonucleotides synthesised by Mycrosynth GmbH (Balgach, Switzerland):

Oligo #1: 5'-CTTCTGTCTGACGAAGAACGCATGGATGCTTTGGAAAATCAACTG AAAGAGGCAAGAATGCTGGCGGA-3';

Oligo #2: 5'-CCACCATTGCGAGCTTGCGTGCCACTTCATCATATTTACGATCTG CCTCTTCCGCCAGCATTCTTGCC-3';

Oligo #3: 5'-GCAAGCTCGCAATGGTGGAAGCAGACCTGGAACGTGCGGAAGAA CGGGCAGAAAGCGGCGAATCCAAG-3'.

After annealing of the 3 oligonucleotides by PCR filling reactions, the cDNA was amplified in a PCR reaction using the primers

forpriNcoI: 5'-CGTCGCCATGGTTCTGTCTGACGAAGAACG-3' and

repriXhoI: 5'-CGTCGCTCGAGCTTGGATTCGCCGCTTTCTGC-3'.

PCR conditions were 94°C for 60s, 54°C for 60s, and 72°C for 60s for 30 cycles, followed by a terminal extension cycle at 72°C for 10 min. The amplification products were digested with XhoI and NcoI (Roche Diagnostics GmbH, Rotkreuz, Switzerland), ligated to XhoI/NcoI-restricted pET 22b(+) vector (Novagen, Madison, WI, USA) and transformed into *E.coli* strain BL21(DE3)pLysS by electroporation. Clones were tested for the presence of Trop Mot DNA by PCR and sequence analysis. A single transformant containing the correct construct was cultivated in 1500ml of LB at pH 7.0 in the presence of ampicilline (100µg/ml), chloramphenicol (34µg/ml) and tetracycline (20µg/ml) at 37°C until an OD at 600nm of 0.6 was reached. Protein expression was induced with 1mM IPTG for 3 h at 37°C and the hexahistidine-tagged Trop Mot was purified from the periplasm using a BD TALON^{TM} Metal Affinity Resin (BD Biosciences) according to the manufacturers instructions. Purity and molecular size of the recombinant Trop Mot were

analysed by SDS-PAGE (15% acrylamide) and Western blotting under denaturing conditions using standard procedures.[23] Western blots were incubated with mouse anti-histidine antibody (Amersham) at 1:1000 overnight at 4°C followed by incubation with a HRP-conjugated sheep anti-mouse IgG antibody (ICN MP Biomedicals, Irvine, Ca, USA) at 1:1000 for 4 h at RT (data not shown).

2.7 IgE binding and inhibition experiments

The specific IgE to MnSOD and MnSOD Mot in patients sera was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA, USA) were coated overnight at 4°C with either MnSOD or MnSOD Mot at a concentration of 5µg/ml. Wells were blocked for 2 h at 37°C with PBS/0.15% casein and incubated with patients sera diluted 1:4, 1:8, 1:16 and 1:32 in blocking buffer for 3 h at 37°C. Bound antibodies were detected by using HRP-conjugated Le27, incubated for 2 h at 37°C at a 1:100 dilution. The 3,3',5,5'-tetramethylbenzidine (Fluka, Buchs, Switzerland) was used as substrate. The absorbance was read after 20 min at 450nm on a Molecular Devices ELISA reader (Paul Bucher, Basel, Switzerland).

For inhibition ELISA, half-well plates were coated and blocked as described. Patients sera were diluted 1:4 and preincubated with 0, 1, 5, and 50 µg/ml of MnSOD or MnSOD Mot for 2 h at 37°C, shaking at 320 rpm. The preincubated sera were transferred to the coated plates and incubated for 2 h at 37°C. Residual IgE-binding capacity was determined as described for ELISA assays.

Specific IgE reactivity to rPen a 1 and Trop Mot was measured by ImmunoCAP and ELISA, respectively. ImmunoCAP measurements were carried out with undiluted sera as prescribed by the manufacturer (Pharmacia). For ELISA, Costar EIA/RIA half-well plates were coated overnight at 4°C with Trop Mot at a concentration of 5µg/ml. Wells were blocked as described and incubated with patients sera diluted 1:2 in blocking buffer for 4 h at 37°C. Bound antibodies were detected by using a HRP-conjugated anti-human IgE antibody (The Binding Site, Schwetzingen, Germany) incubated for 2 h at 37°C at a 1:1000 dilution. Tetramethylbenzidine was used as substrate and the absorbance was read after 5 min at 450nm on a molecular devices ELISA reader.

For inhibition ELISA, half-well plates were coated and blocked as described. Patients sera were diluted 1:4 and preincubated with 0, 0.5, 5, 50 and 100 µg/ml of Trop Mot, chicken tropomyosin or BSA for 2 h at 37°C. The preincubated sera were transferred to the coated plates and incubated for 2 h at 37°C. Residual IgE-binding capacity was detected by incubating 1:100 diluted HRP-conjugated Le27 antibody for 2 h at 37°C and by using tetramethylbenzidine as substrate. The absorbance was read after 5 min as described above.

3. Results

3.1 Identification of allergen motif in MnSODs

Within the 69 allergen motifs found by the *in silico* sequence comparison analysis, the motif 22, containing MnSODs of different organisms such as *Aspergillus fumigatus* (Asp f 6), *Malassezia sympodialis* (Mala s 11), *Hevea brasiliensis* (Hev b 10.0101) (rubber tree) and *Drosophila melanogaster* (Dro m MnSOD) (fruit fly), was chosen. In order to investigate the *in vitro* immunogenicity of a fifty amino acid motif in relation to its corresponding full length and other highly homologous allergens, we selected the motif stretch of the *A.fumigatus* MnSOD as a representative and synthesised it as a peptide. Fig 1 shows an alignment of *A.fumigatus* MnSOD, including the localisation of the motif, with three selected MnSOD sequences of other organisms. The MnSOD of *A.fumigatus* has a total length of 210 amino acids, the motif thus accounts for approximately one fourth of the full length sequence.

3.2 IgE reactivity to MnSOD and MnSOD Mot

To compare the *in vitro* IgE reactivity of the *A.fumigatus* MnSOD motif (MnSOD Mot) with the whole MnSOD protein, full length recombinant MnSOD was produced as a soluble hexahistidine-tagged protein in *E.coli* and purified in two chromatography steps, first by Co^{2+}-chelate metal affinity and then by size exclusion chromatography. When analysed by SDS-PAGE, the purified recombinant MnSOD protein appeared as a single band with an apparent molecular weight in the range of 24 kd, which is in agreement with the calculated value for monomeric *A.fumigatus* MnSOD (23.4 kd) fused to a hexahistidine tag. In Western blot analysis this band showed strong reactivity with a mouse anti-histidine antibody (data not shown).

The IgE reactivity of the recombinant full length MnSOD was tested in ELISA with sera of three groups of patients. A first group of 10 patients suffering from allergic bronchopulmonary aspergillosis (ABPA) was chosen and high IgE reactivity to MnSOD could be detected in 8 of 10 ABPA patients (Fig 2A). This result is in agreement with a recent report showing that ABPA patients are exposed to intracellular proteins and are particularly sensitised to *A.fumigatus* MnSOD.[25] To verify the cross-reactivity between MnSODs of different organisms predicted by our motif analysis, sera of patients with atopic dermatitis (AD), who have recently been recognised to be sensitised to the MnSOD of the skin-colonising yeast *M.sympodialis*,[26] were tested against recombinant MnSOD. Indeed, 3 of 10 AD patients exhibited strong reactivity with *A.fumigatus*

MnSOD, indicating that *A.fumigatus* MnSOD can act as a pan-allergen in individuals sensitised to the fungal MnSOD (Fig 2B). The third patient group, which included 12 sera of individuals sensitised to *A.fumigatus* extract, only showed low to moderate overall reactivity to MnSOD, with only one serum exhibiting a higher IgE binding reactivity (Fig 2C). This result is in accordance with expectations, since it has been shown that the majority of individuals allergic to *A.fumigatus* is not sensitised to Asp f 6. None of the control sera from individuals not sensitised to *A.fumigatus* extract showed binding to MnSOD (Fig 2D).

In order to compare the immunogenicity of the full length MnSOD with the one of the motif, the IgE binding capacity of MnSOD Mot was tested in ELISA using the same groups of patients. Interestingly, 2 of the 8 ABPA sera positive on MnSOD were able to recognise MnSOD Mot (Fig 3A), suggesting that these patients have anti-MnSOD antibodies which bind to an epitope present in the motif region. Of the 3 AD sera positive on full length recombinant MnSOD, only one displayed a weak reactivity with MnSOD Mot (Fig 3B). Also sera of patients sensitised to *A.fumigatus* exhibited only a marginal reactivity with MnSOD Mot (Fig 3C) and no significant binding to MnSOD Mot was observed with the control sera (Fig 3D). Overall, these results indicate that the motif region represents one of the IgE reactive epitopes of MnSOD but only in sera of patients highly sensitised to MnSOD.

3.3 MnSOD Mot represents an epitope of the fungal MnSOD allergen

To verify the specificity of the observed IgE reactivity to MnSOD Mot, inhibition ELISAs were performed using the patient serum of the ABPA and the AD patient group respectively, showing the highest IgE reactivity to MnSOD Mot at a 1:4 dilution (Fig 4). The binding of serum IgE from the ABPA patient to 5µg/ml of solid phase bound MnSOD Mot was tested for inhibition using increasing concentrations of either soluble MnSOD Mot or full length MnSOD. A complete inhibition was already observed with 1µg/ml of MnSOD Mot, confirming the specificity of the previously observed IgE binding to MnSOD Mot (Fig 4A). Moreover, the same amount of preincubated MnSOD was shown to inhibit the IgE binding to MnSOD Mot, indicating that MnSOD Mot indeed embodies one of the epitopes of the full length allergen.

Similar results were observed with the AD patients serum using the same amount of solid phase bound MnSOD Mot (Fig 4B). Preincubation of the serum with increasing amounts of soluble MnSOD Mot resulted in a complete inhibition, already with 1µg/ml of MnSOD Mot, of IgE binding to the coated MnSOD Mot. Furthermore, the reaction could also be inhibited with the same amount of full length MnSOD, suggesting that the motif

represents one of the determinants responsible for cross-reactivity between the MnSODs of *A.fumigatus* and *M.sympodialis*.

In this first example, the motif seems to cover only part of the IgE binding structures that are presented by full length allergen.

3.4 Allergen motif in tropomyosins and designation of consensus sequence

As a second motif we have chosen the motif 6 which has been defined for the highly conserved tropomyosins of different invertebrates. Fig 5 shows the localisation of the motif in the aligned tropomyosins of *Dermatophagoides pteronyssinus* (house-dust mite), *Dermatophagoides farinae* (house-dust mite), *Lepidoglyphus destructor* (storage mite), *Charybdis feriatus* (crab), *Homarus americanus* (American lobster), *Panulirus stimpsoni* (spiny lobster), *Penaeus aztecus* (brown shrimp), *Chironomus kiiensis* (midge), *Blattella germanica* (German cockroach) and *Anisakis simplex* (herring worm). For Pan s 1, the motif starts at amino acid 127 and ends at position 176, for all the other sequences it stretches from position 136 to 186. To further investigate the biological relevance of allergen motifs, we applied an alternative strategy than the one described for the MnSOD motif: instead of selecting one of the tropomyosins as representative, a motif consensus sequence considering all the aligned sequences was generated. The consensus sequence was designed by either selecting from the aligned sequences the most abundant amino acids or the ones which fit the best to the coiled-coil structure of tropomyosin. The majority of the sequences have a total length of 284 amino acids, meaning that the motif accounts for one fifth to one sixth of the whole sequence.

3.5 Trop Mot represents a major epitope of the included full length tropomyosins

To test the relevance of the tropomyosin consensus motif in terms of IgE reactivity, we produced it as a soluble protein in *E.coli* and purified it via the linked His (6) tag epitope. The affinity-purified recombinant Trop Mot protein appeared as virtually pure when analysed by SDS-PAGE and the estimated molecular weight of 6.5 kd is in agreement with the calculated molecular weight of the His-tagged Trop Mot. In Western Blot analysis, the band strongly reacted with a peroxidase-labelled anti-histidine antibody (data not shown).

The IgE binding capacity of Trop Mot was tested in ELISA using sera from donors allergic to shellfish. In order to compare this reactivity to the one obtained with a full length tropomyosin, the same sera were tested for IgE binding to the recombinant shrimp *Penaeus aztecus* tropomyosin (rPen a 1) in ImmunoCAP assay. The same sera were

found to contain IgE against both recombinant proteins. As depicted in Fig 6A, the IgE reactivity to rPen a 1 correlated very well with the IgE binding to Trop Mot (r^2= 0.9606), suggesting that Trop Mot represents a major allergenic epitope of tropomyosin. No IgE binding was observed with control sera from donors sensitised to grass pollen (data not shown).

We further demonstrated the specificity of the ELISA results obtained with Trop Mot by an inhibition assay. One of the sera showing IgE reactivity to both rPen a 1 and Trop Mot was tested for its binding to 5µg/ml solid phase coated Trop Mot after preincubation with different amounts of soluble Trop Mot. As shown in Fig 6B, IgE binding to Trop Mot was completely inhibited by increasing concentrations of the consensus motif, confirming that the reaction to Trop Mot was specific. BSA as control protein did not interfere with IgE binding to Trop Mot. Interestingly, the binding could neither be inhibited by preincubation with increasing amounts of soluble chicken tropomyosin, suggesting that this non-allergenic vertebrate tropomyosin does not cross-react with epitopes present in the allergen consensus motif.

4. Discussion

The knowledge about allergenic proteins and their structures is constantly growing but the molecular characteristics making a protein allergenic are still not known. We have recently described the identification of motifs in allergen sequences by an iterative sequence comparison protocol.[3] The *in silico* analysis has shown that a large number of allergens can be grouped into a limited set of motifs.

In this study we analysed the immunological reactivity of two allergen motifs in relation to the one of their corresponding full length allergens. From a first motif that contains MnSOD sequences of different organisms, the *A.fumigatus* motif was selected as a representative and analysed for IgE binding activity using different groups of patients sera. Previous studies analysing the sequence similarities between MnSODs of different organisms aimed at assigning identical residues which are solvent exposed and thus candidates for IgE binding involvement.[27] For this purpose, the crystal structures of *A.fumigatus* and human MnSOD were analysed with a suitable program to calculate the relative solvent accessibility of the amino acid residues. A number of conserved amino acids was found that are solvent exposed and that are considered to be involved in IgE cross-reactivity of the two proteins. Additionally, sequence alignment of the MnSODs of *A.fumigatus*, man, *S.cerevisiae*, *D.melanogaster* and *E.coli* revealed 48 amino acids to be identical in all the sequences, from them only four were found to be at least 30% solvent exposed. Interestingly, all of these four (Pro^{8}, Pro^{19}, Lys^{32}, and Asn^{39}) are within the MnSOD motif stretch which was defined by our *in silico* analysis. In our IgE binding studies we found specific IgE to MnSOD Mot in sera of ABPA patients having a high reactivity to MnSOD, whereas no reactivity was observed in sera of patients sensitised to *A.fumigatus* extract. This suggests that MnSOD Mot represents a part of the IgE epitopes present on the surface of *A.fumigatus* MnSOD that may be involved in the immune response associated with ABPA. It has been shown that *A.fumigatus* MnSOD represents an intracellular protein and is not secreted by the fungus. However, patients with ABPA have fungus growing in their lung and become exposed to non-secreted proteins as a result of fungal damage due to inflammatory processes. Previous experiments demonstrated that *A.fumigatus* MnSOD is allergenic only in patients suffering from ABPA and not in *A.fumigatus* sensitised individuals. Our data confirm these results by showing a high reactivity to *A.fumigatus* MnSOD in sera of patients with ABPA and a much weaker one in sera of patients only sensitised to *A.fumigatus* extract. Furthermore, a motif region present in MnSOD was identified that carries an IgE binding epitope which is specifically recognised only by sera of ABPA patients.

Our *in silico* analysis has predicted a high degree of sequence homology between the MnSODs of *A.fumigatus* and *M.sympodialis*. The results indicate that 3 of 10 AD patients, who are sensitised to the MnSOD of *M.sympodialis*, displayed IgE binding reactivity to the MnSOD of *A.fumigatus.* This confirms the predicted cross-reactivity and the fact that MnSOD is a pan-allergen that can be recognised by individuals sensitised to different MnSODs. It is also consistent with earlier experiments showing that patients with severe AD have a high rate of specific IgE antibodies which cross-react with MnSODs of different origins.[26]

Moreover, in the present study one serum out of the three *A.fumigatus* MnSOD positive AD sera showed a reaction to MnSOD Mot, which could be inhibited with MnSOD. This is a direct demonstration that the cross-reactivity between the MnSODs of the two fungi is in part attributable to conserved epitopes included in the motif region. The fact that only one patient reacts with MnSOD Mot lets us assume that MnSOD contains additional allergenic epitopes in other parts of the sequence, which may be of equal or higher importance. This assumption is in accordance with previous works on structural analysis reporting that IgE-binding epitopes are scattered over the surface of the MnSOD molecule.[27] Furthermore, it has been shown with other allergens that IgE epitopes can be highly patient specific.[28, 29]

The IgE binding capacity of allergenic motifs was further documented using a second motif that contained tropomyosins of different phylogenetic origin. Ayuso et al. have previously analysed the major shrimp allergen Pen a 1 (tropomyosin) with regard to important IgE-binding regions by screening synthetic peptides spanning the whole length of the molecule for their IgE antibody reactivity with sera of shrimp-allergic subjects.[30] Considering the overall prevalence and intensity of IgE binding in the sera, they identified five major IgE-binding regions. Interestingly, one of these regions overlaps with the 50 amino acids of the tropomyosin motif. In the current study we have shown that the IgE reactivity of sera from shellfish-allergic individuals to recombinant shrimp *Penaeus aztecus* tropomyosin (rPen a 1) is comparable with the one observed for the tropomyosin consensus motif (Trop Mot) in the same sera. Compared to the study mentioned above[30] and other reports using peptide libraries of 10 to 30 amino acid residues long and where specificities of 30 to 80% were reached[31] we obtained 100% specificity in that all the tested sera reacted with our Trop Mot. Therefore we assume that the tropomyosin motif not only binds IgE but most probably represents an allergenic structure comparable to the full length tropomyosin that can act as a marker for serological identification of seafood allergy. This conclusion is supported by the fact that tropomyosin molecules are rather unique molecules which consist of two α-helical chains wound around each other in a coiled-coil formation.[7] The amino acid sequence of

tropomyosin shows repeating patterns of nonpolar and polar amino acids, one repeat consisting of seven residues. Because of its highly repetitive nature, such a structure theoretically could include conformational epitopes with high similarity, which may induce cross-reactive anti-tropomyosin IgE antibodies. Since previous studies have determined the IgE-binding epitopes to consist of approximately 8 amino acids,[32-35] a motif of 50 amino acids might contain several of these epitopes that are involved in the binding of polyclonal IgE.

In comparison to the repetitive arrangement of amino acids in a tropomyosin molecule, the structure of a monomeric MnSOD is rather irregular. It consists of seven α-helices and five strands of β-sheet,[36] and putative IgE-binding epitopes are formed by residues on different surface loops suggesting that correct folding is required for allergenicity.[27] This observation might provide an additional explanation for the relative low reactivity observed with the *A.fumigatus* MnSOD motif which was chemically synthesised as a peptide and most probably did not present the same conformational structure as the native protein.

In summary, we could show for both the MnSOD and the tropomyosin motif, that the *in silico* motif-based analysis was able to predict IgE epitopes in protein sequences. So far, one of the most frequently used method to investigate IgE-binding epitopes is the use of short overlapping peptides covering the whole sequence of an allergen with regard to their IgE reactivity with sera of individuals sensitised to this allergen. This approach may serve to identify crucial residues involved in IgE binding, but since B-cell epitopes are mostly conformational, it is not suitable to determine a complete B-cell epitope. Moreover, as indicated above,[28, 29] the reactivity of IgE antibodies with an epitope can vary from patient to patient and therefore large pools of patients sera need to be tested in order to make a reliable statement about the general IgE binding capacity of the peptides. Another approach for the identification of IgE binding regions is the analysis of solvent accessible areas by considering the tertiary structure of a given allergen. This method takes into consideration the conformational nature of IgE epitopes, but therein also lies its limitation, namely it is only applicable to a limited set of allergens, since the structure has to be known beforehand. Overall, these approaches have been successful in clarifying the question of IgE binding regions for some selected allergens, but a more general, broadly implementable method to predict the allergenicity of protein sequences would be of great importance. In the current study, we report the first *in vitro* application of an *in silico* motif-based allergenicity prediction protocol previously developed at our institute[3] with two selected motifs. Trop Mot was shown to represent a major allergenic structure that contains several epitopes and may be used instead of full length tropomyosin for serological diagnosis of shellfish allergy. In contrast, the MnSOD motif most probably

contains one epitope and is recognised only by a subgroup of patients sensitised to the full length allergen. The fact that epitopes are highly patient specific is thus a limitation to the overall impact of the motif analysis, but this is probably true for any generalised prediction method. Since our motif based allergenicity evaluation method is solely based on comparison of similarities on the sequence level, no information on structural characteristics of an allergen is required, a feature which emphasises the broad applicability of this approach.

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Figure Legends

Fig. 1. Amino acid sequences alignment of the MnSODs from *A.fumigatus* (Asp f 6), *M.sympodialis* (Mala s 11), *H.brasiliensis* (Hev b 10.0101) and *D.melanogaster* (Dro m MnSOD). The region of the motif in Asp f 6 is shown with black bars.

Fig. 2. Binding specificities to MnSOD of serum IgE of three patient groups and control subjects. Wells were coated with 5µg/ml full length MnSOD and incubated with sera diluted 1:4, 1:8, 1:16 and 1:32. Bound IgE was detected using HRP-conjugated Le27 antibody at a 1:100 dilution. **A.** Patients suffering from allergic bronchopulmonary aspergillosis (ABPA) (10 donors). **B.** Patients suffering from atopic dermatitis (AD), who are sensitised to Mala s 11 (10 donors). **C.** Patients tested positive on *A.fumigatus* extract by ImmunoCap (12 donors). **D.** Donors tested negative on recombinant Asp f 6 by ImmunoCap, serving as the control group (12 donors). Results are given as values of optical density measured at 450nm. The dashed line indicates the background level, meaning the binding of the second antibody to the coating antigen without addition of serum.

Fig. 3. Binding specificities to MnSOD Mot of serum IgE of three patient groups and control subjects. Wells were coated with 5µg/ml full length MnSOD Mot and incubated with sera diluted 1:4, 1:8, 1:16 and 1:32. Bound IgE was detected as in figure 2. **A-D.** Patient groups and control subjects as indicated for figure 2. Results are given as values of optical density measured at 450 nm. The dashed line indicates the background level, meaning the binding of the second antibody to the coating antigen without addition of serum. **A-D.** Patient groups and control subjects as indicated for figure 2.

Fig. 4. Inhibition of IgE binding to MnSOD Mot. The patients serum showing the highest binding signal to MnSOD Mot of the ABPA patient group **(A)** and the AD patient group **(B)** was diluted 1:4 and preincubated with 1, 5 and 50 µg/ml of MnSOD Mot or MnSOD, samples were transferred to MnSOD Mot-coated wells , and residual IgE binding was detected using HRP-conjugated Le27 antibody at a 1:100 dilution.

Fig. 5. Amino acid sequences alignment of the tropomyosins from *D.pteronyssinus*, house-dust mite (Der p 10), *D.farinae*, house-dust mite (Der f 10), *L.destructor*, storage mite (Lep d 10), *C.feriatus*, crab (Cha f 1), *H.americanus*, American lobster (Hom a 1), *P.stimpsoni*, spiny lobster (Pan s 1), *P.aztecus*, brown shrimp (Pen a 1), *C.kiiensis*, midge (Chi k 1), *B.germanica*, German cockroach (Bla g 7) and *A.simplex*, herring worm (Ani s

3). The region of the motif in the aligned sequences is shown with black bars and the derived consensus sequence is indicated at the bottom.

Fig. 6. A. Correlation of values of specific IgE to recombinant Pen a 1 (*P.aztecus*, brown shrimp) analysed by ImmunoCap and to Trop Mot analysed by ELISA. Sera of shellfish allergic patients (9 donors) were used undiluted for ImmunoCap and diluted 1:2 for ELISA. Results are given as values in kUnits per liter (kUA/l) and values of optical density measured at 450 nm, respectively. **B.** Inhibition of IgE binding to solid phase coated Trop Mot. One patient serum showing a high binding signal on both recombinant Pen a 1 and Trop Mot was diluted 1:4 and preincubated with 0.5, 5, 50 and 100 µg/ml of Trop Mot, recombinant chicken tropomyosin (Trop Chicken) or BSA, samples were then transferred to Trop Mot-coated wells, and residual IgE binding was detected using HRP-conjugated Le27 antibody at a 1:100 dilution.

Figures

Fig. 1

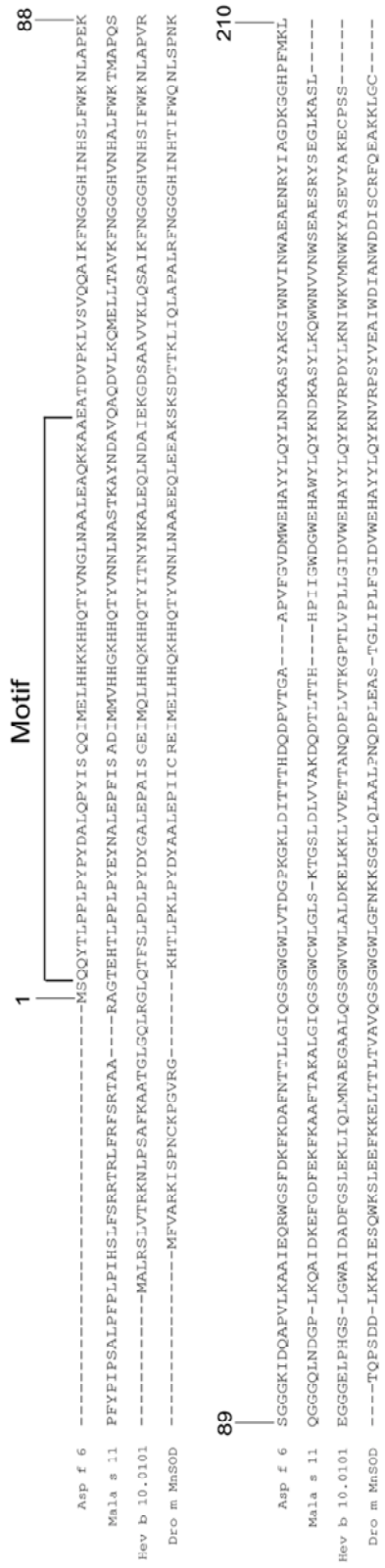


Fig. 2A-D

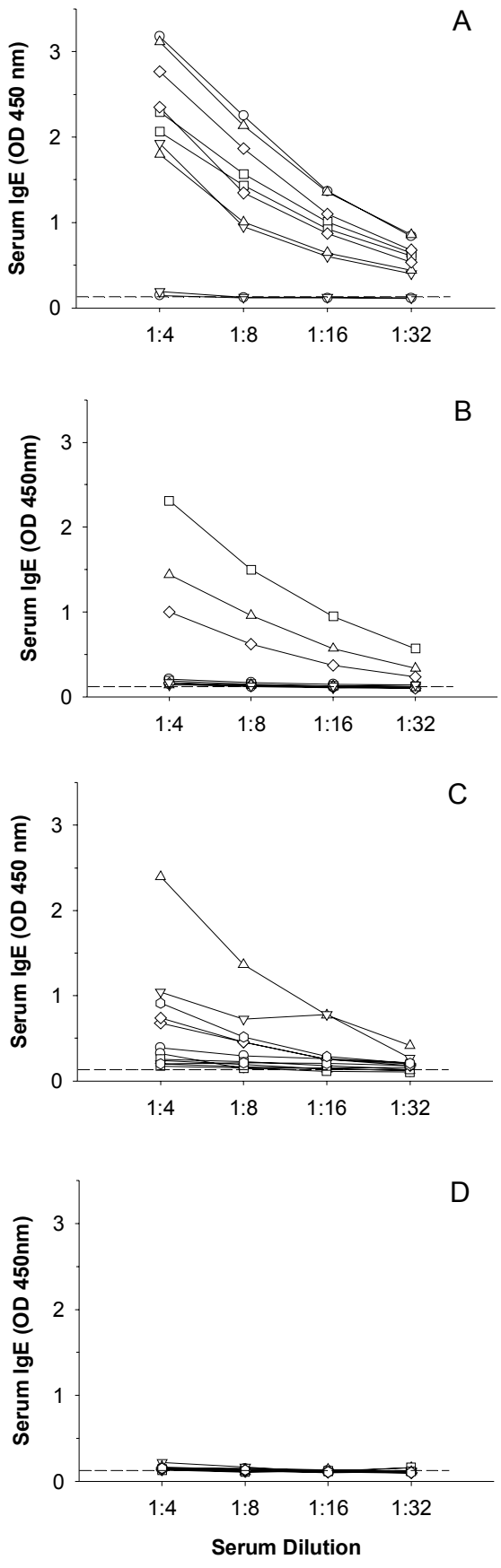
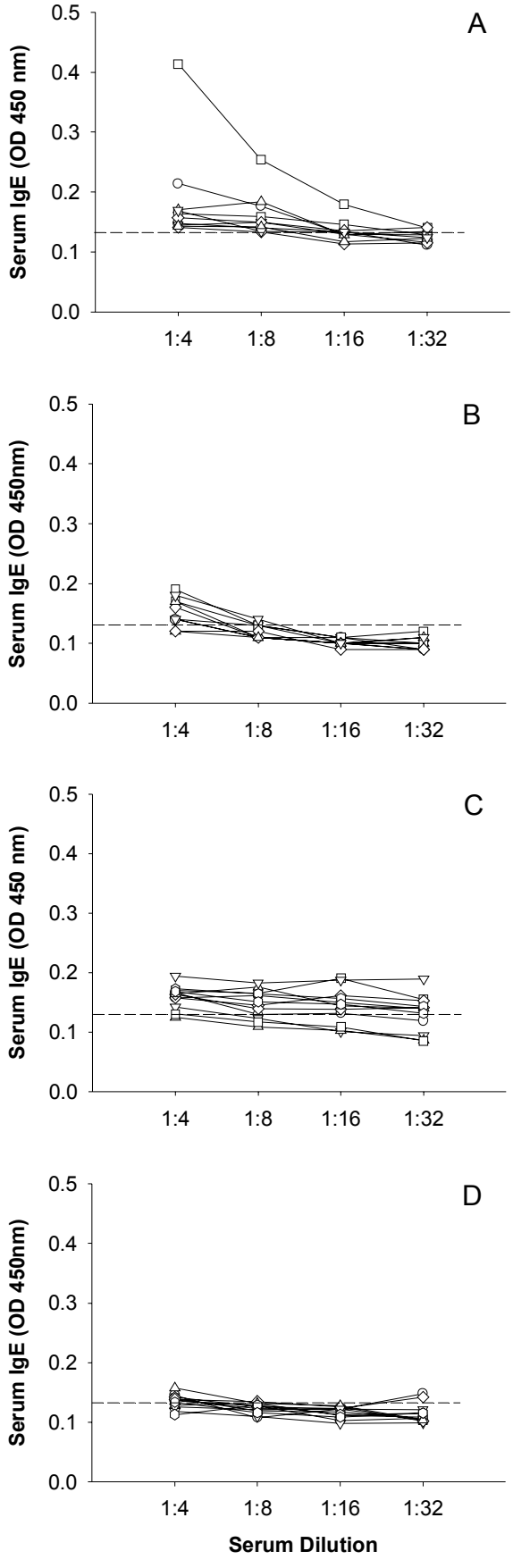

A
B
C
D
Serum IgE (OD 450 nm)
Serum IgE (OD 450nm)
0
1
2
3
1:4
1:8
1:16
1:32
Serum Dilution

Fig. 3A-D


A
B
C
D
Serum IgE (OD 450 nm)
Serum IgE (OD 450nm)
0.0
0.1
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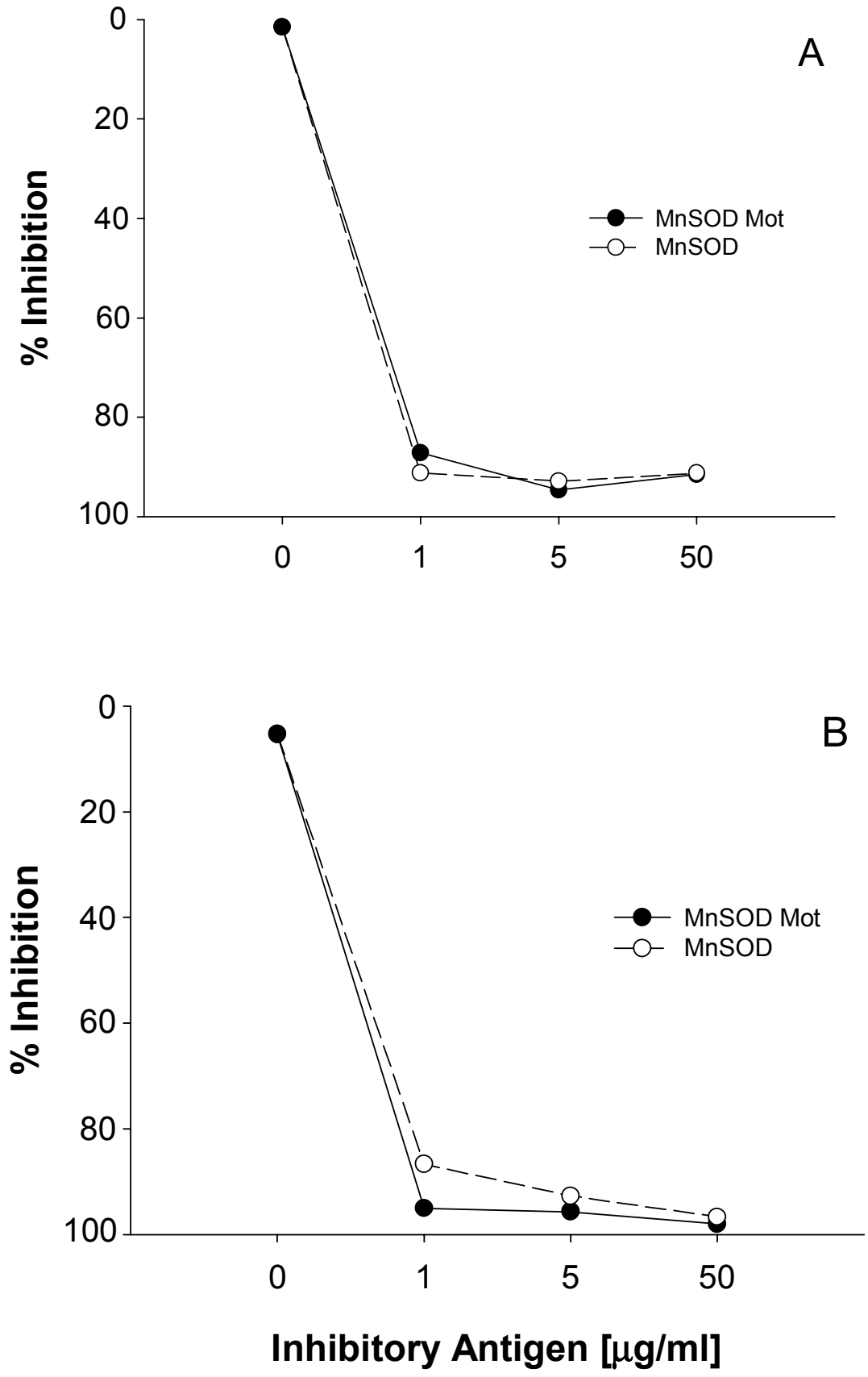
0.3
0.4
0.5
1:4
1:8
1:16
1:32
Serum Dilution

Fig. 4A,B


A
B
% Inhibition
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20
40
60
80
100
0
1
5
50
MnSOD Mot
MnSOD
Inhibitory Antigen [μg/ml]

Fig. 5

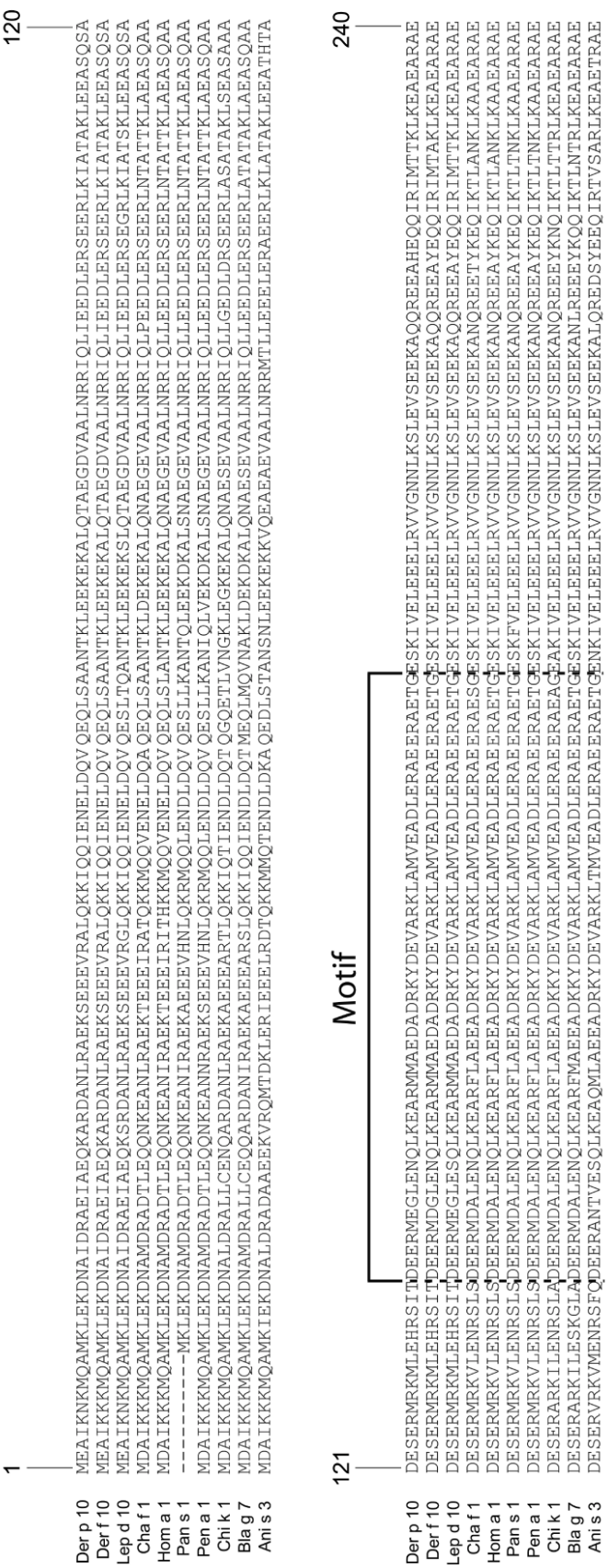
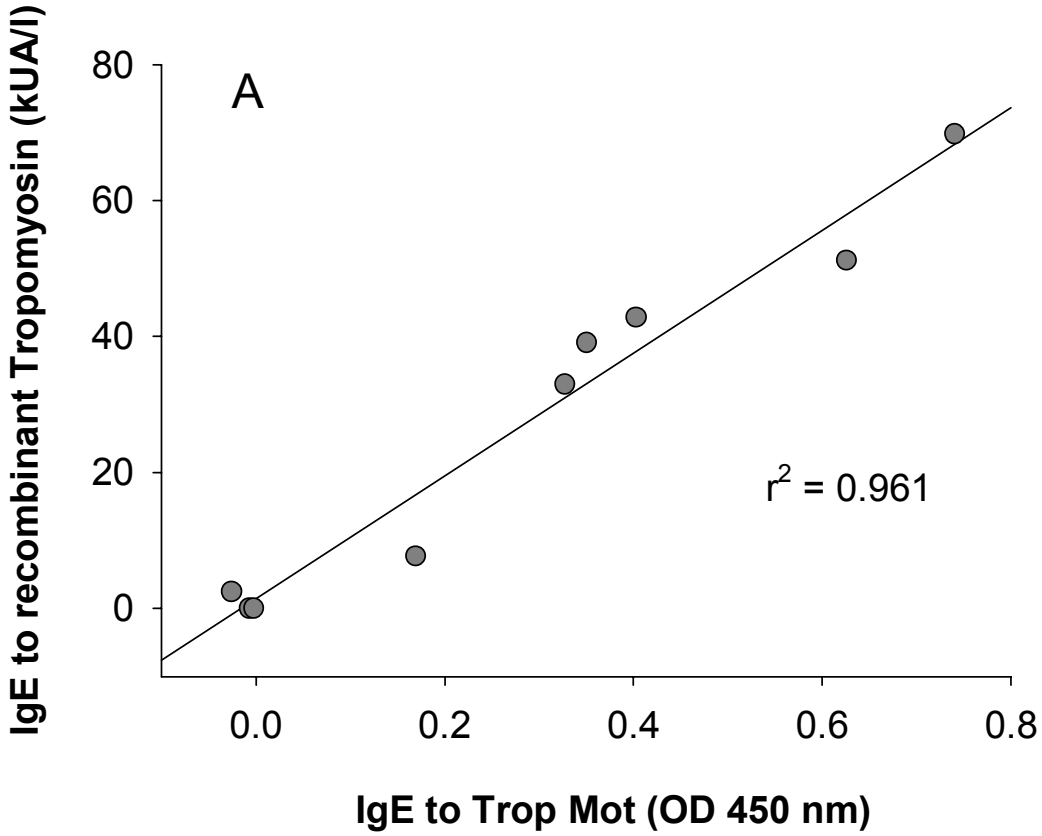
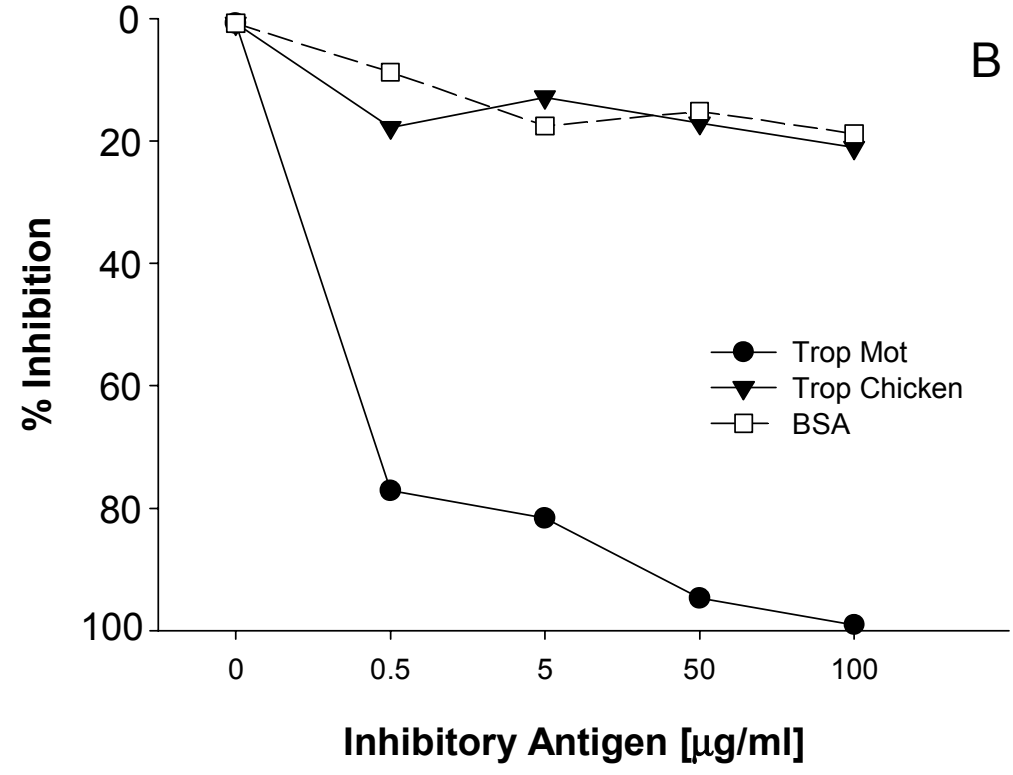

Motif
Consensus: DEERMDALENQLKEARMLAEEADRKYDEVARKLAMVEADLERAEERAESG

Fig. 6A,B


A
IgE to recombinant Tropomyosin (kUA/l)
IgE to Trop Mot (OD 450 nm)
r2 = 0.961
0
20
40
60
80
0.0
0.2
0.4
0.6
0.8


B
% Inhibition
Inhibitory Antigen [µg/ml]
Trop Mot
Trop Chicken
BSA
0
20
40
60
80
100
0
0.5
5
50
100

Dissertation Equivalent II

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Intranasal immunisation using recombinant *Lactobacillus johnsonii* as a new strategy to prevent allergic disease

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Abstract

We have previously demonstrated the induction of a specific anti-IgE response in vivo by parenteral immunisation of rhesus monkeys using short IgE mimotopes or an anti-idiotypic antibody mimicking an IgE epitope. Such specific anti-IgE responses may be of clinical benefit for atopic patients. In this study, we examined the potential for a more convenient therapy via mucosal immunisation using live recombinant *Lactobacillus johnsonii* (Lj) as a vaccine delivery vehicle. Either an anti-idiotypic scFv or an IgE mimotope were expressed on the surface of Lj as fusion proteins using the cell wall anchored proteinase PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus*. The recombinant Lj were shown to express the heterologous fusion proteins and were specifically recognised by the corresponding anti-human IgE monoclonal antibody. Subcutaneous and intranasal immunisation of mice with recombinant Lj, expressing these fusion proteins induced a systemic IgG response against human IgE. Our data suggest that recombinant Lactobacilli expressing IgE epitopes may represent a novel means of vaccination to induce a beneficial anti-IgE response.

Keywords: *Lactobacillus johnsonii*; IgE mimotope; Anti-idiotypic single chain fragment variable

1. Introduction

The incidence of atopic disease has increased considerably in most Western countries during the last 30 years. New therapies are required independent of the initiating allergen which would have a broader application to many different patient groups. A promising approach has been the development of non-anaphylactogenic anti-IgE antibodies aimed at targeting IgE which plays a key role in initiating the symptoms of atopic disease. These anti-IgE antibodies recognize epitopes close to the binding site of IgE on the high affinity IgE receptor (FcεRI) [1,2]. One of these anti-IgE antibodies, omalizumab is currently used to treat allergic patients by passive immunisation [1–8]. However, passive immunisation requires continuous treatments with relatively high amounts of monoclonal antibodies resulting in high costs. Therefore, an active immunisation strategy aimed at neutralising IgE was envisaged by using IgE epitopes to induce a natural autoimmune response, resulting in the formation of autoantibodies that may have the same effect as passively administered non-anaphylactogenic anti-IgE antibodies. In order to isolate therapeutically applicable, immunogenic IgE epitopes a non-anaphylactogenic monoclonal anti-IgE antibody BSW17 was used as a screening tool [9,10] on random peptide phage display libraries [11,12]. Two peptides (Cε3mim and Cε4mim) were isolated which mimicked part of the IgE epitope recognised by BSW17 and were therefore called IgE mimotopes. In addition, an anti-idiotypic Fab (Fab α-Id-B43) was isolated [13] which mimicked the same region as the IgE mimotopes but in addition covered a larger area on IgE. Both the mimotopes [14] and the anti-idiotypic Fab were used for parenteral immunisation of rhesus monkeys and were shown in a passive cutaneous anaphylaxis (PCA) test to completely

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abolish PCA reactivity after allergen challenge (manuscript in preparation).

In this study, we analysed the ability of *Lactobacillus johnsonii* (Lj) to express C*ε*4mim and α-IdscFv2, an anti-idiotypic single chain Fragment variable (scFv) derived from Fab α-Id-B43 to induce an anti-IgE response after active, mucosal immunisation. *Lactobacillus* strains are attractive candidates as carriers for mucosal vaccination. Since centuries, they have been used for food processing and food preservation and are therefore considered to be safe organisms for human consumption. Several systems have been described using *Lactobacillus* as a carrier for expressing foreign antigens and for mucosal immunisation. Zegers et al. [15] have reported the use of *Lactobacillus casei* to express the protective antigen of *Bacillus anthracis* as an oral prototype vaccine against anthrax. In addition, the use of tetanus toxin fragment C (TTFC) as a model antigen for expression on *Lactobacillus plantarum* [16,17] or on *L. casei* [18] and the induction of an anti-TTFC specific immune response after mucosal immunisation has been described. We have reported the use of Lj to express mimotopes fused to the cell wall anchored proteinase PrtB [19] and the induction of a local and a systemic immune response against these heterologous proteins after oral immunisation [20].

In the present study, we show that both an anti-idiotypic scFv and an IgE mimotope could be expressed as a fusion protein with PrtB on the surface of Lj and induced anti-IgE antibodies after subcutaneous and intranasal administration. In contrast to other reports, we demonstrate here that Gram-positive bacteria expressing anti-idiotypic single chain Fragment variable (scFv) may be used to act as immunomodulators. A mucosal anti-IgE vaccine based on recombinant Lj would not only be much cheaper than the currently used passive administration of monoclonal antibodies, but would also avoid disadvantages associated with parenterally administered vaccines.

2. Materials and methods

2.1. Plasmids and oligonucleotides

Plasmid pComb3His was reconstructed based on pComb3H [12] by inserting 6His tags at the *Spe*I/*Nhe*I sites. Plasmid pComb3-α-Id-B43 contains the sequence coding for Fab α-Id-B43. Plasmid pMD112 was constructed by inserting into pNZ124 [21] the cell wall anchored proteinase (prtB) gene [19] of *Lactobacillus delbrueckii* subsp. *bulgaricus* and was isolated from transformed *Lactococcus lactis* as described recently [20]. The linker sequences used for the construction of the anti-idiotypic scFv fragments were designed as described elsewhere [22–25] (Table 1). All oligonucleotides were synthesised by Microsynth GmbH (Balgach, Switzerland).

2.2. Bacteria and reagents

Bacterial strain XL-1 Blue was purchased from Stratagene (San Diego, USA). *L. johnsonii* strain NCC2754 (Lj) were grown in MRS broth (Difco Laboratories, Detroit, USA) containing 1% Glucose. Chloramphenicol (Fluka Chemie AG, Buchs, Switzerland) was used at a concentration of 10 μg/ml. All restriction enzymes were purchased from Roche Diagnostics GmbH (Rotkreuz, Switzerland).

2.3. Antibodies and antigens

Affinity-purified rabbit anti-PrtB antibodies were purchased from Eurogentech (Seraing, Belgium). Rabbit anti-human C*ε*4mim [10] and anti-human IgE antibody BSW17 [9,26] were prepared in house as previously described. Rabbit anti-α-Id-B43 was prepared in our laboratory by immunising rabbits using Fab α-Id-B43 [13]. Horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG was purchased from Cappel (ICN, Switzerland). HRP-conjugated goat anti-rabbit antibodies were obtained from Nordic Immunology Laboratories (Tilberg, The Netherlands). Human myeloma IgE Savasal was a kind gift from Dr. V. Savasal (Pilsen, Czech Republic) and was purified on a MonoQ ion exchange column using an FPLC system (Pharmacia Biotech AG, Dübendorf, Switzerland) and used at a concentration of 10 μg/ml.

2.4. Production of anti-idiotypic scFv fragments in Escherichia coli XL-1 Blue

V_H and V_L sequences of Fab α-Id-B43 [13] and the linker sequences used for the construction of the anti-idiotypic scFv fragments were published elsewhere [22–25] and are shown in Table 1. The V_H and V_L sequences of the anti-idiotypic scFv fragments were amplified separately from plasmid pComb3-α-Id-B43 using primer pairs specific for the N- and C-terminal of either the V_H or the V_L regions. To assemble V_H and V_L fragments, overlapping oligonucleotides encoding different linkers (Table 1) were inserted between heavy and light chain in a PCR reaction. For subsequent cloning, the assembled products were PCR amplified

Table 1
Linkers for the construction of anti-idiotypic scFv fragments

ScFv designation[a]	Linker sequence	Linker description	Reference for linkers
α-IdscFv1	GGGGSGGGGSGGGGS	Flexible linker	[22,23]
α-IdscFv2	PNGASNSGSAPDTSSAPGSQ	β-Turn linker	[24]
α-IdscFv3	YPRSIYIRRRHPSPSLTT	Random linker	[25]

[a] Sequences of V_L and V_H of Fab α-Id-B43 are already published [13].

with flanking primers containing *Sac*I and *Spe*I restriction sites.

The resulting DNA fragments encoding either α-IdscFv1, α-IdscFv2 or α-IdscFv3 were then digested with *Sac*I and *Spe*I restriction enzymes and inserted into plasmid pComb3His digested with *Sac*I and *Spe*I. Ligated plasmid DNA was used to transform *Escherichia coli* XL-1 Blue. Positive clones were tested for the presence of the corresponding α-IdscFv DNA by PCR and sequencing analysis (data not shown) and were subjected to large-scale production. Transformed XL-1 Blue were cultivated in 1000 ml of super broth (SB) at pH 7.0 in the presence of ampicillin (50 μg/ml) at 37 °C until an OD at 600 nm of 1.0 was reached. Subsequently, protein synthesis was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Promega, Madison, WI). The bacteria were further cultured by shaking (220 rpm) for 4 h at 37 °C. The culture supernatant was collected and purified on a BD TALON™ Metal Affinity Resin (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

2.5. Screening of anti-idiotypic scFv fragments by anti-human IgE antibodies

The anti-idiotypic scFv fragments were tested by ELISA using Costar EIA/RIA half-well plates (Costar, Cambridge, MA) coated for 4 h at 37 °C with α-IdscFv1, α-IdscFv2 or α-IdscFv3 at a concentration of 10 μg/ml. IgE Savasal coated at the same concentration was used as a control. Wells were blocked for 2 h at 37 °C with PBS/5% BSA and incubated with either BSW17 (50 μg/ml), rabbit anti-human Cε4mim (10 μg/ml) or rabbit anti-α-Id-B43 (1:1000) for 4 h at 37 °C. Bound antibodies were detected by using HRP-conjugated sheep anti-mouse IgG (Fc) or HRP-conjugated goat anti-rabbit IgG (Fc) antibodies incubated for 1.5 h at 37 °C. Tetramethylbenzidine (3,3′,5,5′-tetramethylbenzidine, Fluka, Buchs, Switzerland) was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher, Basel, Switzerland).

2.6. Generation of Lj transformants expressing PrtB fusion proteins

Recombinant Lj expressing either a tetanus mimotope (LjTT) or a Cε4mim (Ljε4), recognised by the monoclonal mouse anti-human IgE antibody BSW17 [9] have been described elsewhere [20]. Recombinant Lj expressing an α-IdscFv2-PrtB fusion protein (Ljα-IdscFv2) was produced by first subjecting DNA encoding α-IdscFv2 to a PCR reaction using primers containing *Pvu*I and *Nhe*I restriction sites. Plasmid pMD112 was digested with *Pvu*I and *Nhe*I, gel purified and ligated to the PCR product encoding α-IdscFv2. Ligated DNA was used to transform electrocompetent Lj and transformants were plated on MRS plates containing chloramphenicol (10 μg/ml). Clones were tested for the presence of α-IdscFv2 DNA by PCR and sequencing analysis (data not shown).

2.7. Gel electrophoresis and Western immunoblots

Transformed Ljα-IdscFv2 and LjTT bacteria were grown overnight in 25 ml medium containing 10 μg/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 × *g* for 15 min at 4 °C and washed with 5 ml TBS. After recentrifugation, the bacterial pellet was resuspended in 450 μl TBS and 150 μl 4× non-reducing sample buffer (80 mM Tris–HCl, pH 6.8, 2.5% SDS, 0.15% glycerol, 0.005% bromophenol blue). Aliquots (20 μl) were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (6% acrylamide, 0.5 M Tris–HCl, pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 100 V for 60 min. The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories, Reinach, Switzerland) or transferred electrophoretically onto Protran BA 83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After transfer, the membranes were blocked with PBS/5% BSA for 2 h at RT and incubated with affinity purified rabbit anti-PrtB antibodies (1:2000) overnight at RT followed by incubation for 3 h at RT with a 1:1000 dilution of HRP-conjugated goat anti-rabbit IgG. Immunoblots were developed with 4-chloro-1-naphtol for 2 min.

2.8. Catching ELISA using recombinant Lj

Transformed bacteria (Ljα-IdscFv2, Ljε4 and LjTT) and wild type Lj were grown overnight in 50 ml medium containing 10 μg/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 × *g* for 15 min at 4 °C, washed with 5 ml TBS and recentrifuged. Finally, the bacterial pellet was resuspended in 1 ml TBS/10% (v/v) PBS/2% FCS/0.05% Tween 20. Fifty microliters of this bacterial suspension (approximately 5×10^7 cells) were overlain on Costar EIA/RIA half-well plates (Costar, Cambridge, MA) coated with 50 μg/ml anti-human IgE BSW17 overnight at 4 °C. Unbound bacteria were washed off the wells using PBS/0.1% Tween 20. Wells were then incubated with rabbit anti-PrtB antibodies diluted 1:2000 in PBS/2% FCS/0.05% Tween 20 for 4 h at 37 °C. Bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher).

2.9. Immunisation of mice

Groups of five female BALB/c mice (8 weeks old) were immunised subcutaneously with 10^8 live LjTT, Ljε4 or Ljα-IdscFv2 at days 0, 21 and 43. The bacterial suspension used for the immunisation was mixed 1:2 with $Al(OH)_3$. For intranasal immunization, three weekly doses of 10^9 live LjTT, Ljε4 or Ljα-IdscFv2 were administered at days 0, 1 and 2 to

three groups, each of five non-anaesthetized mice in a volume of 12 μl/mouse. Booster immunisations were given at days 21, 22, 23 and 43, 44, 45. Sera were collected by retro-orbital bleeding at days 52 (subcutaneous immunisations) and 56 (intranasal immunisations), respectively [16].

2.10. Evaluation of antibody responses

The antibody response was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated overnight at 37 °C with either 10 μg/ml human IgE Savasal or 10 μg/ml soluble α-IdscFv2. Sera were tested at dilutions of 1:50, 1:100 and 1:200 by incubation on the coated wells at 37 °C for 4 h. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) incubated for 1.5 h at 37 °C. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 6 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher). Serum of non-immunised mice were used as negative controls (background) and subtracted from serum signals. Absorbance in these samples was never higher than OD 0.1.

3. Results

3.1. Production of soluble anti-idiotypic scFv fragments in E. coli XL-1 Blue

We have previously isolated an anti-idiotypic Fab (Fab α-Id-B43), mimicking part of the third and fourth constant domains of human IgE [13]. In order to develop a mucosal anti-IgE vaccine, we expressed Fab α-Id-B43 on the surface of Lj. The binding site of Fab α-Id-B43 was constructed as a single chain fragment variable (α-IdscFv), because Gram-positive bacteria lack an oxidative site, where heavy and light chain of an Fab could assemble.

As the linker used for the construction of a scFv influences its correct folding [24,25], three anti-idiotypic scFv fragments were tested using different linkers. Thus, annealed overlapping oligonucleotides encoding the different linkers for α-IdscFv1 ($(Gly_4\text{-}Ser)_3$-linker [22,23]), α-IdscFv2 (β-turn-promoting linker [24]), or α-IdscFv3 (random linker [25]) were used for assembly of V_H and V_L of α-Id-B43 in a PCR reaction (Table 1). In a subsequent PCR reaction, restriction sites were added and the final PCR products encoding the three α-IdscFv1, 2 and 3 were inserted into the *Sac*I and *Spe*I restriction sites of pComb3His, which contains 6His tags. The recombinant plasmids were transformed into electrocompetent *E. coli* XL-1 Blue. Soluble anti-idiotypic scFv fragments were produced and isolated on a nickel affinity column.

3.2. Screening for correctly folded anti-idiotypic scFv fragments

Analysis of the isolated anti-idiotypic scFv fragments was performed using α-IdscFv1, α-IdscFv2 and α-IdscFv3 as coating antigens in an ELISA. Human IgE was used as a control and was analysed on the same ELISA plate. Fig. 1 shows the recognition of the anti-idiotypic scFv fragments by different antibodies reacting with human IgE. α-IdscFv2, α-IdscFv3 and the control IgE were well recognised by the monoclonal anti-human IgE BSW17 and slightly recognised by the rabbit anti-human Cε4mim serum, whereas the recognition of α-IdscFv1 was only minimal. In contrast, the rabbit anti-α-Id-B43 serum (BSW17-like) only recognised α-IdscFv2 and the control IgE, but not α-IdscFv1 and α-IdscFv3. As the control IgE showed the same pattern of recognition as α-IdscFv2, only α-IdscFv2 was used in the further experiments.

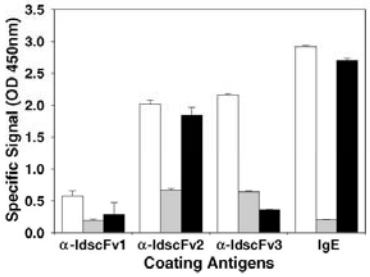

Fig. 1. Detection of the anti-idiotypic scFv fragments by anti-human IgE antibodies. Wells were coated with α-IdscFv1, α-IdscFv2, α-IdscFv3 or with human IgE Savasal at a concentration of 10 μg/ml. Wells were subsequently incubated with either 50 μg/ml of monoclonal mouse anti-human IgE (BSW17, white bars), with a rabbit anti-human Cε4mim serum (diluted 1:1000, grey bars) or a rabbit anti-α-Id-B43 serum (BSW17-like, diluted 1:1000, black bars). Bound antibodies were detected by using either HRP-conjugated sheep anti-mouse IgG (Fc) or HRP-conjugated goat anti-rabbit antibodies. Bars represent mean values (±S.D.) from three individual experiments.

3.3. Transformation of lactic acid bacteria

For development of an oral vaccine using *L. johnsonii* (Lj), the plasmid pMD112 was chosen as the expression vector. This plasmid contains the entire structural gene of the cell wall anchored proteinase (PrtB) from *L. delbrueckii* subsp. *bulgaricus*. DNA encoding α-IdscFv2 was amplified in a PCR using primers containing *Pvu*I and *Nhe*I restriction sites. The final PCR product coding for α-IdscFv2 was inserted at the *Nhe*I and *Pvu*I restriction sites which are located in the active site of PrtB. In addition, an ε4 mimotope (Cε4 mim), an IgE decapeptide [10] and for control purposes a tetanus toxin mimotope (TTmim), a peptide of 11 amino acids derived from tetanus toxin were all cloned in the same way as α-IdscFv2, as described recently [20]. The resulting plasmids pMD112α-IdscFv2, pMD112ε4 and pMD112TT thus code for proteins inserted into the sequence of PrtB that are expressed as fusion proteins on the bacterial cell surface.

Lj was transformed with the recombinant plasmids pMD112α-IdscFv2, pMD112ε4 and pMD112TT (Table 2).

Table 2
Bacterial clones and designations

Clone designation	Bacterial strain	Plasmid used for transformation	Peptide fused to PrtB	Expressed fusion protein[a]
Lj	*Lactobacillus johnsonii*	None	None	None
LjTT	*Lactobacillus johnsonii*	pMD112TT	TTmim	TTmim-PrtB
Ljε4	*Lactobacillus johnsonii*	pMD112ε4	Cε4mim	Cε4mim-PrtB
Ljα-IdscFv2	*Lactobacillus johnsonii*	pMD112α-IdscFv2	α-IdscFv2	α-IdscFv2-PrtB

[a] Expression was monitored by Western blot and ELISA (see Section 3, Fig. 2 and [20]).

DNA was prepared from chloramphenicol resistant transformants and tested by PCR and sequencing analysis for the presence of the recombinant plasmids using primers specific for the different peptides and the wild type proteinase PrtB (data not shown).

3.4. Expression of the recombinant PrtB fusion proteins in Lj

The expression of the α-IdscFv2-PrtB fusion protein in Lj was analysed by SDS–PAGE Western blot and ELISA. Both Ljε4 and LjTT were already shown to express recombinant PrtB fusion proteins [20] and were used as controls in addition to wild type Lj. Fig. 2A demonstrates that α-IdscFv-PrtB fusion protein was substantially less expressed than TTmim-PrB. This in contrast to ε4mim-PrtB which has been shown in a previous study to be expressed in a comparable amount as TTmim-PrtB. However, breakdown products with molecular weights between 90 and 210 kDa were observed in either recombinant LjTT or Ljα-IdscFv2 transformants. On the Western blot, these bands showed reactivity with rabbit-anti PrtB serum (Fig. 2B), indicating that the PrtB protein was expressed by the Lj bacteria and that these bands correspond to full length (MW 190 kDa for LjTT and 212 kDa for Ljα-IdscFv2) and to fragments of PrtB. As α-IdscFv2 is a conformational epitope, the bacterial clones were tested for correctly folded α-IdscFv2-PrtB fusion protein in a catching ELISA. BSW17 was coated onto ELISA plates and was overlaid with either Lj, LjTT, Ljε4 or Ljα-IdscFv2. Unbound bacteria were washed off the wells, and bound bacteria were detected using rabbit anti-PrtB antibodies. As shown in Fig. 2C (third and fourth bar), only Ljε4 and Ljα-IdscFv2 bound to BSW17 coated on the solid phase, indicating that α-IdscFv2 was expressed as a fusion protein with PrtB. Only a marginal reactivity was observed with either wild type Lj or recombinant LjTT.

3.5. Subcutaneous immunisation of mice with Ljα-IdscFv2

Sera of mice immunised subcutaneously with wild type and recombinant *L. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2 (Fig. 3A) or human myeloma IgE ("Savazal") on the solid phase (Fig. 3B). Fig. 3A shows that different dilutions of sera of mice immunised with Ljα-IdscFv2 recognise soluble α-IdscFv2. Weaker reactivities to soluble α-IdscFv2 were

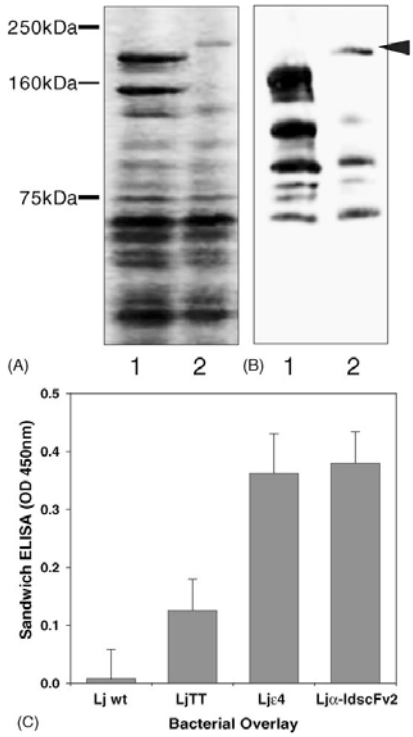

Fig. 2. Recombinant expression of α-IdscFv2-PrtB fusion protein in Lj. (A and B) Whole bacterial cell lysates from overnight cultures of LjTT (lane 1) or Ljα-IdscFv2 (lane 2) were separated by SDS–PAGE 6% and stained with Bio-Safe Coomassie Stain (A) or transferred to nitrocellulose filters and incubated with affinity purified rabbit anti-PrtB antibodies (B). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. The arrow indicates the height of full-length α-IdscFv2-PrtB. (C) Wells were coated with BSW17 (anti-human IgE) at a concentration of 50 μg/ml and overlaid with 5×10^7 wild type Lj (Lj wt), or recombinant Lj bearing either a tetanus-derived mimotope (LjTT), an IgE (Cε4) derived mimotope (Ljε4) or α-IdscFv2 fused to PrtB (Ljα-IdscFv2). Unbound bacteria were washed off and wells were incubated with affinity purified rabbit anti-PrtB antibodies. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. Bars represent mean values (±S.D.) from three individual experiments.

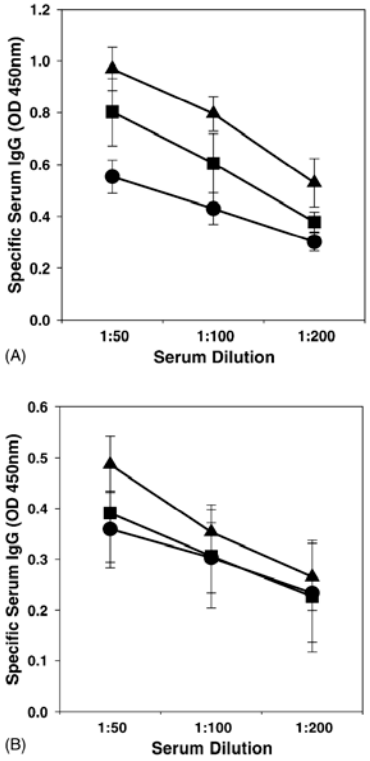

Fig. 3. Binding specificities of serum IgG from mice subcutaneously immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10 μg/ml soluble α-IdscFv2 produced in *Escherichia coli* XL-1 Blue (A) or with human IgE Savasal at a concentration of 10 μg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised subcutaneously with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice. In (A) values obtained with the three dilutions (1:50, 1:100, 1:200) of sera of mice immunized with either Ljα-IdscFv2 ($p < 0.01$) or Ljε4 ($p < 0.01$) are significantly higher than those obtained with LjTT. In (B) only the value obtained with one dilution (1:50) of sera of mice immunised with Ljα-IdscFv2 ($p < 0.01$) is significantly higher than that obtained with LjTT.

also observed with sera of mice immunised either with Ljε4 or with the control strain LjTT. According to the Student's *t*-test the reactivities observed with sera of mice immunised with either Ljα-IdscFv2 or Ljε4 were significantly different from the ones observed with sera of mice immunised with LjTT. These results indicate that a specific response was induced against α-IdscFv2 after immunisation with either Ljα-IdscFv2 or Ljε4. As shown in Fig. 3B only sera of mice immunised with Ljα-IdscFv2 react with human IgE. The reactivity observed with the sera of mice immunised with Ljε4 was not statistically different from that obtained using sera of mice immunised with LjTT. Thus, a specific reaction against IgE was induced only upon immunisation of mice with Ljα-IdscFv2.

3.6. Intranasal immunisation of mice with Ljα-IdscFv2

Sera of mice immunised intranasally with wild type and recombinant *L. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2

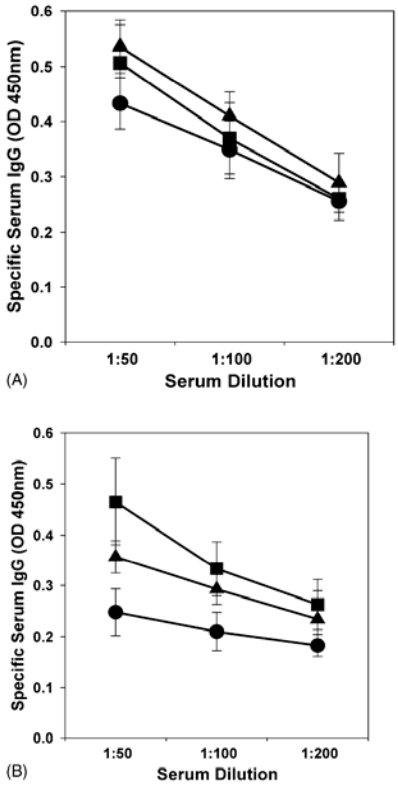

Fig. 4. Binding specificities of serum IgG from mice intranasally immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10 μg/ml soluble α-IdscFv2 produced in *Escherichia coli* XL-1 Blue (A) or with human IgE Savasal at a concentration of 10 μg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised intranasally with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice. In (A) values obtained with two dilutions (1:50 and 1:100) of sera of mice immunized with Ljα-IdscFv2 ($p < 0.01$ for 1:50 and $p < 0.05$ for 1:100) and with one dilution (1:50) of sera of mice immunized with Ljε4 ($p < 0.05$) are significantly higher than those obtained with LjTT. In (B) values obtained with the three dilutions (1:50, 1:100 and 1:200) of sera immunized with either Ljα-IdscFv2 ($p < 0.01$ for 1:50 and 1:100 and $p < 0.05$ for 1:200) or Ljε4 ($p < 0.01$ for dilutions 1:50, 1:100 and 1:200) are significantly higher than those obtained with LjTT.

(Fig. 4A) or human IgE Savazal on the solid phase (Fig. 4B). As shown in Fig. 4A soluble α-IdscFv2 reacts with sera of mice immunised intranasally with Ljα-IdscFv2 but also to a lesser extent to sera of mice immunised with either Ljε4 or the control strain LjTT. Based on the Student's *t*-test, only sera of mice immunised with either Ljα-IdscFv2 or Ljε4 showed a specific reaction to soluble α-IdscFv2. Ljα-IdscFv2 induced a stronger specific antibody response to α-IdscFv2 than Ljε4 as shown by serial dilution of the serum. In contrast, Fig. 4B shows that human IgE was better recognised by sera of mice immunised with Ljε4 than with Ljα-IdscFv2 indicating that intranasally Ljε4 induced a stronger anti-IgE response than Ljα-IdscFv2. Sera of mice immunised with the control strain LjTT also reacted weakly with human IgE but according to a Student's *t*-test reactivities to IgE were specific for sera immunised with either Ljα-IdscFv2 or Ljε4.

4. Discussion

We have previously described the use of *L. johnsonii* as a mucosal vaccine delivery vehicle. The cell surface proteinase PrtB isolated from *L. delbrueckii* subsp. *bulgaricus* was used as a model antigen as our previous studies had shown that PrtB was immunogenic after intragastric delivery of recombinant Lj [20]. In this study, Lj was engineered to express PrtB linked to either an anti-idiotypic single chain fragment variable (α-IdscFv2), or a Cε4 mimotope (Cε4 mim). Both the Cε4mim and the binding site of α-IdscFv2 were recognised by the protective monoclonal anti-IgE antibody, BSW17 and were able to induce a systemic anti-human IgE antibody response after subcutaneous and intranasal immunisation.

The original antibody providing the starting point for the α-IdscFv2 was our previously isolated anti-idiotypic Fab (Fab α-Id-B43) which mimics the epitope on human IgE recognised by BSW17 [13]. Additionally, parenteral immunisation of rabbits using Fab α-Id-B43 was shown to induce high-affinity antibodies with the same characteristics as BSW17. In order to target an antibody molecule onto the surface of Lj, the binding site of Fab α-Id-B43 was constructed as a single chain fragment variable (α-IdscFv), because Gram-positive bacteria lack an oxidative site, where heavy and light chain of an Fab could assemble. However, there are many examples in the literature describing scFv's which were unstable or showed suboptimal binding properties [27–29]. In addition, it is also known, that the nature of the linker may have an influence on the correct folding of a scFv [30,31]. Thus, three different linkers were tested for optimal assembly of the variable heavy and light chain regions of Fab α-Id-B43. The linker of α-IdscFv2, bearing a β-turn motif on both ends [24], proved to be the most appropriate for cloning the anti-idiotypic scFv into PrtB as it was the only scFv recognised by both BSW17 and the rabbit anti- Fab α-Id-B43 (BSW17-like) antibodies. Interestingly, IgE Savazal only weakly reacted with the rabbit anti-human Cε4mim serum. This might be explained by the fact that anti-Cε4 serum was made against a linear peptide of nine amino acids which carries a different conformational structure compared to the to the full-length native IgE molecule. This conformational difference might prevent the translation of anti-peptide reactivity into specific recognition of native IgE.

This is the first report of engineering gram-positive bacteria to express an anti-idiotypic scFv (α-IdscFv2) that can be used for active immunisation. Other recent reports have also described the use of Gram-positive bacteria as carriers for the expression of scFv proteins. Gunneriusson et al. [33] have reported the use of *Staphylococci* to surface display scFv antibodies in order to develop a whole-cell diagnostic device and for using as an alternative to filamentous phages in scFv libraries [32]. More recently, *Lactobacillus zeae* producing scFv antibodies, recognising the streptococcal antigen I/II were used to deliver passive immunity to *Streptococcus mutans* in a rat model of caries development [33]. However, in contrast to these studies we were interested to use the recombinant Lj for active immunisation.

Both recombinant bacterial clones (Ljα-IdscFv2 and Ljε4) induced after subcutaneous or intranasal immunisation a specific anti-IgE response. However, the magnitude of the anti-IgE response was strongly influenced by the route of immunisation and depended on the recombinant bacterial clone used. Thus, both recombinant bacterial clones (Ljα-IdscFv2 and Ljε4) were first tried by feeding mice intragastrically. In that case, an antibody response was induced against Lj but only low antibody titers against scFv and ε4 suggesting that both proteins fragments were weakly immunogenic via the oral route. In this study, upon subcutaneous immunisation, the α-IdscFv2 expressing clone (Ljα-IdscFv2) induced a higher anti-IgE immune response than the Cε4mim expressing clone (Ljε4) indicating that the α-IdscFv2 is more immunogenic than the small Cε4 mim. However, after intranasal immunisation the opposite was the case. This is consistent with the data obtained by oral delivery of phage particles expressing the same antigens [34]. In this study, Cε4mim expressing M13 bacteriophages also induced a higher anti-IgE titer than Fab α-Id-B43 expressing phages. These results might be explained by the more complex conformational structure of the anti-idiotypic antibody fragments in comparison to the Cε4 mim. Correctly folded anti-idiotypic antibodies are therefore more dependent on the route of immunisation where different conditions prevail. The nasal route of immunisation is thought to be especially restrictive for antibody structures, as the nasal cavity is known to harbour proteases specific for antibody structures [35]. Such proteases, produced by resident aerobic bacteria [36], might lead to the digestion of α-IdscFv2. Thus, Poethke et al. [37] have shown the presence in various human secretions such as the nasal mucus of a proteolytic enzyme which reacts with the Fab portion of immunoglobulin G and inhibits the binding of IgG to its antigen.

Our results show the induction of an anti-IgE response after intranasal immunisation with recombinant Lj, expressing

IgE epitopes. The question remains however, whether these antibodies are protective and result in reduction of allergic symptoms in atopic patients. Immunisation of monkeys using soluble Cε4mim or Fab α-Id-B43 have shown that both IgE epitopes behaved as surrogate antigens and induced an anti-IgE response able to inhibit an in vivo passive cutaneous anaphylaxis test [38]. Thus, it can also be expected that recombinant Lj bearing the same antigens on their surface would induce a qualitatively similar anti-IgE immune response preventing binding of IgE to the FcεRI, and consequently inhibit the sensitisation of the effector cells implicated in allergic disease.

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