Dissertation

DISSECTION OF THE IgE INTERACTOME ON A MOLECULAR LEVEL

zur Erlangung des Doktorgrades der Naturwissenschaften

der Fakultät für Mathematik, Informatik und Naturwissenschaften
der Universität Hamburg

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Hamburg 2011

Der experimentelle Teil der vorliegenden Arbeit wurde von Dezember 2007 bis September 2011 am Institut für Biochemie und Lebensmittelchemie der Universität Hamburg, Fachbereich Chemie, Abteilung für Biochemie und Molekularbiologie, im Arbeitskreis von Herrn Prof. Dr. Reinhard Bredehorst durchgeführt.

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Datum der Disputation:	22.12.2011

ABSTRACT

In the developed western world more than 25 % of the population is adversely affected by allergic diseases. These include allergic reactions that are caused by aeroallergens as well as injective allergens. Aeroallergens include grass and tree pollen, house dust mite fecal matter, and animal dander, and induce conjunctivitis, allergic rhinitis, and asthma. In contrast, anaphylactic reactions constitute the most serious reaction e.g. to hymenoptera venom. Both types of allergens have a detrimental impact on the quality of life, and even a life-threatening outcome.

In general, allergic reactions are based on the cross-linking of mast cell and basophil bound, allergen specific IgE antibodies. Apart from hypersensitivities IgE has been suggested as a natural defense mechanism involved in anti-parasitic and even anti-tumor immune responses. Nevertheless, the molecular understanding of antibody/antigen binding as well as effector mechanisms remains scarce due to the lack of human monoclonal IgE antibodies.

The aim of this work was the generation of human monoclonal IgE antibodies and the detailed analysis of the interaction with their corresponding epitopes for fostering the molecular understanding of binding and clinical outcome.

Thus, recombinant human/mouse chimeric as well as fully human i.a. IgE monoclonal antibodies with specificity for a variety of antigens were generated. These cover the major timothy grass allergen Phl p 5, the major birch pollen allergen Bet v 1, and the tumor associated EGFR on a protein level as well as the glycotopes α1,3-core fucose and alpha-Gal.

The monoclonal antibodies were employed in diverse artificial and authentic approaches. The immunoreactivity of the obtained antibodies and their corresponding epitopes was analyzed by biochemical, immunological, and biophysical methods.

By such analyses, the epitope of Phl p 5 was successfully assigned to a looped stretch that is exclusively present in Phl p 5a, and represents the first authentic human IgE epitope on the major timothy grass allergen Phl p 5. Analogously, by an engineered human IgE anti-Bet v 1 antibody selected from an immune repertoire library, its

corresponding epitope was identified as the terminal helix. This epitope seems to be conserved in tree pollen PR-10 homologues of *Fagales*, but less in PR-10 homologues from food.

These two identified epitopes constitute the first IgE epitopes of authentic human IgE antibodies, on major pollen allergens.

In contrast to proteinic epitopes, CCDs provide epitopes of unique characteristics. Generally, CCDs comprise identical glycotopes of limited size that occur on a variety of proteins. Hence, defined CCD phenotypes might provide further insights into hymenoptera venom specific IgE reactivities and contribute to an improved understanding of molecular recognition properties. In order to assess the relevance of cross-reactive carbohydrates in immunoreactivity, the polyclonal detection of α1,3-core fucose as an IgE epitope was proven to be truly monospecific and solely dependent on α1,3-core fucose, even though present on diverse allergens of different origins. Therefore, Api m 1 with a single α1,3-core fucose as CCD was generated as recombinant non-fucosylated protein. Its reactivity was compared with native Api m 1 by the use of polyclonal human patient serum. This approach was suitable for the dissection of specific IgE to CCDs and the allergenic protein itself.

Additionally, the xenobiotic IgE epitope alpha-Gal was assessed. Therefore, a human/mouse chimeric anti-alpha-Gal IgE antibody was generated. Supplementary, alpha-Gal specific antibodies were affinity purified from polyclonal human serum. The binding characteristics of the monoclonal antibody and the obtained polyclonal antibodies were assessed by STD NMR. The epitope on alpha-Gal could be primarily assigned to the two galactose units, and to a weaker extent to the residues downstream of the Gal-Gal. The obtained results represent the first example of an epitope, that simultaneously act as an authentic IgG epitope as well as an IgE epitope.

In order to address the potential of recombinant IgE antibodies, the effector mechanisms of EGFR specific human IgE and IgG1 (225-IgG1 and 225-IgE) antibodies were compared. In contrast to IgG, IgE may recruit alternate immune competent cells to inaccessible tumor sites. Comparative examination revealed that the EGFR specific IgE has superior tumoricidal activity compared to IgG1, and therefore, may provide novel concepts in tumor therapy.

In summary, the results of this work demonstrate the feasibility of recombinant technologies for the elucidation of antibody/antigen interaction, in particular for IgE epitopes and IgE effector mechanisms. Furthermore, detailed insights into epitope recognition may bridge the gap between IgE and IgG antibodies. These findings may help to understand the still enigmatic biology of IgE and provide insights into IgE related pathologies which are associated with IgE.

ABSTRACT

In den Staaten der industrialisierten westlichen Welt sind mehr als 25 % der Bevölkerung von allergischen Erkrankungen betroffen. Diese schließen allergische Reaktionen ein, welche sowohl durch Inhalationsallergene als auch durch Injektionsallergene verursacht werden. Zu den Inhalationsallergenen werden Gräser und Baumpollen, Kot von Hausstaubmilben sowie Tierhaar und Hautschuppen gezählt, welche bei Atopikern Konjunktivitis, Heuschnupfen und Asthma auslösen können. Im Gegensatz dazu stellen anaphylaktische Reaktionen z.B. auf Insektengifte, sehr viel schwerwiegendere Reaktionen dar. Inhalations- und Injektionsallergene sind für Hypersensitivitätsreaktionen vom Typ I (Sofort-Typ) verantwortlich, haben nachteilige Auswirkungen auf die Lebensqualität und können lebensbedrohliche Ausmaße annehmen.

Generell basieren allergische Reaktionen auf der Quervernetzung von auf Mastzellen und Basophilen gebundenen spezifischen IgE Antikörpern. Abgesehen von ihrer Rolle in allergischen Reaktionen wird angenommen, dass IgE Antikörper einen natürlichen Abwehrmechanismus gegen Parasiten und Tumore darstellen. Dennoch sind Erkenntnisse zu Antikörper/Antigen-Bindungen und deren Effektormechanismen auf molekularer Ebene nach wie vor rar, was in der mangelnden Verfügbarkeit humaner monoklonaler IgE Antikörper begründet ist.

Das Ziel dieser Arbeit war die Generierung von humanen monoklonalen IgE Antikörpern sowie die detaillierte Analyse ihrer Interaktionen mit ihren entsprechenden antigenen Determinanten, um Erkenntnisse über Bindungseigenschaften und die klinischen Folgen auf molekularer Ebene zu gewinnen.

Zu diesem Zweck wurden sowohl rekombinante human/murine chimäre als auch vollständig humane Antikörper unter anderem als IgE Isotyp generiert, welche Spezifitäten für eine Vielzahl von Antigenen aufweisen. Diese umfassen stellvertretend für Proteinepitope das Hauptallergen Phl p 5 aus dem Wiesen-Lieschgras, das Hauptallergen Bet v 1 aus der Birke und den Tumor-assoziierten EGF-Rezeptor sowie für Glycotope die α1,3-core Fukose and das alpha-Gal. Die monoklonalen Antikörper

wurden unter Verwendung diverser artifizieller und authentischer Ansätze angewandt. Die Antikörper/Antigen-Immunoreaktivitäten wurden mittels biochemischer, immunologischer und biophysikalischer Methoden analysiert.

Durch derartige Analysen konnte das Epitop auf Phl p 5 auf einen Loop eingegrenzt werden, welcher ausschließlich auf Phl p 5a zu finden ist, und das erste authentische humane IgE Epitop auf Phl p 5 darstellt. In Analogie wurde für einen humanen anti-Bet v 1 IgE Antikörper, welcher aus einer immunogenen Repertoire-Bibliothek selektiert wurde, das entsprechende Epitop als die terminale Helix von Bet v 1 identifiziert. Dieses Epitop scheint in PR-10 Homologen aus Baumpollen der Ordnung *Fagales* konserviert zu sein, jedoch weniger in PR-10 Homologen aus Lebensmitteln.

Diese zwei identifizierten Epitope stellen die ersten IgE Epitope von authentischen humanen IgE Antikörpern auf Pollenhauptallergenen dar.

Im Gegensatz zu Proteinepitopen besitzen CCDs Epitope mit einzigartigen Eigenschaften. Generell stellen CCDs identische Glycotope mit einer limitierten Größe dar, welche auf einer Vielzahl von Proteinen vorkommen. Daher könnten definierte CCD Phänotypen weiterführende Einblicke in Insektengift-spezifische IgE Reaktivitäten geben und zu einem besseren Verständnis der molekularen Erkennungsmechanismen beitragen. Die Relevanz der Immunoreaktivität kreuzreaktiver Carbohydrat-Determinanten als IgE Epitop wurde mittels polyklonaler Detektion von α1,3-core Fukose untersucht. Diese beruht tatsächlich auf einer monospezifischen und ausschließlich α1,3-core Fukose abhängigen Reaktivität, auch wenn diese auf Proteinen unterschiedlichen Ursprungs vorkommt. Daher wurde Api m 1, welches in nativer Form nur ein singuläres N-Glycan mit α1,3-core Fukose als CCD trägt, als nicht fucosyliertes Protein rekombinant hergestellt. Dessen Reaktivität wurde unter Verwendung von polyklonalem humanem Patientenserum mit der von nativem Api m 1 verglichen. Diese Anwendung erwies sich als geeignet für differenzierte Analysen CCD- und Protein-spezifischer IgE.

Zusätzlich wurde das xenobiotische alpha-Gal Epitop untersucht. Hierfür wurde ein human/mouse chimärer anti-alpha-Gal IgE Antikörper generiert. Weiterhin wurden alpha-Gal spezifische Antikörper aus polyklonalem humanem Serum affinitätschromatographisch gereinigt. Die Bindungscharakteristika des monoklonalen Antikörpers sowie der erhaltenen polyklonalen Antikörper wurden mittels STD NMR

untersucht. Das Epitop auf alpha-Gal konnte primär den zwei Galaktoseeinheiten und zu einem geringeren Maß den nachfolgenden Resten zugewiesen werden. Die hier erhaltenen Ergebnisse repräsentieren das erste Beispiel eines Carbohydrat-Epitops, welches gleichzeitig ein authentisches IgG als auch IgE Epitop darstellt.

Um das volle Potenzial der rekombinanten IgE Antikörper evaluieren zu können wurden weiterhin die Effektormechanismen von EGFR-spezifischen humanen IgE und IgG1 (225-IgG1 und 225-IgE) Antikörpern miteinander verglichen. Im Gegensatz zu IgG könnten IgE andere Populationen immunkompetenter Zellen zu unzugänglichen Tumorlokalitäten rekrutieren. Vergleichende Versuche machten deutlich, dass EGFR-spezifische IgE, gegenüber EGFR-spezifischen IgG1, überlegene destruktive Eigenschaften gegen Tumore besitzt und damit neuartige Konzepte für die Tumortherapie denkbar macht.

Zusammenfassend demonstrieren die Ergebnisse dieser Arbeit die Anwendbarkeit rekombinanter Technologien für die Aufklärung von Antikörper/Antigen Interaktionen, vor allem für IgE Epitope und IgE Effektormechanismen. Weiterhin können erhaltene Einblicke in die Epitoperkennung die Kluft zwischen IgE und IgG Antikörpern schließen. Die hier gewonnenen Erkenntnisse könnten zum Verständnis der nach wie vor enigmatischen Biologie des IgE beitragen und tiefere Einblicke in Krankheitsbilder geben, deren Pathologie IgE assoziiert ist.

INDEX

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is a complex network of biological components and comprehensive processes which facilitate protection of individual organisms against extrinsic and intrinsic pathogens. To fulfill this challenge accurately it is subdivided into innate and adaptive immune system each comprising beneficial and unique properties.

Even though the innate immune system is often designated as non-specific due to the participation of diverse immune cells expressing pattern recognition receptors (PRRs) which enable the organism to fight a broad variety of pathogens like microorganisms, self antigens or allergens [1].

In contrast, the adaptive immune system, also termed specific immune system, is based on T and B lymphocytes [2] expressing a diverse repertoire of antigen specific receptors. Upon activation which not solely depends on direct recognition of antigens but additionally on signals resulting from the innate immune system [2] antigen specific memory responses are constituted [3].

1.1.1 THE INNATE IMMUNE SYSTEM

The innate immune system constitutes the first-strike of host defense against infection caused by pathogens such as viruses, non-commensal bacteria, parasites, self antigens and allergens [4], and has been perceived as an evolutionary older defense mechanisms in contrast to the younger acquired immune system [5].

Despite the biochemical cytolytic cascade of the complement system a variety of cell types are responsible for recognition of and reply to pathogens however, contemporaneously able to discriminate self from non-self. The main cell types of the innate immune system are mast cells, dendritic cells, macrophages, natural killer cells, and neutrophils. These cells interact in innate immunity through mechanisms such as phagocytosis and the release of inflammatory mediators like cytokines. This defense mechanisms are activated by molecular structures which are exclusively present on

microorganisms. Stimuli can be mannose, teichoic acids, and lipopolysaccharides (LPS), that represent the pathogen-associated molecular patterns (PAMPs) that interact with PRRs to which the Toll-like receptors (TLRs) belong.

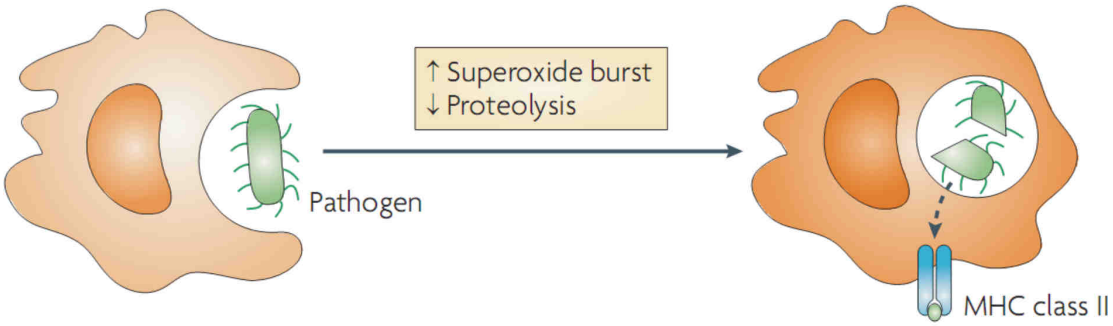


Fig. 1.1: Activation of macrophages due to phagocytosis of a pathogen [6]
Toll-like receptors expressed by macrophages recognize molecules of microorganisms which are then internalized. Recruitment to the phagosome is followed by the degradation of the pathogen, and subsequently presentation of pathogen derived peptides by MHC II.

After recognition, pathogens are uptaken by phagocytic effector cells such as macrophages, and internalized into phagosomal vesicles that fuse to phagosomes. In the absence of inflammatory stimuli the primary role of macrophages is the removement of cellular debris, for instance from apoptotic cells, by proteolysis. In contrast, macrophages that are activated by TLR-agonists kill microbes based on the production of nitrogen intermediates as well as reactive oxygen species [6]. Thereafter, peptides derived from the degraded pathogens are presented by MHC II complexes, now involving the adaptive immune system (Fig. 1.1).

1.1.2 THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system is a complex defense system which is dependent on lymphocytes expressing different antigen receptors, whose diversity is achieved by somatic recombination to ensure an appropiate defense action [7]. This somatic diversication is one of the benefits of the adaptive immunity [7], in addition to the memory function which enables a rapid response of the organism to previously encountered pathogens.

In contrast to the cells of the innate immune system, cells belonging to the acquired immune system derive from lymphoid progenitors that include B cells as part of the humoral immune response, and diverse T cells as part of the cell-mediated immune response, expressing B cell receptors (BCRs) and T cell receptors (TCRs), respectively.

1.1.2.1 B CELLS

B cells are $CD19^+$ lymphocytes which are engendered in a sophisticated microinvironment in the bone marrow [8] and generated throughout life [9]. Activation of mature B cells owing to the specific recognition of an antigen by the BCR expressed as membrane bound antibodies leads to the secretion of corresponding soluble immunoglobulins. The previous rearrangement or more precisely the somatic occasion of V(D)J (Variable, Diverse, and Joining) recombination of immunoglobulin genes occurs during B cell differentiation, and antigen specifity is improved by somatic hypermutation events.

1.1.2.2 T CELLS

Both, T and B cells are lymphocytes but in contrast to B cells T cells are, when mature, either $CD4^+$ or $CD8^+$, and develop in the bone marrow as immature ancestors which migrate to the thymus where they undergo processes of positive and negative selection. Activation of naïve T cells by presentation of antigen derived peptides through major histocompatibility complexes (MHCs) expressed on antigen presenting cells (APCs) by V(D)J recombination derived TCRs is an example of the interplay of the innate and the adaptive immune system finally leading to cytokine induced differentiation of naïve T cells. The mature T cell subsets are defined by the expression of the suitable co-receptor CD4 or CD8. The MHC I molecules are bound by CD8, whereas the MHC II molecules are bound by CD4 [10].

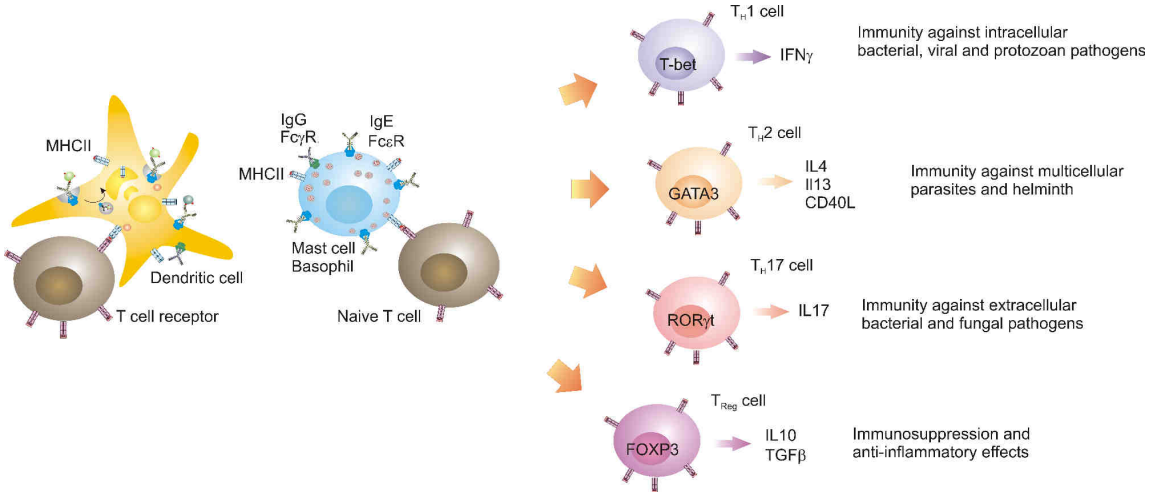


Fig. 1.2: Differentiation of T_H cells into their subsets
Depicted is the differentiation of naïve $CD4^+$ T cell and the resulting subsets of activated T helper cells all of them specialized for a destined defense mechanism.

Naïve T cells possess the potential to differentiate into the most intensively studied T helper cell T_H1 and T_H2 subsets (Fig. 1.2) which orchestrate critical stages of the adaptive immune system [11], as well as into recently described T_H17 and T_{reg} cells (Fig. 1.2) which are also defined by their particular cytokine pattern.

1.1.2.3 T_H1 IMMUNITY

The recognition of pathogens like bacteria or viruses by cells of the innate immune system such as dendritic cells (DCs) or macrophages in the context of IL-12 leads to the polarization of $CD4^+$ naïve T cells into T_H1 secreting interferon-γ (IFN-γ), lymphotoxin (LT), and interleukin-2 (IL-2) then capable of further activating macrophages, natural killer (NK) cells and $CD8^+$ T cells [12] eliminating the causative agent. This differentiation program of the cellular immune response is dependent on the transcription factor STAT4 (signal transducer and activator of transcription 4), and the transcription factor T-bet (T-box expressed in T cells) [13], the master regulator of the T_H1 lineage.

Several inflammatory and autoimmune diseases like multiple sclerosis [14], type I diabetes [15] and rheumatoid arthritis [16] are considered to be associated with a T_H1 predominance.

1.1.2.4 T_H2 IMMUNITY

In contrast to the recognition of micropathogens in the case of T_H1 immunity, the identification of multicellular macropathogens such as helminths which mostly do not replicate in the host but present altered antigens throughout different stages of development to the host immune system, demands a different recognition pattern. This may explain why such pathogens induce a type of immune response distinct from that of the T_H1 immunity [17]. Naïve $CD4^+$ T cells that are activated by cells of the innate immune system, differentiate into T_H2 cells which secrete IL-4, IL-5 and IL-13, then activating B cells to induce immunoglobulin class switching [11].

Additionally, T_H2 cytokines are able to induce and achieve key pathophysiology features of asthma and IgE production in an allergic context [18].

1.1.2.5 T_H17

Among $CD4^+$ T helper cells, an IL-17 (IL-17A) producing subset, characterized by unique transcription factors [19] exists, and develops via cytokines which are distinct

from the more common T_H1 and T_H2 lineages [20]. These T_H17 cells are known to promote inflammation in the context of autoimmune pathologies [19, 21, 22] like chronic rheumatoid arthritis [23] or encephalomyelitis [24] and play a role in anti-tumor as well as pro-tumor events [19].

1.1.2.6 T_{REG}

Apart from the effector T_H cells, a regulatory subset of $CD4^+$ T cells can be activated to suppress responses of the adaptive immune system and therefore, preventing autoimmunity [20] and inflammation incidents [25]. These regualtory T cells (T_{reg}) constitute an essential element for the maintenance of self tolerance and immune homeostasis [26] and typically express the transcription factor forkhead box P3 (FOXP3). They are able to suppress effector cells such as APC, $CD4^+$, $CD8^+$, natural killer (NK), and B cells to regulate and control immune responses. However, T_{reg} cells are also known to suppress anti-tumor responses and therefore, promote tumor progression [26].

1.2 IMMUNOGLOBULINS

Immunoglobulins (Fig. 1.3) belong to the immunoglobulin superfamily and in this context are known as antibodies or B cell receptors. Monomeric immunoglobulins consist of two identical heavy (H) and light (L) chains which are covalently linked by disulfide bonds and provide a bivalent binding pattern [7]. The paratope that shapes the antigen binding site is characterized by the complementarity determining regions (CDRs) from which the highly diverse CDR3 of the heavy chain variable (V_H) domain is thought to play a key role in the formation of the binding site [27, 28]. Additionaly, the isotype of an immunoglobulin is defined by its H chain from which five different exist in humans [7].

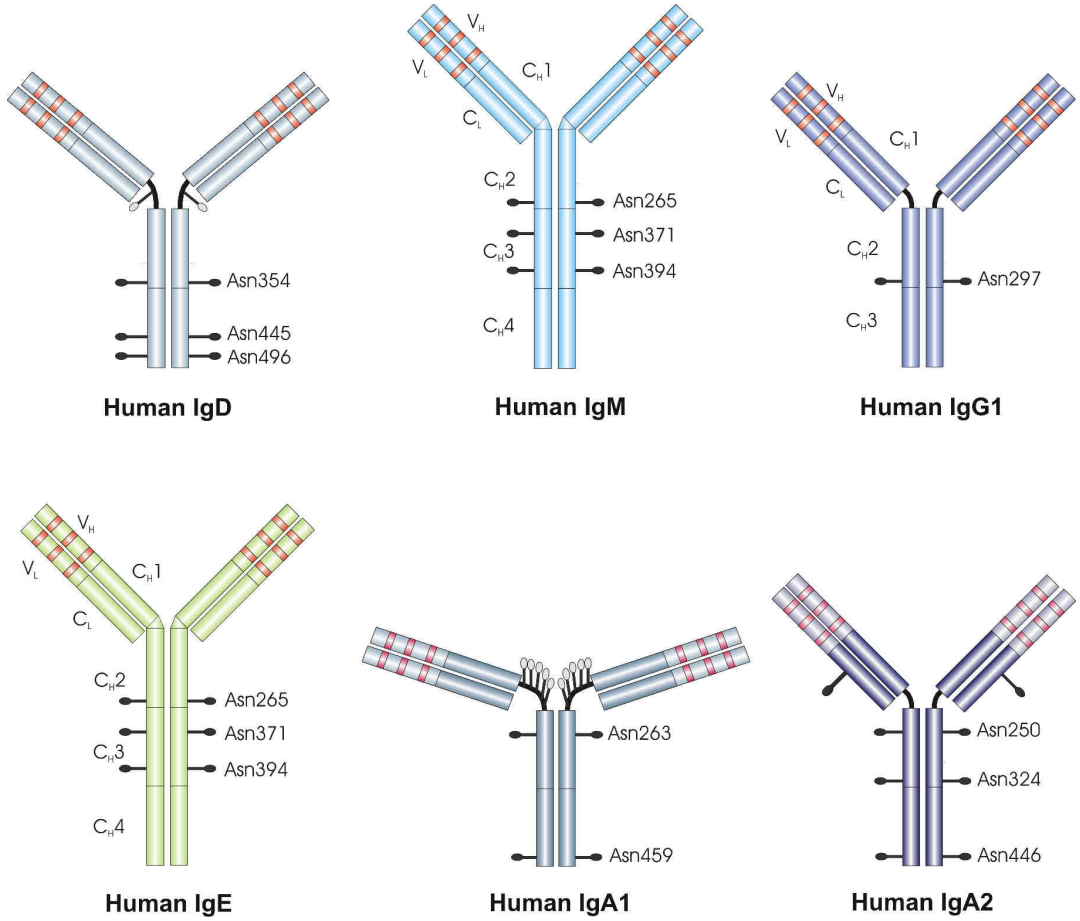


Fig. 1.3: Human immunoglobulins
Depicted is the subset of human Immunoglobulins. Shown are the isotypes IgD, IgM, IgG1 (representative for all IgG subclasses, since IgG3 differs only in the length of its hinge region from the others), IgE, IgA1 (highly O-glycosylated in its hinge region), and IgA2.

1.2.1 IGG

The immunoglobulin isotype G (IgG) constitutes about 75 % of immunoglobulins in human serum and is primarily responsible for the recognition, neutralization, and elimination of pathogenic antigens. It mediates pro- and anti-inflammatory activities through the interaction of its Fc (fragment, crystallizable) moiety with distinct Fcγ receptors (FcγR) [29]. There are four IgG subclasses, namely IgG1, IgG2, IgG3, and IgG4, which exhibit a sequence homology of 95 %, and, show unique effector functions. Despite the different γ-chains the most conspicuous difference appears in the hinge region of the heavy chain constant region 1 (C_H1) that contains different numbers of disulfide bonds between the two heavy chains.

Amongst all IgG subclasses the most abundant is IgG1 (Fig. 1.3) with about 60 %. It demonstrates robust Fc-mediated effector functions [30], particularly its capacity to induce strong effector functions in humans, such as antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) [31]. IgG1 also predominates in anti-viral immune responses in humans [32].

The natural role of IgG2 which accounts for about 25 % of all IgG is typically the response toward carbohydrate antigens such as repetitive patterns on bacterial surfaces and its binding may enhance protective properties [33]. Due to reduced affinity binding to human FcγRs this subtype exhibits reduced effector functions and is known to form covalently joined dimers [33].

About 10 % of all IgG in humans is represented by IgG3 which differs from the other subclasses by its unique hinge region that is much more extended, and augments the intramolecular distance between the Fab (fragment, antigen binding) and the Fc moiety. Additionally, IgG3 is susceptible for cleavage by proteolytic enzymes like pepsin or trypsin. Within the IgG subclasses, IgG3 is the most polymorphic and appears first in the course of infection most notably in conjunction with anti-viral responses, and additionally, is the most effective complement activator [34].

IgG4 represents 4 % of all IgG and is generated as a response upon persistent [33] or repeated long term antigenic stimulation, and specific IgG4 levels may arise in allergic responses [32]. Due to the structure of its Fc part as well as the sterical hindrance of the complement binding site by the Fab moiety IgG4 is inable to activate complement.

Interestingly, IgG4 antibodies are able to exchange heterodimers of half-molecules [33, 35], and hence, evolved functional monovalency [33]. This may explain inhibitory effects of autoantibodies of other subclasses by steric blockade concurrent without generating harm to the organism [33] and affects any therapeutic strategies based on IgG4 [36].

1.2.2 IgM

The immunoglobulin isotype IgM (Fig. 1.3) is the most ancient isotype, that, as transmembrane type, defines the B cell lineage [7]. Onto stimulation of a B cell by an antigen and the contribution of engaged T cells, soluble pentameric IgM is secreted into the plasma, hence, the first antibody produced during and due to an immune response

[37]. Compared with other isotypes IgM provides low affinities [32] but compensates this seemingly drawback with a high valency that confers a remarkable advantage in mediating agglutination of invading organisms [37].

1.2.3 IGD

Together with IgM, IgD (Fig. 1.3) is expressed in association with early immune responses by B cells as BCR but the reason why dual expression of both is required remains enigmatic. Soluble IgD is detectable in low concentrations in human serum, and upon maturation due to the encountering of pathogens, B cells transcriptionally downregulate membrane bound IgD [38].

1.2.4 IGA

IgA is the major isotype in secretions, particularly in the mucosa of the respiratory and the intestinal tract it protects epithelial cells and therefore provides a first line defense against pathogens [32]. There are two subtypes of IgA that exist in different concentrations in human serum. IgA1 (Fig. 1.3) makes up about 90 % of IgA in human serum, and IgA2 (Fig. 1.3) is mostly found in a secreted form in mucosa associated tissues. IgA monomers bind to their specific FcαR which is expressed by immune competent cells like monocytes and macrophages, and additionally, are covalently polymerized by disulfide bonds formed by a J (joining) chain to dimers or unusual trimers.

1.2.5 IGE

The concentration of IgE (Fig. 1.3) in human serum is the lowest of all immunoglobulin isotypes but the total IgE level in the context of allergy is also influenced by the immune status and environmental factors. Increased IgE levels are also seen during parasitic infections, hematologic malignancies or primary immunodeficiency diseases [39]. Circulating IgE bind to FcεR on mast cells and basophils, and the engagement by multivalent antigens leads to effector cell activation [32] (Fig. 1.4).

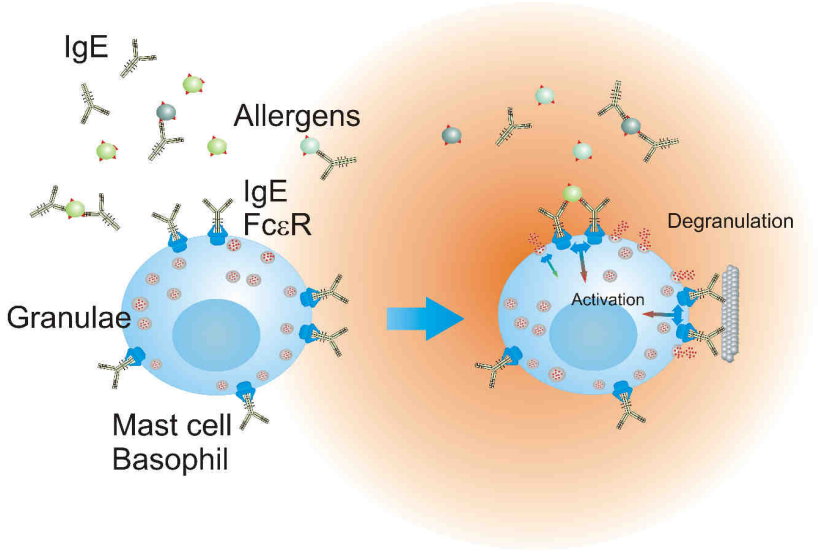


Fig. 1.4: Mast cell and basophil activation
In IgE-associated immune responses to allergens or parasites (top right), the activation of mast cells via crosslinking of IgE bound to high-affinity receptors for IgE (FcεRI) on the cell surface by bi- or multivalent antigens results in rapid exocytosis of the cytoplasmic granules (degranulation), and the production of lipid mediators (such as leukotrienes and prostaglandins), and the more sustained secretion of many cytokines, chemokines and growth factors [40].

A notable benefit of IgE is its capability to enter tissues after recruitment of FcεR expressing immune cells, a fact that constitutes an inherent advantage over all other isotypes, particularly IgG in antibody mediated anti-tumor therapies.

1.3 ALLERGY

Some individuals react in a disproportional manner with environmental substances that are usually harmless [41]. About 25 % of the population in the developed world is affected by atopic disorders such as eczema, hay fever, asthma, and, even worse, by live-threatening, anaphylaxis [41]. These hypersensitivity reactions upon a persistent or repetitive exposure to allergens results in chronic allergic inflammation that in turn causes long term modifications in the arrangement of the affected organs and detrimental deviations of their functions [41].

Allergic immune responses show the same characteristics like infections caused by macropathogens like helminths. In both cases T_H2 cells and antigen-specific IgE are involved [41]. In the case of allergy, a partial downregulation of immunosuppressive and anti-inflammatory effects by Il-10 secreting Treg cells which usually occurs while infection to overcome tissue damage is proposed [41]. Early childhood infections with

micropathogens such as bacteria and viruses provide a T_H1 stimulus for the T_H2 skewed immune system of humans because children are born with a T_H2 biased immune system that normally diminishes during the first two years of life in non allergic individuals [42]. The hygiene hypothesis claimes that such infections usually promote an immune response with bias towards T_H1 cells rather than T_H2 cells, while reduced exposure to infections or increased exposure to otherwise harmless environmental allergens leads to the development of a T_H2 type response in certain predisposed individuals [41].

The different immunopathologic mechanisms of allergic reactions or hypersensitivities, respectively, were classified in the early 60s into four types (Tab. 1.1).

Tab. 1.1: Classification of hypersensitivity reactions

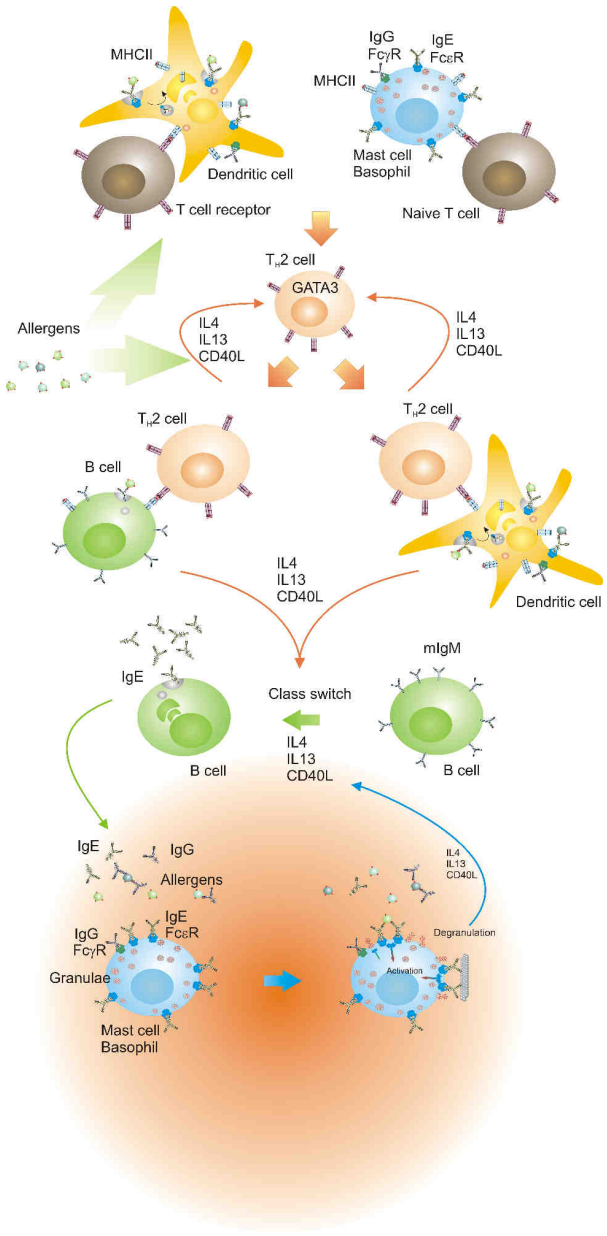
Type	Alternative Name	Associated Diseases	Mediators
I	Immediate Hypersensitivity	Atopy Asthma Anaphylaxis	IgE
II	Antibody-mediated Hypersensitivity	Autoimmune Hemolytic Anemia Erythroblastosis fetalis	IgG IgM Complement
III	Immune Complex-mediated Hypersensitivity	Serum Sickness Lupus Nephritis Arthus Reaction	IgG Complement
IV	Delayed Hypersensitivity	Tuberculosis Contact Dermatitis Transplant Rejektion	T cells Macrophages

1.3.1 TYPE I HYPERSENSITIVITY

Type I hypersensitivity (Fig. 1.5) is also known as immediate or anaphylactic hypersensitivity which is characterized by the presence of IgE and triggered by external antigens [43]. The primary cellular components of this type of reaction are mast cells and basophils, and individuals generate T_H2 based responses with an increased secretion of IL-4 and IL-13 which in turn favor class switching to IgE.

The immediate hypersensitivity is subdivided into an early and a late phase both are classified by the participation of specific inflammatory cells. The early phase is characterized by the presence of activated mast cells and basophils whereas a

pronounced influx of $CD4^+$ T_H2 cells and eosinophils is observed in the late phase response [44].



Initially, a sensitization phase marks the beginning of an allergic inflammatory response where allergen exposure results in IgE formation as well as induction of the humoral response [45] by APCs, in particular dendritic cells (DCs) [46], that take up allergens followed by degradation and presentation of the resulting peptides via MHC to naïve T cells.

Fig. 1.5: Hypersensitivity type I
Mechanism of allergic inflammation. APCs such as DCs take up allergens that are processed to short peptides, and then presented to naïve T cells via MHC II. In the presence of early Il-4 that is secreted by mast cells, basophils, NKs, and eosinophils, naïve T cell become activated and acquire T_H2 status. The transcription factor GATA3 (trans-acting T cell specific transcription factor GATA3, GATA binding protein 3, GATA3) mediates the secretion of relevant chemokines. In the presence of IL-4 and IL-13 produced by T_H2 cells as well as costimulatory molecules like CD40L (CD154) B cells undergo immunoglobulin class switching resulting in the production of specific IgE (sIgE). These sIgE bind with high affinity to FcεRs on tissue resident mast cells or basophils and induce allergen mediated degranulation of inflammatory mediators.

In the early phase of allergic inflammation which occurs within minutes after exposure of an individual to an allergen and primarily reflects the secretion of mediators by mast cells which already bear allergen specific IgE (sIgE) via their expressed FcεR. Polyvalent antigens are able to crosslink IgE receptor monomers to aggregates which trigger signalling processes from the outside to the inside. As a consequence, cytoplasmic granules inside the mast cell fuse with the plasma membrane and preformed mediators such as biogenic amines like histamine and serine proteases like tryptase are released by degranulation, this time

from the inner to the outside. The rapid and systemic release of such basophil or mast cell derived mediators accounts for most of the pathology associated with anaphylactic reactions [41].

The late phase reaction commonly occurs in allergic rhinitis and asthma typically hours after allergen challenge and is represented by the recruitment of B cells, T cells, and particularly eosinophils by T_H2 cell secreted cytokines. Eosinophils contain potent mediators, including major basic cationic protein, neurotoxin, and peroxidase all of which can induce airway damage and contribute to airway hyperresponsiveness. This immune response is IgE mediated and has been associated with the severity of disease determined by recruited eosinophils, change of reactivity, and progression of the atopic disease state from a localized target organ to a more systemic atopic disorder. In general, the late phase should not be confounded with delayed hypersensitivity Type IV allergic reactions [47].

1.3.2 TYPE II, III, AND IV HYPERSENSITIVITIES

Type II is known as a cytotoxic and cytolytic hypersensitivity reaction and depends on the generation of immunoglobulins of the antigen-specific IgG and IgM isotype which as part of the humoral response may harmfully effect a variety of tissues [43]. Endogenous as well as exogenous chemicals (so called haptens) can also serve as a stimulus of this type of immune response.

Type III is a hypersensitivity reaction due to antigens that are assembled as immune complexes deposited in tissues, and activate the complement system. The immunglobulins that are involved in this type of immune response are mostly of the IgG isotype, except of IgG4, and recognize soluble antigens that are of exo- or endogenous origin.

Type IV is known as a delayed type of hypersensitivity that is mediated by immune competent cells such as T cells, macrophages, and monocytes which are recruited and activated by $CD4^+$ helper T cells through secreted chemokines. $CD8^+$ cytotoxic T cells on the other hand are known to cause tissue damage [43].

1.4 CROSS-REACTIVE CARBOHYDRATE DETERMINANTS

Carbohydrate determinants are the most frequently encountered putatively epitope structures which might play a pivotal role in allergic hypersensitivity reactions. Particular N-glycans have been namend cross-reactive carbohydrate determinants (CCDs) [48] due to previous observations that IgE in human sera reacted with an epitope present in a large number of otherwise unrelated foods like wheat or peanut [49]. Additionally, such anti-CCD IgE have been noticed to react with hymenoptera venom from wasp or bee *in vitro* [49, 50].

The key item of CCDs is the core α1,3-core fucose (Fig. 1.6) that occurs in insect glycoproteins, and in addition to that, a β1,2-xylose (Fig. 1.6) in plant glycoproteins [51]. The binding of IgE to Api m 1 (honeybee venom phospholipase A2) from sera of patients with bee venom allergy was shown to be inhibited by glycopeptides from pineapple stem bromelain, a protease with both β1,2-xylose and α1,3-core fucose residues [52]. These CCD specific IgE can be induced after stings of hymenoptera and are capable to cross-react with CCDs on pollen, and are considered to have low or no biological activity, but can cause false results in allergy diagnostics [49, 53, 54]. The basic principle to diagnose e.g. pollen allergy is the skin prick test (SPT), however, CCD-sIgE are neither known to be associated with a positive SPT, nor with a positive clinical outcome [54, 55]. Additionally, β1,2-xylose and α1,3-core fucose residues have been found in helminthic parasites [48, 56]. Immunological phenomena of infections due to parasites and atopic diseases are similar, however, the clinical outcome referring to the implication of the in both cases induced T_H2 responses is quite different [57].

Due to the scarce information of structure and composition of complex glycan chains in association with antibody binding, in particular of IgE, the inability of carbohydrate polymers to trigger allergic reactions in CCD-sIgE positive individuals remains enigmatic [58].

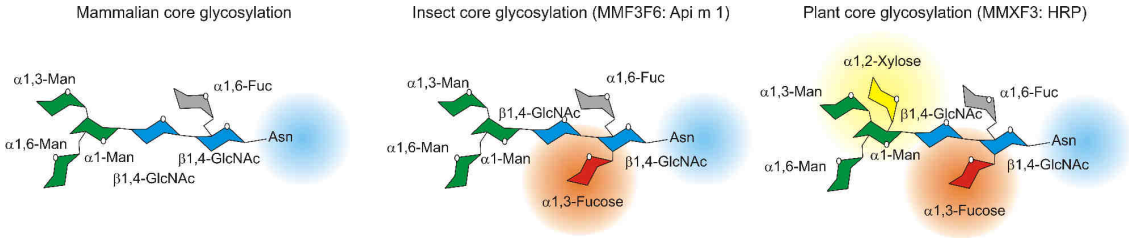


Fig. 1.6: Core glycosylation pattern of N-glycans with cross-reactive carbohydrate determinants
Depicted are the core glycosylation patterns of N-glycans with their CCDs (middel and right) in comparison with the mammalian core glycosylation (left). Highlighted in red is the α1,3-core fucose and in yellow the β1,2-xylose.

Recently, another type of CCD, namely α1,3-gal (alpha-Gal, galactose-α-1,3-galactose), provided final evidence for the harmfull potential of glycans, thus, alpha-Gal dependent allergic immune responses that are IgE mediated become important.

About 1 % of IgG antibodies in human serum are anti-Gal specific, in particular IgG2 [59] is considered to act as blocking antibody. The alpha-Gal epitope (Fig. 1.7) is expressed on cells of non-primate mammals, prosimians and New World monkeys [60] but not on cells of Catarrhini (Old World monkeys and apes) due to mutational inactivation of the α1,3-galactosyltransferase (α1,3-GT) gene followed by the elimination of the alpha-Gal epitope in these primates [61, 62]. Probably, this event occurred as a result of a protective immune response against alpha-Gal epitope expressing pathogens which were detrimental to ancestral Old World monkeys and apes [59, 60, 63].

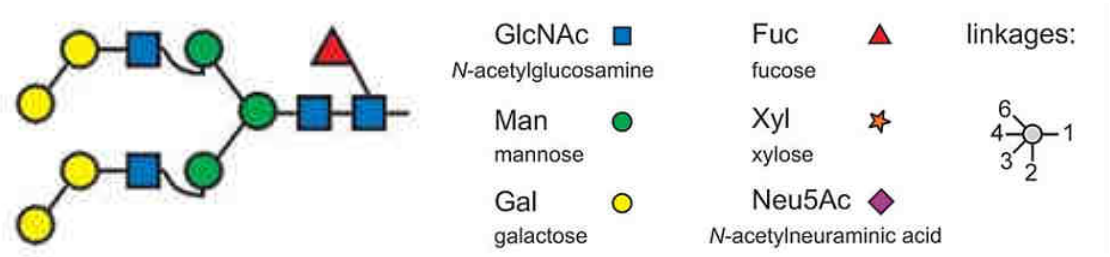


Fig. 1.7: N-glycan as it appears on non-primate mammals, prosimians and New World monkeys [48]
Depicted is a mammalian diantennary N-linked carbohydrate of the complex type (left) and the corresponding Consortium for Functional Glycomics (CFG) nomenclature for carbohydrates.

The clinical relevance is well documented for non-primate mammal red blood group antigens and xenotransplantation triggering hyperacute xenograft rejection [64] as well as for red meat induced allergic reactions [65]. Additionally, a correlation between tick

bites of particular species and allergy was observed in individuals within a restricted area of the U.S. [66]. Interestingly and in contrast to IgE specific for β1,2-xylose and α1,3-core fucose residues on helminthic parasites, sIgE that are induced by tick bites in the U.S. and that bind to the alpha-Gal epitope, provide a clinical relevance due to the provoked symptoms in affected individuals [66]. Induced anti-CCD IgE from atopic patients have shown capability to trigger mediator release from basophils and again raise the question of a clinical significance in allergic disease [52, 67]. IgE mediated anaphylactic reactions were observed in patients receiving the therapeutic chimeric monoclonal anti-EGFR antibody cetuximab (Erbitux®) bearing the alpha-Gal epitope in its V_H (Fig. 1.8) [68].

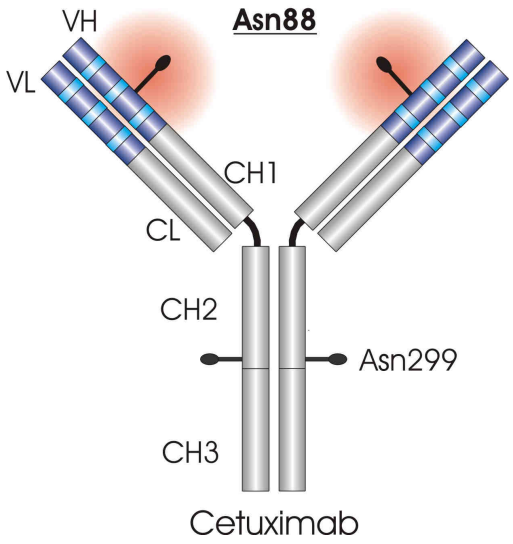


Fig. 1.8: Monoclonal antibody cetuximab
Schematic representation of the therapeutic monoclonal antibody cetuximab. Shown are the murine V_H and V_L (purple), and the constant chains (gray). Highlighted in red are the two N-linked α-Gal epitopes which are located at Asn88.

Structural and molecular data referring to the interaction of immunoglobulins, particularly of IgE, with carbohydrates are still scarce, and therefore, require further investigation.

1.5 ONCOLOGY

Oncology deals with the study of tumors, more precisely with diseases of malignant neoplasms summarized as cancer, all of them involving abnormal cell growth. The formation of malignant tumors includes irrepressible segmentation and invasion of

distant parts of the organism by spreading (metastasizing) through the blood stream and the wide network of the lymphatic system. There are benign tumors which are not cancerous and after removal usually appear anti-recurrent and the more deleterious life-threatening malignant tumors which in contrast are cancerous what is characterized by aggressive metastasizing. Some kinds of malignant cancers do not form tumors such as leukemia.

In general, cancer can be subdivided in five main categories due to its origin, namely carcinomas that begin in the skin or related epithelial tissues, sarcomas that start in connective or supportive tissues, leukemias that begin in bone marrow derived cells, cancers that are associated with the immune system such as lymphoma and myeloma, and finally cancers that effect the central nervous system, and start in the brain and spinal cord.

Epithelial cancer such as skin and colorectal cancer accounts for the most frequent cancer in humans [69]. In colorectal cancer the epidermal growth factor receptor (EGFR) has been found to be overexpressed in more than 80 % of tumors, hence, validating EGFR a clinically relevent target. At present, two different monoclonal antibodies directed against the extracellular part of the EGFR receptor are in use in a clinical setting, namely cetuximab and panitumumab. Both of these monoclonal antibodies bind to the extracellular domain of the EGFR preventing its activation [70].

1.5.1 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The highly relevant tumor associated antigen EGFR (ERBB1; ERBB for its similarity to the avian erythroblastosis oncogene B) belongs amongst others to the family of the closely related [71] receptor tyrosine kinases (RTK), in particular RTK type I, that includes EGFR (ERBB1), HER2 (HER2/neu, ERBB2), HER3 (ERBB3) and HER4 (ERBB4) [72]. Each receptor of this family type is composed of an extracellular ligand-binding domain, a α–helical transmembrane domain and an intracellular RTK domain, and all of them except ERBB3 comprise substantial RTK activity [72] (Fig. 1.9).

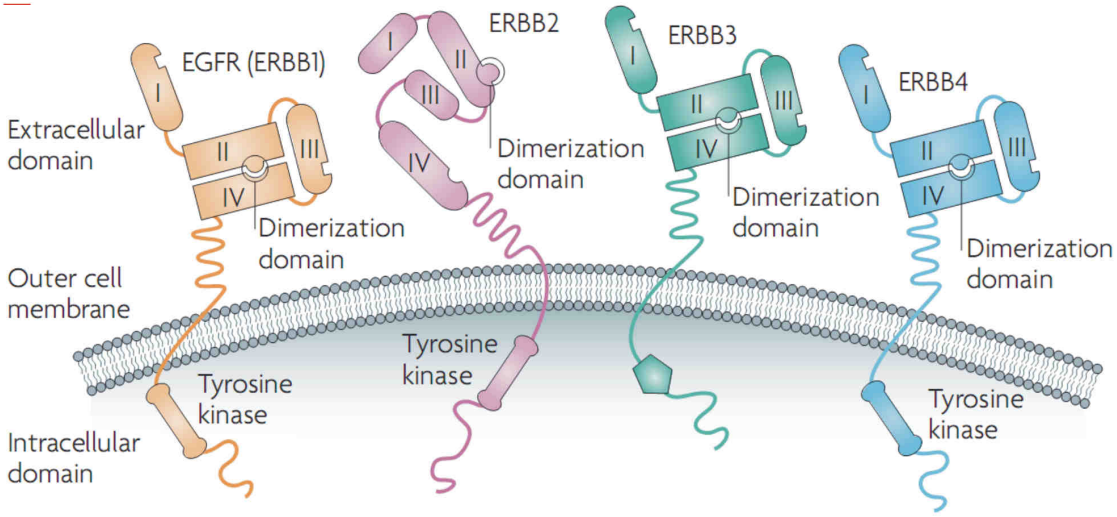


Fig. 1.9: Receptor tyrosine kinases [71]
Shown are the four ERBB members of the RTK family type I. Each receptor monomer comprises an extracellular domain subdivided into the subdomains I - IV, a transmembrane domain and an intracellular domain that includes the tyrosine kinase domain. Except for ERBB2 all receptors exist in a tethered conformation in which the dimerization domain is not available.

The ERBB receptors are expressed on various tissues of epithelial, mesenchymal and neuronal origin and controlled by the spatial and temporal occurrence of their ligands under common physiological conditions. Binding of ligands, such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) to these receptors, leads to conformational changes (Fig. 1.10) that facilitate the formation of homo- and heterodimers and activates a signal cascade starting with the activation of the intrinsic kinase domain by intermolecular autophosphorylation through the transfer of γ-phosphates from bound adenosine triphosphate (ATP) to specific tyrosine residues within the catalytical cytoplasmic tail serving as binding sites for a range of proteins [73]. Activation of EGFR, triggers pathways including RAS-RAF-MEK-MAPK, PLC-gamma/PKC PI-3K/AKT, and activation of STAT or NFκB, resulting in dysregulation of cell growth, and proliferation.

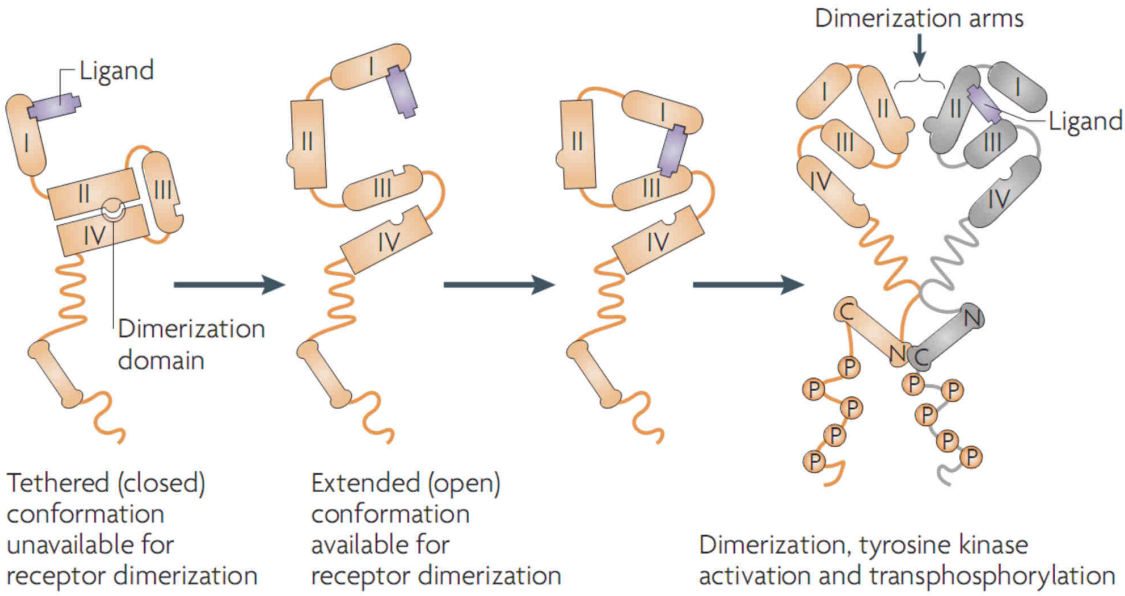


Fig. 1.10: **Changes in receptor conformation on ligand binding [71]**
Upon ligand binding to ERBB receptors, with the exception of ERBB2, they undergo a conformational change and as a result the dimerization domain, namely subdomain II, is exposed. This step is required for dimer formation and functional activation of the kinase domain interaction, which is asymmetric, namely, with the aminoterminal portion of one tyrosine kinase interacting with the carboxyterminal portion of the other.

Individuals with sarcoma derived cancer which show alterations of ERRB expression tend to have a more aggressive disease that unfortunately is associated with poor clinical outcome. On this account, ERBB receptors have been intensely studied as therapeutic targets. The selective blockade of EGFR and ERBB2 has been shown to be an effective therapeutic approach against multiple epithelial cancers. Two major therapeutic agents, namely small molecule RTK inhibitors that compete with ATP binding within the RTK domain, and monoclonal antibodies which bind to the extracellular portion of ERBB are the main classes [73].

1.5.2 MONOCLONAL ANTIBODIES

Antibodies possess various immunomodulatory properties due to their linkage to effector functions by the Fc moiety that is on the one hand capable of initiating CDC resulting in the formation of (tumor-) cell lysing pores by the membrane attack complex (MAC), and, additionally, in the production of highly chemotactic complement components that activate immune effector cells such as mast cells, basophils, macrophages, and eosinophils [74]. On the other hand there is the interaction of the Fc domains of immunoglobulins and the corresponding Fc receptors expressed on effector

cells such as natural killer (NK) cells which mediate ADCC [75-78] an important mechanism of action for several monoclonal antibodies used in cancer immunotherapy [74]. Monoclonal antibodies and related products are the fastest growing class of therapeutic agents and have been approved for use in various indications [79].

A potent inhibitor of cancer is the monoclonal antibody cetuximab which is the chimeric derivative of the murine mAb IMC-225 that is approved for treatment of metastatic colorectal carcinoma. It is composed of murin variable regions and human constant kappa light chains as well as of human constant heavy chains of the IgG1 isotype. In conjunction with EGFR this antibody competes with endogenous ligands like EGF and transforming growth factor (TGF-α) [80] and shows effective inhibition of downstream signalling within the affected cell, however, the mechanism of anti-tumor activity of individual anti-ERRB receptor mAbs is not yet entirely understood.

Additionally, complement mediated cell killing for solid tumors in homologous systems has rarely been shown and CDC apparently cannot be mediated via cetuximab as IgG alone [78, 81]. Hence, there is a need to modify treatment modalities in order to increase the number of patients who benefit from therapy.

1.5.3 ALLERGOONCOLOGY

Epidemiological evidence has revealed an inverse correlation between allergy and the risk of cancer [82-85], therefore IgE has been proposed as a possible natural defense mechanism involved in anti-tumor immune responses [86-88] prompting the field of allergooncology [85].

There is a potential association between atopic diseases, in particular hypersensitivity type I reactions, and uncrontrolled cell growth in cancer [84]. For example, patients who suffer from glioblastoma and exhibit elevated IgE levels showed a nine months longer survival compared to patients with normal or borderline IgE levels [83, 86], and the chimeric mAb MOv18 IgE, directed against folate binding protein, together with human peripheral blood mononuclear cells (PBMCs) appears superior to MOv18 IgG1 [89] in the context of ADCC.

These findings suggest IgE to be considered a new perspective. Consequently approaches to specifically harness this isotype with specificity for tumor associated antigens become a major goal.

2 MATERIALS AND METHODS

2.1 GENERATION AND EPITOPE ANALYSIS OF HUMAN MONOCLONAL ANTIBODY ISOTYPES WITH SPECIFICITY FOR THE TIMOTHY GRASS MAJOR ALLERGEN PHL P 5A

2.1.1 EXPRESSION OF RECOMBINANT PHL P 5A AND PHL P 5B CONSTRUCTS

For expression of recombinant Phl p 5 in *E. coli*, the pMal system (New England Biolabs) was used. Expression clones containing the coding sequence of Phl p 5a and b were kindly provided by Arnd Petersen (Research Center Borstel, Borstel, Germany). Both isoforms were purified from E. coli lysates by affinity chromatography using an amylase column and 10 mM maltose in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) for elution. Phl p 5 fragments were obtained by introducing particular PCR fragments into the vector pTXB1 (New England Biolabs) and expressed as N-terminal fusion proteins with an intein sequence and a chitin binding domain (CBD). Purification from the *E. coli* cell lysates was performed using chitin beads and elution from the column by addition of 50 mM dithiotreitol (DTT, SigmaAldrich) according to the recommendations of the manufacturer.

The oligonucleotides used for generation of the fragments were as follows: F1 a: gatccatatggcaggtaaggcgacgaccgag (Nde I for) and gatcgctcttccgcagccggcgat-gatgcggag (Sap I back); F2 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctctt-ccgcagccggcgatgatgcggag (Sap I back); F3 a: gatccatatggcaggtaaggcgacgaccgag (Nde I for) and gatc gctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F4 a: gatccatatgaagtaca-ggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F5 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcactcggc-tgtcttgtaggcgagc (Sap I back); chimeric F1: gatcccatggcctgttccaacaaggccttcgcggagg (Nco I for) and gatc gcgatcgcacaggaggtgagcgcggccttg (AsiS I back).

2.1.2 PRODUCTION OF RECOMBINANT ANTIBODIES

Heterotetrameric IgG and IgE immunoglobulins were produced using recently established vector systems (22). The variable regions V_H and V_L were amplified using oligonucleotides containing restriction sites at the 5´- and 3´-termini of the V_H (gatcatttaaatgtgtccagtgtgaggtgcagctggtgg and gatcctcgagacggtgaccagggt) and V_L (gatccctgcagggtgccagatgtgagctcacccagtctccatc and gatcgcgatcgcacgtttgatttccacc) by PCR, respectively. IgA2 heavy chain constant regions were amplified from total cDNA of human PBMCs and restriction sites were introduced at the 5´- and 3´-termini of the C_H (gatcctcgagcgcatccccgaccagcc and gatcggcccagccggcctcaatggtggtgatggt-agcaggtgccgtccacc) and lambda C_L (gatcgcgatcgcacagcccaaggctgccc and gatctctagactatgaacattctgtaggggc) by PCR, respectively, in a way that a 4 x His-Tag is generated at the C-terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using a protein A-medium (SureMAb columns, GE Healthcare) or Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

2.1.3 AMPLIFICATION AND CLONING OF FCεRI-IGY FC AND CD64-IGY FC

The cloning and expression of the soluble IgE Fc receptor FcεRI-IgY Fc has been described elsewhere [90]. The human CD64 extracellular domains were amplified without the original signal sequence using one PCR primer containing a Pfl23 II site (gatccgtacgtgtgggcaagtggacaccacaaaggc) and another primer containing an Sgs I site (gatcggcgcgccatgaaaccagacaggagttgg) and introduced into pcDNA3.1/zeo providing a rat immunoglobulin leader sequence and avian Fc regions [91].

2.1.4 ASSESSMENT OF IMMUNOREACTIVITY IN ELISA AND IMMUNOBLOT

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml diluted with 2 % MPBS) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 5 % MPBS at RT for 1 h. Thereafter, the recombinant immunoglobulins (1 µg/ml diluted with 2 % MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with human Ig isotype specific antibodies conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm. The immunoreactivity of immunoglobulins with their particular Fc receptors was demonstrated by Sandwich ELISA. Therefore, FcεRI-IgY Fc or CD64 IgY Fc (1 µg/ml diluted with 2 % MPBS) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 5 % MPBS at RT for 1 h. Thereafter, hIgE, cIgE and hIgG1 (1 µg/ml diluted with 2 % MPBS) were added to the wells, incubated at RT for 1 h ed according to established protocols and detected with a chicken IgG specific antibody antibody conjugate and pNPP as a substrate at 405 nm. For immunoblot procedures, the particular recombinant antibodies were separated by SDS-PAGE. Visualisation was then performed with human Ig isotype specific antibodies conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP). Reactivity with Phl p 5 in immunoblot was performed after separation of aqueus timothy grass pollen extract (Siemens Healthcare Diagnostics) by SDS-PAGE. Recombinant IgE antibodies were applied at 1 µg/ml in 2 % MPBS and visualised human Ig isotype specific antibodies conjugated to alkaline phosphatase and (NBT/BCIP).

2.1.5 *IN VITRO* MEDIATOR RELEASE ASSAY WITH RAT BASOPHIL LEUKEMIA CELLS (RBL-SX38)

In vitro degranulation was analysed as described previously [92]. Soluble MBP-Phl p 5a was biotinylated using TFPA-PEG3-Biotin (Tetrafluorophenylazide-(triethyleneglycol)-Biotin (Pierce) according to the recommendations of the manufacturer and incubated with streptavidin-coated Roti-MagBeads (Roth). After sensitisation of RBL-SX38 cells with IgE and washing with incomplete Tyrode´s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$), Phl p 5a-coated beads were added to the wells and incubated for 60 min at 37 °C. As a positive control,

cross-linking was achieved by addition of polyclonal anti-human IgE serum (1 µg/ml from goat, Bethyl). β-hexosaminidase release of viable versus lysed cells was assessed with p-nitrophenyl N-acetyl-glucosaminide (Sigma Aldrich) as a substrate.

2.1.6 HYBRIDOMA GENERATION

The establishment of the Phl p 5-specific hybridoma line was achieved by immunisation with Phl p 5a (Biomay, Vienna, Austria) according to standard protocols by Biogenes GmbH (Berlin, Germany).

2.1.7 OTHER METHODS

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [93]. Monoclonal anti-human IgE (BD Biosciences), polyclonal anti-human IgG (Fc-specific, Sigma Aldrich), polyclonal anti-human IgA (Sigma Aldrich) or polyclonal anti-chicken IgG (Rockland Immunotech) conjugated to alkaline phosphatase were used for specific detection in ELISA and immunoblot. Quantitation of allergen specific IgE was performed using an ImmunoCAP 250 analyser (Phadia, Uppsala, Sweden) and an Immulite 2000 analyser (Siemens Healthcare Diagnostics) according to the recommendations of the manufacturers.

2.2 A HUMAN MONOCLONAL IGE DERIVED FROM HYBRID REPERTOIRE LIBRARIES DEFINES AN EPITOPE GENUINE FOR BET V 1 AND *FAGALES* PR-10 PROTEINS

2.2.1 GENERATION OF THE HYBRID SCFV LIBRARIES

Total RNA prepared from human peripheral blood mononuclear cells of three allergic individuals (10^7 cells) was prepared according to standard protocols. In order to amplify the human IgE V_H repertoire an epsilon heavy chain-specific oligonucleotide was used for reverse transcription and oligonucleotides covering the entire human V_H repertoire were used to amplify the V_H from gene-specific cDNA. V_H were subcloned and

individual clones subjected to sequence analysis to ensure epsilon-specific amplification. Subsequently, V_H domains were amplified using a set of PCR primers providing a 5´- Sfi I site and a 3´- Xho I site for cloning purposes. For generation of the hybrid libraries the human synthetic antibody library Griffin-1 was used, comprising scFv-formatted variable regions of human origin in the phagemid pHEN2, providing a diversity of approximately $2x10^9$ individual clones. The synthetic V_H were replaced by donor-derived IgE-specific V_H regions via Sfi I and Xho I. Transformation of *E. coli* TG1 cells yielded libraries of 10^7 independent clones for each individual donor.

2.2.2 SELECTION OF ANTIBODY LIBRARIES

Selection of the libraries and identification of reactive antibody fragments were performed according to established protocols. Briefly, after rescue of the library immunotubes coated with Bet v 1a (Biomay, Austria) were incubated with a total of 10^{12} to 10^{13} phages for 90 min under continuous rotation. After washing, bound phages were eluted by addition of 1 mL of 100 mmol/L triethylamine followed by neutralization using 0.5 mL 1 mol/L Tris-HCl, pH 7.4. After reinfection of *E. coli* TG1 and overnight growth on agar plates, the phages were rescued by superinfection with M13KO7 helper phages and overnight growth. Phages were then directly subjected to the next round of selection. Immunoreactivity of phages was assessed in ELISA using anti-M13 horseradish peroxidase conjugate (Amersham).

2.2.3 RECONVERSION INTO BIVALENT ANTIBODY FORMATS AND EXPRESSION IN MAMMALIAN CELLS

Recombinant immunoglobulins were produced using vector systems previously established in our lab. The variable regions V_H and V_L were amplified using oligonucleotides containing restriction sites at the 5´- and 3´-termini of the V_H (gatcatttaaatgtgtccagtgtgaggtgcagctggtgg and gatcctcgagacggtgaccagggt) and V_L (gatccctgcagggtgccagatgtgagctcacccagtctccatc and gatcgcgatcgcacgtttgatttccacc) by PCR, respectively. IgA2 heavy chain constant regions were amplified from total cDNA of human PBMCs and restriction sites were introduced at the 5´- and 3´-termini of the C_H (gatcctcgagcgcatccccgaccagcc and gatcggcccagccggcctcaatggtg gtgat ggtag caggtg-ccgtccacc) by PCR, respectively, in a way that a 4 x His-Tag is generated at the C-

terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich).

2.2.4 ASSESSMENT OF IMMUNOREACTIVITY IN ELISA AND ALABLOT

For assessment of immunoreactivity in ELISA the particular proteins (diluted with 20 g/L MPBS) were applied to microtiter plates coated with particular antigens at 4 °C overnight and blocked with 50 g/L MPBS. Thereafter, ELISA was performed according to established protocols. For immunoblotting the particular recombinant antibodies were diluted using DMEM supplemented with 100 mL/L heat-inactivated fetal calf serum and applied to AlaBLOTs (Siemens Healthcare Diagnostics), providing native allergens after separation by SDS-PAGE and transfer onto NC-membranes. Visualization of IgE was performed with anti-human IgE conjugated to alkaline phosphatase (AP).

2.2.5 RECOMBINANT PRODUCTION OF BET V 1 ISOFORMS AND DERIVED VARIANTS

The different PR-10 proteins, isoforms and mutants were assembled by using oligonucleotides in PCR. For expression in *E. coli*, the Impact system (NEB, Bad Schwalbach, Germany) was used. In brief, the coding sequences were introduced via EcoR I and Hind III cleavage sites into the vector pMAL-c2X and expressed as N-terminal MBP fusion proteins. Purification of the fusion proteins was performed using an amylose resin according to the recommendations of the manufacturer.

Amino acid substitutions were performed by Quikchange mutagenesis (Agilent, Waldbronn, Germany) according to the recommendations of the manufacturer.

2.3 EVALUATION OF DIFFERENT GLYCOFORMS OF HONEYBEE VENOM MAJOR ALLERGEN PHOSPHOLIPASE A2 (API M 1) PRODUCED IN INSECT CELLS

2.3.1 MATERIALS

Crude HBV collected by electrostimulation and native purified PLA2 were purchased from Latoxan (Valence, France). Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate, anti-mouse IgG AP conjugate and bromelain from pineapple stem was obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). The MUXF-HSA conjugate was kindly provided by Siemens Healthcare Diagnostics (Los Angeles, CA, USA). The rat basophilic leukemia cell line RBL-SX38 was kindly provided by Prof. J.-P. Kinet (Harvard University, Boston, MA, USA). Sera with a positive test to HBV and/or YJV (sIgE >0.35 kU/L) were obtained from the institutional serum bank. All patients had given their informed written consent to draw an additional serum sample.

2.3.2 CLONING OF CDNA

Total RNA was isolated from honeybee (*Apis mellifera carnica*) venom sacks using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to synthesize cDNA. The Api m 1 mature peptide coding region was amplified using Pfu DNA polymerase (Fermentas, St. Leon-Rot, Germany) and the primers 5'-ATA ATA TAT CCA GGA ACG TTA TGG TG-3' and 5'-ATA CTT GCG AAG ATC GAA CCA TTG-3' and subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector. A C-terminal V5 epitope and a 10-fold His-tag as well as 5' BamH I and 3' Not I restriction sites were added by PCR and the PCR product was subcloned into the pAcGP67-B baculovirus transfer vector (BD Pharmingen, Heidelberg, Germany) after restriction digest with BamHI and NotI.

2.3.3 SITE DIRECTED MUTAGENESIS

For generation of the inactive Api m 1 H34Q, histidine 34 was substituted by glutamine by using the QuikChange site directed mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturers' recommendations and employing the primers 5'-GCA TGC TGT CGA ACC CAA GAC ATG TGC CCG GAC G-3' and 5'-CGT CCG GGC ACA TGT CTT GGG TTC GAC AGC ATG C-3'. For the non-glycosylated form of Api m 1 H34Q N13Q, asparagin 13 was changed to glutamine by employing the primers 5'-GGT GCG GGC ATG GTC AAA AGT CGT CCG GCC C-3' and 5'-GGG CCG GAC GAC TTT TGA CCA TGC CCG CAC C-3'.

2.3.4 RECOMBINANT BACULOVIRUS PRODUCTION AND EXPRESSION

Recombinant baculovirus was generated by cotransfection of *Spodoptera frugiperda* (Sf9) cells (Invitrogen) with BaculoGold bright DNA (BD Pharmingen) and the baculovirus transfer vector pAcGP67-B Api m 1 H34Q or Api m 1 H34Q N13Q, respectively, according to recommendations of the manufacturer. High titer stocks were produced by three rounds of virus amplification. Optimal multiplicity of infection (MOI) for subsequent protein expression was determined empirically by infection of Sf9 cells with serial dilutions of virus stocks.

2.3.5 EXPRESSION IN BACULOVIRUS-INFECTED SF9 CELLS AND PROTEIN PURIFICATION

High titer stocks of recombinant baculovirus containing the Api m 1 coding DNA were used to infect Sf9 or HighFive cells (Invitrogen) (1.5-2.0 × 106 cells per ml) in a 2000 ml suspension flask (400 ml suspension culture). For protein production the cells were incubated at 27 °C and 110 rpm for 72 h. Cellular supernatants were then applied to a nickel-chelating affinity matrix (Ni NTA-agarose, Qiagen, Hilden, Germany). After washing with NTA-binding buffer (50 mM sodium phosphate, pH 7.6, 500 mM NaCl) the protein was eluted with NTA-binding buffer containing 300 mM imidazole.

2.3.6 IMMUNOREACTIVITY OF HUMAN SERA

For assessment of specific IgE immunoreactivity, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with recombinant allergens, nApi m 1, HBV and the CCD marker MUXF-HSA (10 µg/ml) at 4 °C overnight and blocked with 40 mg/ml skimmed milk powder in PBS at room temperature. Human sera were diluted 1:2 in PBS and applied for 4 hours at room temperature. Wells were rinsed 4 times with PBS and incubated with an alkaline phosphatase conjugated mouse anti-human IgE antibody (BD Pharmingen, clone G7-26) diluted 1:1000 in 20 mg/ml skimmed milk powder in PBS. Wells were rinsed 4 times with PBS and substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) was added. After 30 minutes absorption was measured at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs.

2.3.7 *IN VITRO* DEGRANULATION ASSAY WITH RAT BASOPHIL LEUKEMIA CELLS (RBL-SX38)

In vitro degranulation was analyzed essentially as described [92]. In brief, RBL-SX38 cells were cultivated in 96-well plates (1 x 10^5 cells per well) for 24 h at 37 °C in 5 % CO2. After washing with Tyrode´s buffer (137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.4 mM NaH_2PO_4 and 10 mM Hepes, pH7.4, BSA 0,1 % w/v) cells were incubated with native PLA2 or PLA2 H34Q (0.01-100 µg/ml in Tyrode´s buffer) at 37°C for 1 h and then washed twice with Tyrode´s buffer. β-hexosaminidase release was assessed using 4-nitrophenyl N-acetyl-glucosaminide as a substrate. The supernatant of stimulated cells was incubated for 1 h at 37 °C and the enzymatic reaction was stopped by adding carbonate buffer (0,1 M, pH 10.0). Absorbance was measured at 405 nm. Cells were washed twice as described before and total cell lysate was obtained by addition of 0,1 % Triton-X-100 in Tyrode´s buffer and incubation for 1 h at 37 °C. β-hexosamindidase release was assessed as the percentage of total cell content which was ascertained by processing the supernatant of the cell lysate in the same way as the supernatant of the activated cells. All measurements were performed as triplicates and subjected to statistical analysis via student's t-test.

2.3.8 OTHER METHODS

SDS-PAGE, Western blotting, and standard procedures in molecular biology were performed according to established protocols [93]. Lectin blots (DIG Glycan Differentiation Kit, Roche Diagnostics, Mannheim, Germany) were performed according to the recommendations of the manufacturer.

2.4 CLOSE-UP OF THE IMMUNOGENIC ALPHA-1,3-GAL EPITOPE AS DEFINED BY A MONOCLONAL CHIMERIC IGE AND HUMAN SERUM USING STD NMR

2.4.1 PRODUCTION OF RECOMBINANT ANTIBODIES

For establishing chimeric mouse/human antibodies, the V_H and V_L sequences of the alpha-Gal-specific antibody M86 was used as template for gene synthesis [94]. Variable regions were assembled in form of a single chain Fv (scFv) and introduced into phagemid vectors allowing prokaryotic production as both soluble fragment and scFv-displaying phage.

Homodimeric IgG1 and IgE and heterotetrameric IgE immunoglobulins were produced using recently established vector systems [95]. The variable regions V_H and V_L were amplified using oligonucleotides containing restriction sites at the 5´- and 3´-termini of the V_H (gatcatttaaatgtgtccagtgtgaggtgaaactggag and gatcgtcgaccccgagacagtg-acagaagttcc) and V_L (gatccctgcagggtgccagatgtgatgtggtgatgacac and gatcggcgcgcc-cacagtccgtttgatttcgag) by PCR, respectively, in a way that a 4 x His-Tag is generated at the C-terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 3 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich). The secreted immunoglobulins were purified from the culture medium by affinity

chromatography Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

2.4.2 AMPLIFICATION AND CLONING OF FCεRI-IGY FC AND CD64-IGY FC

The cloning and expression of the soluble IgE Fc receptor FcεRI-IgY Fc has been described elsewhere [90]. The human CD64 extracellular domains were amplified without the original signal sequence using one PCR primer containing a Pfl23 II site (gatccgtacgtgtgggcaagtggacaccacaaaggc) and another primer containing an Sgs I site (gatcggcgcgccatgaaaccagacaggagttgg) and introduced into pcDNA3.1/zeo providing a rat immunoglobulin leader sequence and avian Fc regions [91].

2.4.3 ASSESSMENT OF IMMUNOREACTIVITY IN ELISA

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 4 % MPBS at RT for 1 h. The recombinant immunoglobulins (1 µg/ml diluted with 2 % MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with human Ig isotype specific antibody conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm.

The immunoreactivity of immunoglobulins with their particular Fc receptors was demonstrated by Sandwich ELISA. Therefore, bovine thyroglobulin (50 µg/ml) was applied to microtiter plates, incubated at 4 °C overnight and blocked with 4 % MPBS at RT for 1 h. Thereafter, hIgE, cIgE and hIgG1 (1 µg/ml diluted with 2 % MPBS) were added to the wells, incubated at RT for 1 h and subsequently incubated with FcεRI-IgY Fc or CD64-IgY Fc (1 µg/ml diluted with 2 % MPBS) according to established protocols and detected with a chicken IgG specific antibody conjugate and pNPP as a substrate at 405 nm.

For immunoblot procedures, the particular recombinant antibodies were separated by SDS-PAGE. Visualization was then performed with human Ig isotype specific antibodies conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP).

2.4.4 *IN VITRO* MEDIATOR RELEASE ASSAY WITH HUMANIZED RAT BASOPHILIC LEUKEMIA CELLS (RBL-SX38)

In vitro degranulation was analyzed as described previously [92]. Soluble alpha-Gal proteins were biotinylated using TFPA-PEG3-Biotin (Tetrafluorophenyl¬azide-(triethylene-glycol)-Biotin (Pierce) according to the recommendations of the manufacturer and incubated with streptavidin-coated Roti-MagBeads (Roth). After sensitization of RBL-SX38 cells with IgE and washing with incomplete Tyrode´s buffer (10-mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2), alpha-Gal-carrying proteins or alpha-Gal-coated beads were added to the wells and incubated for 60 min at 37 °C. As reference, cross-linking was achieved by addition of polyclonal anti-human IgE serum (1 µg/ml from goat, Bethyl). β-hexosaminidase release of viable versus lysed cells was assessed with p nitrophenyl N-acetyl-glucosaminide (Sigma Aldrich) as a substrate.

2.4.5 SURFACE PLASMON RESONANCE ANALYSIS

The interaction affinity of cetuximab, alpha-Gal-HSA, thyroglobulin and immobilized IgE as well as the affinity of a TNP-specific mouse IgE (clone C38-2) and immobilized TNP-BSA have been determined by surface plasmon resonance (SPR) measurements using the SPR-2 affinity sensor from Sierra Sensors, Hamburg, Germany. The IgE was covalently coupled to a total of 2400 resonance units on a carboxymethylated sensor chip surface (SPR-2 affinity sensor) using standard NHS/EDC coupling procedure via primary amines and capping by ethanolamine. The uncoupled surface served as reference. Measurements were performed at 20 °C in buffer containing 10 mM monosodium phosphate, 40 mM disodium phosphate and 100 mM NaCl, pH 7.5 with 0,01 % Tween-20. For the kinetic analyses, increasing concentrations of the alpha-Gal carrying antigens (7 – 167 nM for cetuximab, 121 – 564 nM for alpha-Gal-HSA, 15-120 nM for thyroglobulin) were injected at a flow rate of 25 µl/min. The association phase was monitored for 120 seconds, the dissociation phase for 90 seconds. Sensor surfaces were regenerated after each binding cycle by two subsequent injections of 50 mM Tris buffer, pH 10. After subtracting reference cell signals, resulting binding data were fitted to a Langmuir 1:1 binding model by using global fit analysis

(evaluation software, Sierra Sensors) and the dissociation constant at equilibrium KD was calculated.

2.4.6 STD NMR

Buffer exchange against deuterated PBS and concentrating of affinity-purified, anti-Gal-specific antibodies to 450 µg/ml was performed by using AMICON Ultra-4 10K centrifugal filter devices. Oligosaccharides were obtained from Dextra (Reading). The Gal-Gal disaccharide was purchased as methylglycoside and as biotinylated carbohydrate.

Saturation transfer difference (STD) NMR experiments were performed at 298 K on a Bruker 500 MHz spectrometer equipped with a 5 mm inverse triple resonance probe head and a Bruker 700 MHz spectrometer equipped with a 5 mm inverse triple resonance probe head with cryo technology. The PBS NMR buffer contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 176 mM KH_2PO_4 in D_2O and was adjusted to pH 7.4. The on resonance pulse for antibody saturation was set to 0 Hz (500 MHz) or -500 Hz (700 MHz), respectively, and the off resonance pulse to 28500 Hz (500 MHz) or 28000 Hz (700 MHz). Saturation was achieved by a train of 90° Gaussian-shaped pulses of 50 ms yielding a total saturation time of 2 s with an attenuation of 45 dB (500 MHz) or 40 dB (700 MHz). The spectra were acquired with a spectral width of 8000 Hz, 64k time domain data points and 2 transients using a pseudo-2D Bruker standard pulse sequence (stddiff.3, 500 MHz). A relaxation delay of 4 s was applied. For suppression of protein background a T1ρ-filter was used applying a field strength of 11.5 kHz and a duration of 15 ms. Each experiment was performed with a total of 1024 scans. STD experiments on serum were performed using a pseudo-2D Bruker standard pulse sequence (stddiffesgp2d) that contained the excitation sculpting sequence for the suppression of residual HDO (700 MHz). The spectra were recorded with a spectral width of 7000 Hz, 32k time domain data points and 2 transients. The FIDs of the on and off resonance spectrum were stored and processed separately. Subtraction of the on and off resonance spectrum resulted in the STD NMR spectrum. Protein:ligand ratios of 1:260 (4.81 µM of the M86 antibody with 1.25 mM of the Gal-Gal-OMe disaccharide) and 1:200 (5.84 µM of the purified serum antibodies with 1.17 mM of the Gal-Gal-OMe disaccharide) were used.

2.4.7 AFFINITY PURIFICATION OF ALPHA-GAL-SPECIFIC IMMUNOGLOBULINS

For purification of anti alpha-Gal-specific antibodies from human serum an alpha-Gal-specific affinity resin was generated. Therefore, 1 mL Affi-Gel 10 (BIO-RAD) activated matrix was rinsed with 3 volumes of ddH_2O for 20 minutes followed by an incubation step with 4 volumes of 100 mM HEPES buffer, pH 7.5, containing bovine thyroglobulin (70 mg/mL) for 4 h at 4 °C on a roller-mixer. Remaining active esters were quenched subsequently by the addition of 100 µL ethanolamine (1 M). Prior to first use the affinity resin was washed with 60 volumes PBS, pH 7.4.

To isolate alpha-Gal-specific antibodies, 15 mL human serum of an alpha-Gal-positive donor was subjected to the forecited anti-Gal specific affinity matrix. Unbound proteins were removed by washing with 100 volumes of PBS, pH 8.0, 500 mM NaCl, and alpha-Gal-specific antibodies were subsequently eluated into 300 µL of 1 M Tris-HCl, pH 7.5, for immediately neutralization using 700 µL of 0.1 M glycine buffer, pH 2.0. Immunoreactivity was subsequently assessed by ELISA.

2.4.8 OTHER METHODS

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [93].

2.5 COMPARISON OF TUMORICIDAL ACTIVITIES MEDIATED BY HUMAN ANTI-EGFR IGG VERSUS IGE ISOTYPES

2.5.1 AMPLIFICATION AND CLONING OF 225-IGE AND 225-IGG1 ANTIBODIES

Human constant regions were amplified from a human cDNA library derived from PBMC. ε heavy chain constant regions (accession no. L00022) were amplified using the primers gatcctcgagcgcgagccccacatcgcc and gatcggcccagccggcctcaatggtggtgatggt-gtttaccgggatttacagacaccg providing a C-terminal 5 x His Tag. The γ1 heavy chain constant regions (accession no. X14735) were amplified using the primers

gatcctcgagcgcctccaccaagggccc and gatcggcccagccggcctcaatggtggtgatgtttacccggagac-aggga providing a C-terminal 4 x His Tag. The κ light chain constant domain (accession no. X14736) was amplified using the primers gatctctagactaacactctcccctgttgaag and ggcgcgccatctgtcttcat and introduced into a vector backbone containing Ig signal sequences as described previously [95]. The Asc I site connecting the variable and constant heavy chain regions was converted to an Xho I site, and the AsiS I site connecting the variable and constant light chain domains was converted to an Sgs I site via site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Stratagene) to avoid the incorporation of additional amino acids. The murine cetuximab variable regions were amplified from cDNA of the mouse hybridoma cell line 225 (ATCC no. HB-8508). The primers gatcatttaaatgtgtccagtgtcaggtgcagctgaagcagtcag and gatcctcgagccgacagtgaccagagtcccttg were used for amplification of the variable heavy chain and incorporated an Swa I site and an Xho I site. The variable light chain was amplified using the primers gatccctgcagggtgccagatgtgacatcttgctgactcagtctc and gatcggcgcgcctttcagctccagcttggtccc, providing an Sbf I site and an Sgs I site.

2.5.2 TISSUE CULTURE

Tissue culture reagents and cell lines were obtained from Life Technologies. Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) and A431NS (ATCC no. CRL-2592) cells were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Transfections were carried out using jetPEI according to the recommendations of the distributor (PeqLab). The rat basophilic leukaemia mast cell line RBL-SX38 expressing the human FcεRIreceptor subunits [96] was maintained in MEM supplemented with 15 % FCS, 250 µg/mL Geneticin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. All cells were maintained at 37 °C in 5 % CO_2in a humidified atmosphere.

2.5.3 BIACORE BINDING ASSAY

SPR based real-time monitored assays were performed using the BiacoreT100 instrument (Biacore, Freiburg, Germany) and carboxylated dextran sensor chips (Sensor

chip CM5, Biacore). Streptavidin was covalently coupled to a total of 1000 resonance units on a carboxymethylated sensor chip surface (CM5, research grade) using standard NHS/EDC coupling procedure via primary amines. Soluble EGFR was isolated from the supernatant of A431NS cells [97], biotinylated using photoactivatable tetrafluorophenyl azide-PEG_3-biotin (TFPA-Biotin, Pierce), and then bound to the streptavidin surface to a total of 330 resonance units. A streptavidin-covered surface served as negative control. Measurements were performed at 25 °C in buffer containing 50 mM sodium phosphate, 150 mM NaCl, pH 7.5. For kinetic analyses, increasing concentrations of 225-IgE, 225-IgG1 and Erbitux® were injected at a flow rate of 10 µl/min. Contact time was monitored for 4 min and dissociation time was monitored for 10 min. Sensor surfaces were regenerated after each binding cycle by injection of 0,1 M acetic acid and 50 mM NaCl, pH 2.8 for 30 sec. After subtracting reference cell signals, resulting binding data were fitted to a Langmuir 1:1 binding model by using global fit analysis (Biacore T100 Evaluation Software). From the apparent association (Ka) and dissociation (Kd) rate constants equilibrium binding constants were calculated according to K_D = Kd/Ka.

2.5.4 FLOW CYTOMETRIC ASSESSMENT OF REACTIVITY WITH IGE RECEPTORS AND SOLUBLE EGF RECEPTOR (SEGFR)

Flow cytometric analyses were performed on a FACSCalibur equipment and analyzed using CellQuest software (Becton Dickinson). Labeling of sEGFR with fluorescein isothiocyanate (FITC) (SigmaAldrich) was performed according to standard protocols. Briefly, $6.25x10^4$ RBL-SX38 cells were resuspended in minimum essential medium (MEM) containing 15 % fetal calf serum and dispersed into 1.5 ml tubes. Cells were spun down and 225-IgE antibodies were added at a concentration of 1 µg/ml in 500 µl culture medium. After incubation for 1 h at 4 °C on a rocker platform cells were washed three times with 500 µl incomplete Tyrode´s buffer(10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mMKCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$) and FITC-labeled sEGFR was added at a concentration of 10 µg/ml in 500 µl culture medium. Cells were incubated for 1 h at 4 °C on a rocker platform. After three washing steps with 500 µl incomplete Tyrode´s buffer cells were resuspended in 500 µl complete Tyrode´s buffer (incomplete Tyrode´s

buffer supplemented with 5.6 mMGlucose, 0.1 % BSA). Controls consisted of cells incubated with sEGFR-FITC only.

2.5.5 FLOW CYTOMETRIC ASSESSMENTS OF ANTIBODY BINDING TO MEMBRANE BOUND RECEPTORS

For this procedure, $4.2x10^5$ A431NS cells were resuspended in DMEM containing 10 % fetal calf serum and transfered into 1.5 ml tubes. Cells were spun down and 225-IgE or 225-IgG1 antibodies were added at a concentration of 1 µg/ml in 500 µl culture medium. After incubation for 1 h at 4 °C on a rocker platform, cells were washed three times with 500 µl incomplete Tyrode´s buffer and FITC-labeled anti-human IgE or tetramethyl rhodamine isothiocyanate (TRITC)-labeled anti-human IgG antibodies were added at a concentration of 1 µg/ml. Cells were incubated for 1 h at 4 °C on a rocker platform. After three washing steps with 500 µl incomplete Tyrode´s buffer, cells were resuspended in 500 µl complete Tyrode´s buffer. Controls consisted of cells incubated with neither primary nor secondary antibody and cells incubated with secondary antibody only.

2.5.6 IMMUNOSTAINING OF A431NS CELLS

Briefly, 3.2×10^4 A431NS cellswere grown on glass slides, dryed and fixed with 3.5 % formaldehyde. Cells were washed three times with PBS and free binding sites blocked with blocking reagent (Dako) for 20 min. 225-IgE and 225-IgG1 were added in a final volume of 300 µl at a concentration of 2.4 µg/ml and 0.45 µg/ml, respectively.After incubation for 1 h, cells were washed three times for 2 min with PBS. A second blocking step was perfomed using blocking reagent (Dako) for 20 min and FITC-labeled anti-human IgE or TRITC-labeled anti-human IgG antibodies were added in 300 µl antibody dilution buffer (Dako) at a dilution of 1:500 for 1 h. Prior to examination cells were washed with PBS and stained with 4′,6-Diamidin-2-phenylindol (DAPI) for 2 min followed by two washing steps with PBS.

2.5.7 *IN VITRO* DEGRANULATION ASSAY WITH RAT BASOPHILIC LEUKEMIA CELLS (RBL-SX38)

In vitro degranulation was analyzed as described previously [92]. Soluble EGFR (sEGFR) was biotinylated using TFPA-Biotin (Pierce) according to the recommendations of the manufacturer and incubated with streptavidin at a molar ration of 4 : 1 or directly coated on DynalBeads (Life Technologies). After sensitization of RBL-SX38 cells with 225-IgE and washing with incomplete Tyrode´s buffer either sEGFR:streptavidin (Sigma), sEGFR coated beads or A431NS cells were added to the wells and incubated for 60 min at 4 °C. β-hexosaminidase release of viable versus lysed cells was assessed with p-nitrophenyl N-acetyl-glucosaminide (Sigma) as a substrate.

2.5.8 CELL VIABILITY ASSAY

Tumor cell viability was analyzed by reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, Calbiochem). A total of 5×10^3 cells/well were seeded in 96-well plates and allowed to adhere overnight. Cells were exposed to 0.15 to 10 µg/mL Erbitux®, 225-IgG1 and 225-IgE antibodies over 72 h. Control groups received media alone or 0.9 % v/v Triton-X-100 for 10 min prior to addition of MTT (5 mg/ml in water).Twenty-five microlitres ofMTT solution were added per well and cells were incubated for 3 h prior to solubilization of formazan crystals with Sorensen´s glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). Absorbance was measured at 550 nm. Cell viability was expressed as a percentage ofcontrols (%CT).

2.5.9 OTHER METHODS

The flow cytometric ADCC/ADCP assay was performed as described previously [89]. Statistical analyses of *in vitro* ADCC/ADCP assays were performed by means of the unpaired two-tailed Student's *t* test, and significance was accepted at $P < 0.05$.

2.6 HUMAN MONOCLONAL IGE AND IGG ANTIBODIES AGAINST MBP AS TOOLS OF DEFINED SPECIFICITY FOR DIAGNOSTIC APPROACHES IN ALLERGY

Target protein for selection was bacterial maltose binding protein (NEB); control allergens fused to MBP were produced according to the recommendations of the manufacturer. Anti-M13-horseradish peroxidase conjugate (Amersham) anti-mouse IgG AP conjugate was from Sigma (Taufkirchen, Germany). AlaBLOTs were obtained from Siemens Healthcare Diagnostics (Bad Nauheim, Germany), Quickline assay from Milenia Biotech (Gießen, Germany).

2.6.1 SELECTION OF THE SEMISYNTHETIC SCFV LIBRARY

For generation of monoclonal antibody fragments used the human synthetic antibody library Griffin-1, which comprises scFv-formatted variable regions of human origin, providing a diversity of approximately 2 x 10^9. The allergens were employed for selection according to established protocols. Briefly, immunotubes coated with the particular allergen and blocked with 20 g/L milk powder phosphate buffered saline (MPBS; 50 mmol/L sodium phosphate, 100 mmol/L sodium chloride, 20 g/L milk powder, pH 7.4) were incubated with a total volume of 4 mL of 20 g/L MPBS containing 10^{12} to 10^{13}phages for 90 min under continuous rotation at room temperature. The immunotubes were then washed, 1 mL of 100 mmol/L triethylamine was added to elute the bound phages, and neutralization was performed by addition of 0.5 mL of 1 mol/L Tris-HCl, pH 7.4. After reinfection of *E. coli* TG1 and overnight growth on agar plates, the phages were rescued by growth of scraped cells in liquid culture, infection with M13KO7 helper phage, and another period of overnight growth. Phages were then subjected to the next round of selection. Immunoreactivity of polyclonal or monoclonal phages was assessed in ELISA with anti-M13 horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt for detection.

2.6.2 CLONING OF THE IMMUNOGLOBULINS

Transfer of the particular scFv into the scFv CH2-3 or scFv CH2–4 format was performed by introduction by PCR of a BsiW I site at the N-terminus and an Asc I site at the C-terminus. Cloning of heterotetrameric IgG was performed by introducing of Sgf I and the Sbf I sites, or the Asc I and Swa I sites, respectively, at the C- and N-terminus of the variable regions. Subsequently, the scFv or the particular variable regions were inserted into expression vectors recently established [95].

2.6.3 TRANSFECTION OF HEK-293 CELLS

HEK-293 cells (ATCC number CRL-1573) were cultivated in DMEM supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Invitrogen Life Technologies. HEK-293 cells were transfected with 2 µg of the particular expression vector by use of polyethyleneimine (Sigma). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using protein A-agarose (Santa Cruz Biotechnologies.) or Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

2.6.4 ASSESSMENT OF IMMUNOREACTIVITY IN ELISA AND IMMUNOBLOT

For assessment of immunoreactivity in ELISA the particular proteins (diluted with 20 g/L MPBS) were applied to microtiter plates coated with particular antigens at 4 °C overnight and blocked with 50 g/L MPBS at room temperature for 1 h. Thereafter, ELISA was performed according to established protocols. For immunoblot procedures, the particular recombinant antibodies were separated by SDS-Page and subsequently blotted on a nitrocellulose membrane.Visualization of IgE was then performed with monoclonal antihuman IgE conjugated to alkaline phosphatase (AP) and nitrotetrazolium blue chloride/ 5-bromo-4-chloro-3-indoyl phosphate according to recommendations of the manufacturer.

2.7 OTHER METHODS

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [93].

3 RESULTS

3.1 GENERATION AND EPITOPE ANALYSIS OF HUMAN MONOCLONAL ANTIBODY ISOTYPES WITH SPECIFICITY FOR THE TIMOTHY GRASS MAJOR ALLERGEN PHL P 5A

It is well established that the development of several immune-mediated diseases is linked to circulating concentrations of IgE, the antibody class responsible for allergic hypersensitivity [98]. IgE antibodies bound to their high affinity receptor (FcεRI) on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. Long-term exposure to higher concentrations of allergens or therapeutic intervention by specific immunotherapy (SIT) results in a T-helper cell type-1 (T_H1) shift in the immune response, leading to an increase in production of allergen-specific Ig, particularly of the IgG4 and IgA2 subclasses [99, 100]. These antibody isotypes are thought to exert their function by blocking the IgE/allergen interaction [101-103], by recruitment of Fcγ receptors inhibiting FcεR mediated activation [104, 105], or by inhibition of IgE-facilitated allergen presentation [106]. Their activity may rely either on a molar excess or on affinity maturation during vaccination [107]. However, the exact interplay of antibodies with their cognate allergens still remains unclear.

50 % of patients suffering from type I allergy are sensitised to grass pollen proteins. These allergens are potent elicitors of clinical symptoms, such as rhinitis, conjunctivitis and asthma [108]. Phl p 5 represents one of the major pollen allergens of timothy grass (Phleum pratense) and accounts for IgE binding in up to 60 % of patients [109]. Two isoforms, Phl p 5a and Phl p 5b exhibit high sequence similarity and differ only slightly in molecular masses and biochemical behaviour [110]. These isoproteins are composed of two alanine-rich (AR) repeats, which form four alpha-helices each and thereby,

group 5 and 6 allergens represent the distinct protein class of 4-helix-bundle allergens [111].

Although the interaction of allergens with polyclonal serum-derived IgE has broadly been studied detailed analyses of the role of allergen-specific antibodies in pathophysiology as well as their interplay on a molecular level have been hampered by two critical limitations, the low IgE levels in serum and the lack of monoclonal allergen-specific antibodies of different isotypes. Approaches to generate human allergen-specific IgE-secreting hybridomas from immunised donors have not been successful so far [112]. Furthermore, conventional hybridoma technology is often limited by a low immunogenic potential of allergens [113] suggesting that allergens have less preferable binding sites for IgG antibodies compared to IgE antibodies. Additionally, resulting murine antibodies are neither compatible with established human specific assay formats nor recognise authentic IgE epitopes.

During the last decade sophisticated antibody technologies ranging from the generation of antibody fragment libraries to the design of tailor-made immunoglobulins have become tools for dissection of human immune responses. The establishment of combinatorial approaches has enabled the selection of monoclonal antibody fragments from synthetic library formats [114, 115] or immune repertoires against an almost unlimited panel of target molecules [116-118]. The former approach is particularly attractive for the generation of antibodies against the vast variety of allergenic molecules [95]. In contrast, the isolation of antibodies from libraries on the basis of lymphoid sources [119-121] is high in effort due to the evanescent number of IgE-producing cells, but imperative for identifying allergy-related authentic antibodies. Hence, only scarce studies reported cloning of allergen-specific IgE antibody fragments [122-126].

The aim of this present work was to gain access to authentic allergen-specific human antibody isotypes allowing insights into the molecular basis of their interaction. Therefore, antibody fragments of varying origin and produced recombinant IgE, IgA and IgG antibodies were employed. On this basis the IgE epitope on the major timothy grass pollen allergen Phl p 5a was assigned and characterised by different techniques. The approach pursued here may facilitate the access to allergen-specific recombinant

antibodies and contribute to the elucidation of the complex molecular interactions in allergy.

3.1.1 GENERATION OF ALLERGEN-SPECIFIC HUMAN ANTIBODY ISOTYPES

For establishing fully human, authentic antibodies, the sequence of a Phl p 5a-specific Fab fragment selected from an immune library was used as template for gene synthesis [122]. In an alternative approach, a murine hybridoma line was generated using Phl p 5a as immunogen. Antibody purification and N-terminal sequencing yielded the information needed for cloning of the variable regions from hybridoma cDNA (data not shown).

For expression of antibody fragments as different Ig isotypes in mammalian cells, fusion proteins were generated by use of modular cassettes containing signal sequences and restriction sites for cloning of variable regions and heavy and light chain constant regions of the human allergy-related immunoglobulins IgG1, IgG4, IgE, IgA1, and IgA2.

SDS-PAGE and immunoblotting of proteins isolated from culture supernatants of stably transfected HEK-293 cells verified the secretion of all immunoglobulins. Identity of the immunoglobulins and the presence of the particular Ig chains were confirmed by SDS-PAGE and immunoblotting (Fig. 3.1).

Molecular masses were found to be 160 kDa, 170 and 200 kDa for the heterotetrameric IgG, IgA and IgE antibodies, respectively. The expected molecular masses in SDS-PAGE suggest that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE and IgA antibodies. The triple bands obtained for the IgA2 correlate with those reported previously for recombinant IgA2 m [98] and are considered to represent different associates of heavy and light chains [127].

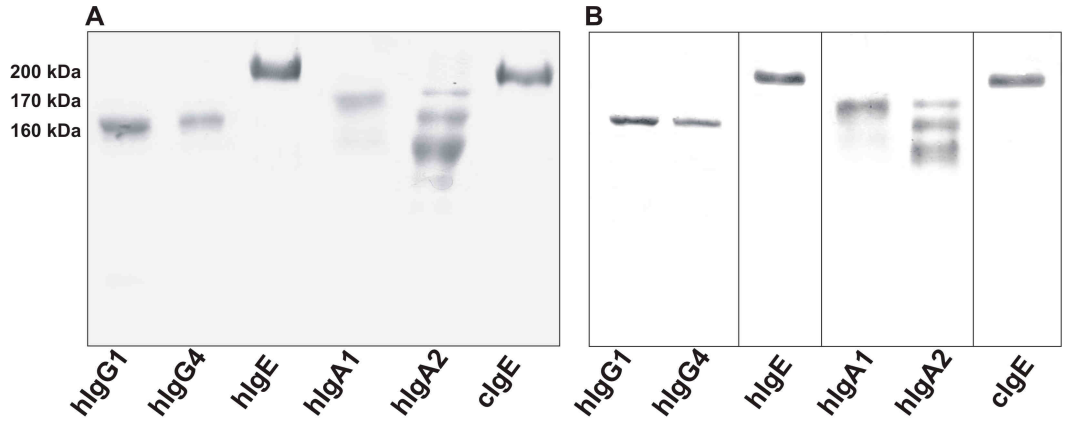


Fig. 3.1: SDS-PAGE and immunoblot analysis of IgG, IgE, and IgA antibodies
Purified proteins were assessed under non-reducing conditions by Coomassie staining (A), or immunoblot (B); the different isotypes were visualised using anti human IgG, IgE, and IgA antibodies conjugated to alkaline phosphatase.

3.1.2 CHARACTERISATION OF THE HUMAN ISOTYPES

Characterisation of the recombinant proteins was pursued by different approaches to demonstrate both antigen and receptor binding. All recombinant antibody isotypes detected Phl p 5a in ELISA (Fig. 3.2 A). Interestingly, Phl p 5b was neither bound by the human isotypes nor the chimeric 3-7 antibody, hinting at an epitope that is exclusively present in Phl p 5a.

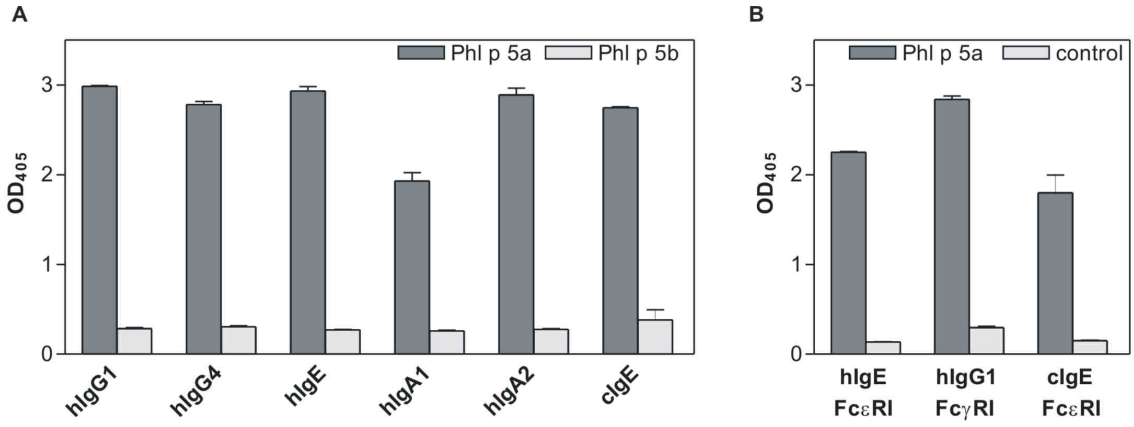


Fig. 3.2: Immunoreactivity with allergens and Fc receptor molecules
A: The immunoreactivity of the different recombinant human and chimeric antibodies was assessed in ELISA using MBP-Phl p 5a, and MBP-Phl p 5b, respectively, and isotype specific antibodies conjugated to alkaline phosphatase. B: Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high affinity Fc receptors and anti chicken IgG conjugated to alkaline phosphatase for detection.

Furthermore, soluble Ig Fc receptor constructs were produced to confirm proper folding and glycosylation of the Fc domains. Therefore, the extracellular domains of the ligand-binding α-chains of the human high affinity receptors FcεRI and FcγRI (CD64) were fused with chicken IgG (IgY) Fc domains and produced in mammalian cells [90]. Recombinant IgE as well as IgG specifically bound to their soluble Fc receptors (Fig. 3.2 B). These data underline that the recombinant isotypes reflect the natural interaction with both the antigen and the cellular receptors and therefore should mediate comparable effects.

Applicability of the recombinant IgE antibodies in routine diagnostic approaches was demonstrated by detection of Phl p 5a using commercial AlaBLOT immunoblot strips that contain extracted timothy pollen antigens (Fig. 3.3 A) and the laboratory analysers ImmunoCAP 250 and Immulite 2000 (Fig. 3.3 B,C).

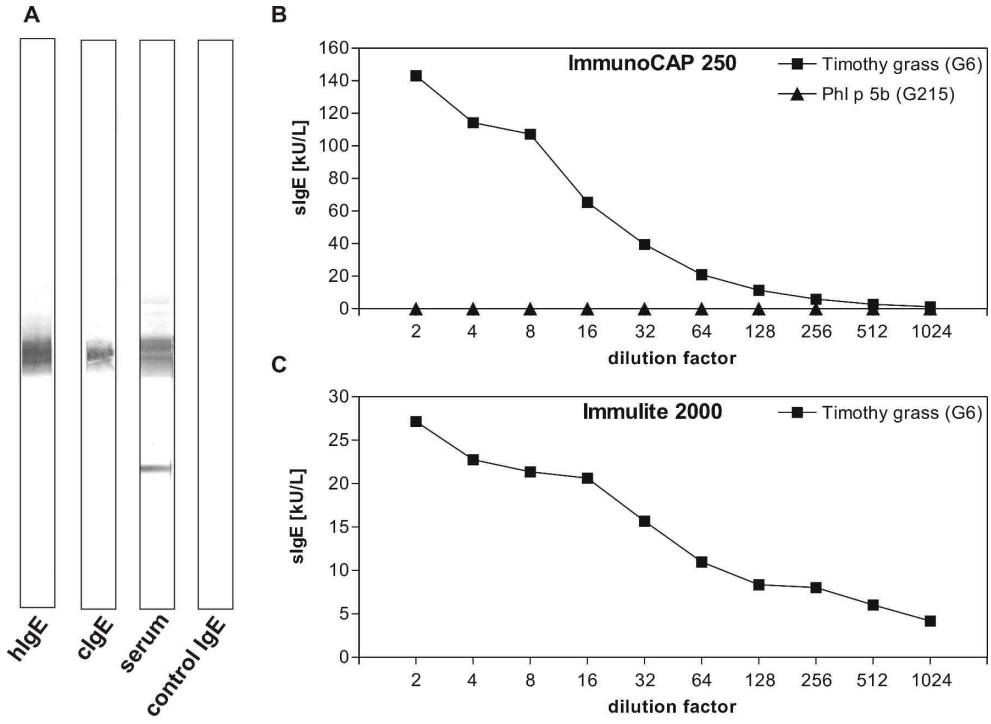


Fig. 3.3: Diagnostic applicability of recombinant IgE

A: Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a grass pollen allergic donor to AlaBLOTs according to the manufacturer´s recommendations. Detection was performed with anti human IgE conjugated to alkaline phosphatase.

B,C: Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE according to established protocols to the particular allergen preparations.

Interestingly, serial dilutions of recombinant IgE yielded up to 143 kU/L corresponding to class 6 on the ImmunoCAP 250 and 11 kU/L, corresponding to class 3 on the Immulite 2000 equipment. The single Phl p 5 allergen (allergen code G215) provided by Phadia, however, was not recognised at all, implying either steric hindrance or an isoform bias in the preparations used by the manufacturers.

3.1.3 ASSESSMENT OF THE CELLULAR ACTIVATION BY RECOMBINANT IGE

IgE-mediated cross-linking of the FcεRI and degranulation of RBL-SX38 cells was assessed by determination of β-hexosaminidase release (Fig. 3.4). Allergen-specific cellular activation is difficult to achieve since monoclonal IgE are not capable of cross-linking cellular receptors due to the availability of only one epitope per allergen. Thus, a multivalent molecule is needed to bridge two identical paratopes, an approach that has never been shown for recombinant monoclonal IgE. This issue was addressed by generating Phl p 5 microspheres using biotinylated allergen clustered with either soluble streptavidin (Fig. 3.4 A,C) or immobilized on streptavidin-coated particles (Fig. 3.4. B,D).

Monovalent allergen used as control was not able to activate RBL-SX38 cells as expected (data not shown). By contrast, using the soluble and the particle-based Phl p 5a microspheres efficient activation was demonstrated for hIgE and cIgE (Fig. 3.4. C). Upon sensitisation with hIgE, Phl p 5b microspheres did not induce activation, but cIgE and particle-based microspheres mediated degranulation. Mediator release, however, was observed to a reduced extent suggesting an avidity effect overcoming a putatively reduced affinity. These data suggest that both IgE exhibit an intrinsic potential to cross-link the FcεRI and activate effector cells.

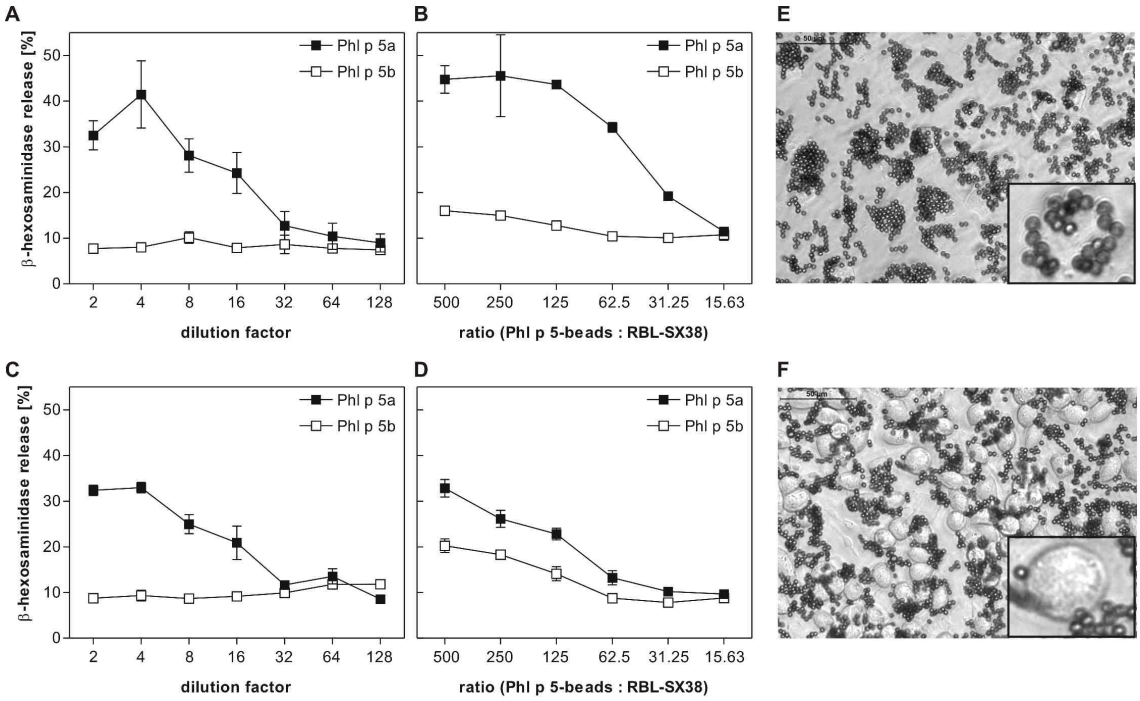


Fig. 3.4: Mediator release of humanised RBL-SX38 by Phl p 5 microspheres

RBL-SX38 cells providing the human Fc□RI were sensitised with both hIgE (A, B) and cIgE (C, D). Degranulation was induced by addition of biotinylated Phl p 5a and b fusion proteins complexed with either soluble streptavidin (A, C), or immobilised to streptavidin coated beads (B, D). Degranulation was monitored by □-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements. E, F: Light microscopic images of RBL-SX38 cells sensitised with hIgE and beads coated with Phl p 5a and Phl p 5b, respectively (E, F).

Given the recognition of non-overlapping, sterically available epitopes, however, monovalent soluble allergens are sufficient for receptor cross-linking. Sandwich ELISA using the hIgE and the cIgE as capture antibodies and the complementary antibody in form of another isotype for detection readily pointed to epitopes with an overall architecture enabling simultaneous binding of the hIgE and cIgE (Fig. 3.5 A).

Consequently, simultaneous use of both the fully human IgE and the chimeric IgE in mediator release assays also resulted in robust activation of RBL-SX38 cells using Phl p 5a in soluble, spatially non-clustered form (Fig. 3.5 B). By contrast, Phl p 5b did not induce any activation. These data show that the two IgE antibodies not only mimic the characteristics of natural IgE antibodies but also allow comparison with the natural situation in tissues using antibodies of different affinities.

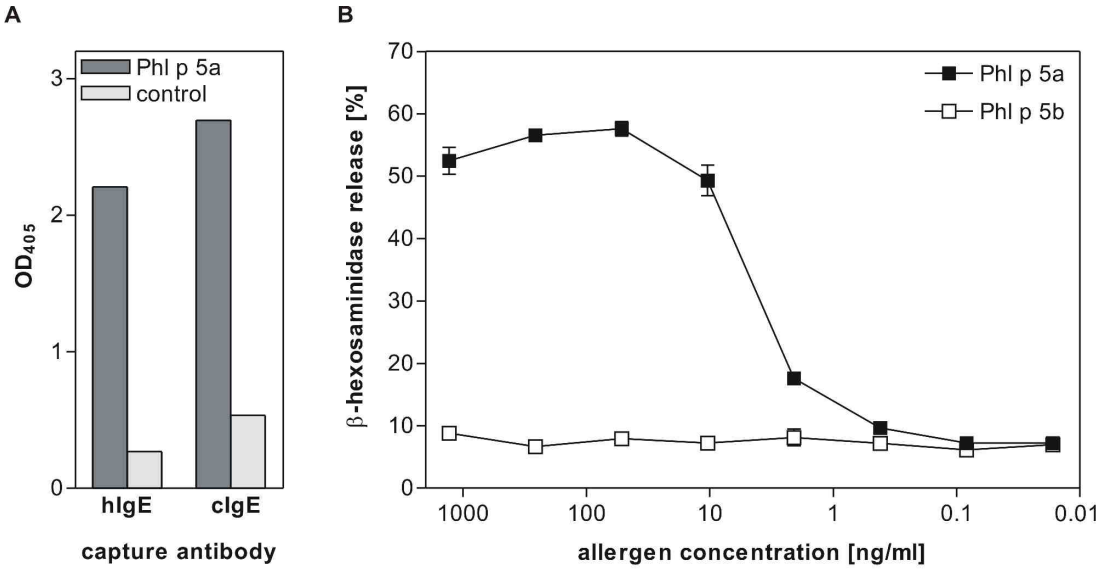


Fig. 3.5: Analysis of epitope distribution by ELISA and mediator release of RBL-SX38 cells
A: The epitope architecture was assessed in sandwich ELISA using the hIgE and the cIgE as capture antibody, and the complementary human and murine IgG isotypes for detection. B: Functional availability of the epitopes was verified by using RBL-SX38 cells sensitised simultaneously with hIgE and cIgE. Degranulation was induced by addition of an anti human IgE serum. Degranulation was monitored by β-hexosaminidase activity released into culture supernatants.

3.1.4 EPITOPE ANALYSIS

As evident from the ELISA and mediator release assays both antibodies apparently exhibit specificity for Phl p 5a. Having this pattern in mind, Phl p 5a and b fragments and chimeras were generated to dissect the interplay of allergens with antibodies on a molecular level (Fig. 3.6 A). These fragments represent variations of different elements of the N-terminal 4 helix bundle. Prokaryotic expression of allergens tends to intracellular aggregation yielding insoluble protein fractions, therefore, these proteins were fused with MBP (full length Phl p 5a and b) or CBD (all fragments including chimeric F1) to avoid detrimental effects on folding and solubility, and purified by affinity chromatography. Subsequent immunoblot analyses demonstrated hIgE reactivities with the fragments F1, F2, and F3, but not with F4 and F5 (Fig. 3.6 B).

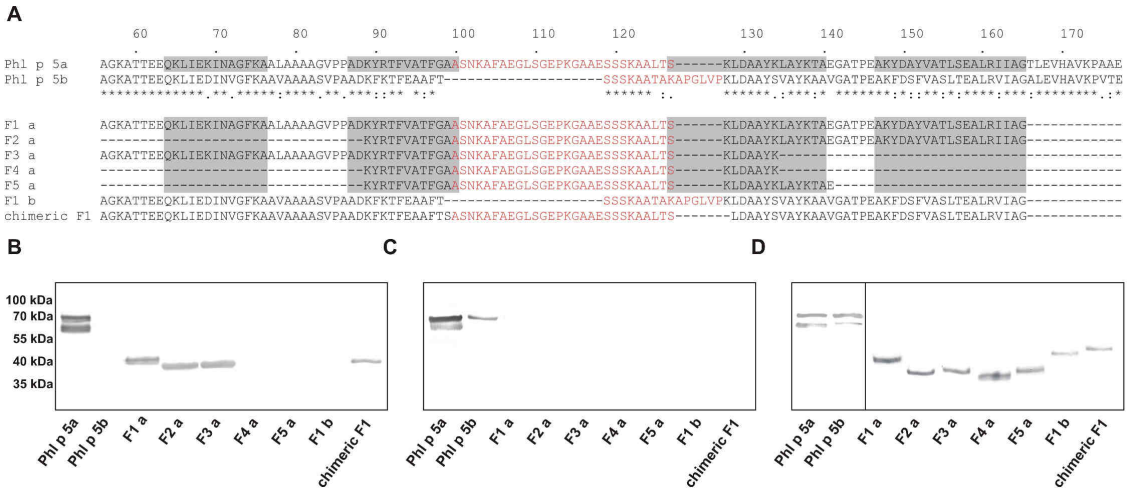


Fig. 3.6: Epitope analyses using fragments and mutants of Phl p 5 in immunoblot

A: Alignment of the N-terminal region of Phl p 5a and Phl p 5b (A, top) in comparison with generated fragments (F1 a - F5 a, and F1 b, chimeric F1) (A, bottom) was developed using ClustalW2. Total and partial conservation is indicated with stars, colons, and dots. Sequences forming predicted helix bundles are highlighted with a grey background. Residues of the Phl p 5a stretch grafted into the framework of Phl p 5b are depicted in red. B,C,D: Immunoreactivities of the fusion proteins shown in A in immunoblotting using hIgE (B), cIgE (C), and anti-fusion protein antibodies as loading controls. Detection was performed using the particular secondary antibodies conjugated to alkaline phosphatase.

These findings suggested that the unique insertion present in Phl p 5a functions as an IgE epitope. In order to verify this observation the particular amino acids of Phl p 5a were grafted into the non-reactive Phl p 5b framework which resulted in de novo establishment of pronounced IgE reactivity. This conversion includes the entire loop region according to the secondary structures observed in NMR analyses [128] and suggests that this stretch is sufficient and essential for epitope formation and IgE reactivity. Most prominent amino acid variations are located in a stretch of 20 amino acids at the N-terminus of the loop. This finding is consistent with the reactivities of the different allergen fragments and mutants.

In contrast to hIgE, the cIgE recognised the Phl p 5 full length proteins only (Fig. 3.6 C,D). The pattern revealing a reduced reactivity with Phl p 5b suggests an epitope within the C-terminal domain that is conserved in Phl p 5b to a certain degree only.

The hIgE epitope itself is defined by the second loop of the N-terminal first 4 helix bundle domain as evident after modelling both isoforms by using the structure of Phl p 6 as template (Fig. 3.7). Although not fully in accordance with the NMR data, this model is helpful to exemplify the structural basis of the epitope.

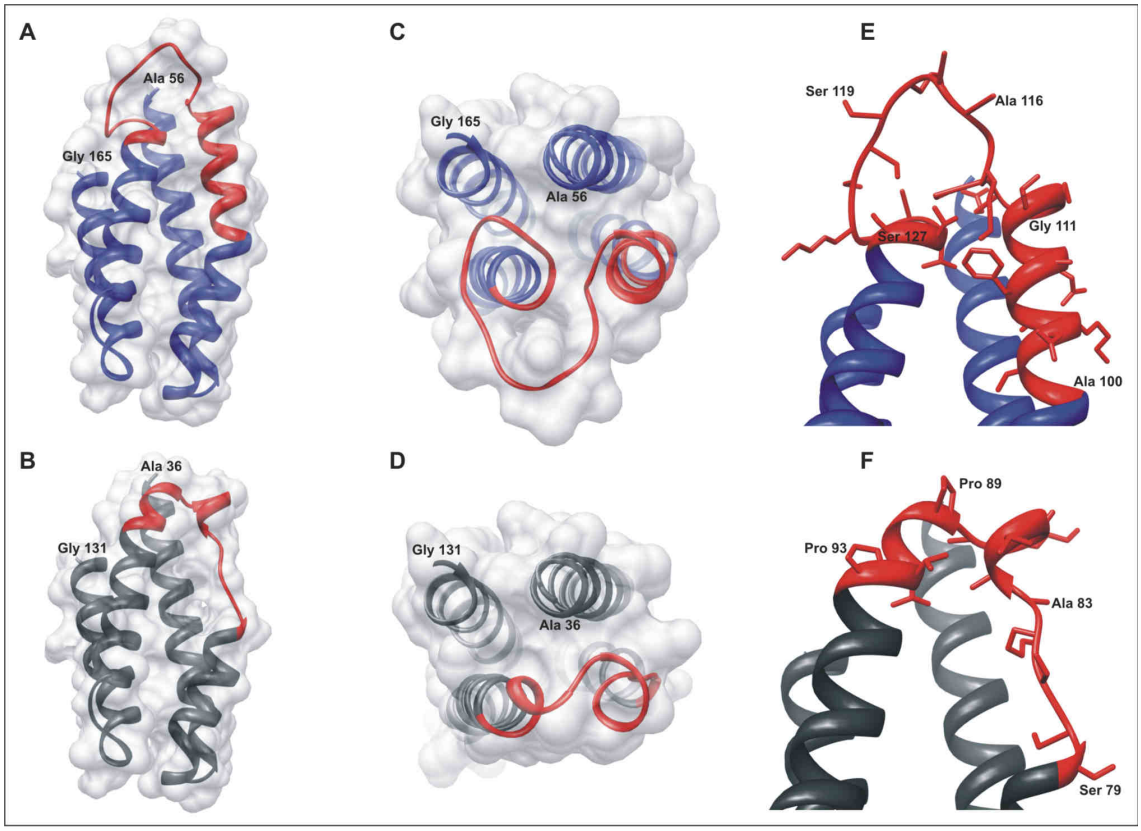


Fig. 3.7: Molecular modelling and IgE epitope mapping on the surface of F1 a (A,C,E) and F1 b (B,D,F)

The modelling was performed using the structure of Phl p 6 as template (front view in the A,B; top view in C,D; close up in E,F). The assignment of helical elements shown here does not coincide with that deduced from the NMR structure of the first AR repeat of Phl p 5a. Amino acids that correspond to the identified stretch are highlighted in red. The secondary structures and the surface representations visualise the variations of the molecular topology.

The loop forming stretch significantly protrudes from the protein and represents a unique feature of Phl p 5a (Fig. 3.7 A,C,E). Insertion of additional amino acids results in an elongation of the helical element in Phl p 5b (Fig. 3.7. B,D,F) which is accompanied by a drastically increased exposure of the loop region. This region contains an elevated number of polar residues including lysine, serine, glutamate and asparagine residues. Although a continuous stretch within the primary structure, its secondary structure appears to be crucial for reactivity. As suggested by the IgE reactivities of F1-F5, interference with the folding of the two flanking helices into antiparallel strands abolishes the IgE reactivity.

3.2 A HUMAN MONOCLONAL IGE DERIVED FROM HYBRID REPERTOIRE LIBRARIESDEFINES AN EPITOPE GENUINE FOR BET V 1 AND *FAGALES* PR-10 PROTEINS

Birch pollen are a major cause of type I allergies in the temperate climate zone of the northern hemisphere. Over 95 % of the tree pollen sensitized patients display IgE binding to Bet v 1, and 60 % react exclusively to this allergen [129]. It is also considered the prototype of the PR-10 protein superfamily [130]. Pollen of *Fagales* species [131] and several foods [132-134] contain homologues sharing epitopes with Bet v 1 and cross reactive IgE are fundamental for the oral allergy syndrome. Hence, Bet v 1 represents a dominant major antigen in immunity and allergy, and has been extensively characterized. Its crucial role in sensitization also renders Bet v 1 an outstanding target for development of therapeutic strategies [135].

Structural analyses [136] and immunological findings [137, 138] illustrated the relevance of conformational epitopes for Bet v 1. A dissection of IgE epitopes of this outstanding allergen on a molecular level nevertheless remains to be done. The characterisation of allergen/IgE interactions however is unequally difficult due to the evanescent abundance of IgE rendering hybridoma technologies difficult [112]. The paucity of successful isolations of specific IgE antibody fragments [122, 124] reflects the fact that the establishment of IgE-derived immune repertoires is highly ambitious. Another quandrary is the vast predominance of IgG and IgM derived light chains. In order to gain access to authentic human allergen-specific antibodies and their epitopes IgE-derived hybrid libraries were established. On the basis of a library-derived antibody the corresponding IgE epitope on the surface of Bet v 1 and other PR-10 proteins was assigned. This approach may contribute to the elucidation of allergen/IgE interaction on a molecular level.

Analyses of the molecular basis underlying allergenicity and allergen cross-reactivity, as well as improvement of allergy diagnostics and therapeutics are hampered by the lack of human monoclonal IgE antibodies and knowledge about their epitopes. Here, the consecutive generation and epitope delineation of a human monoclonal IgE against the prototype allergen Bet v 1 from birch pollen is reported. Immune/synthetic hybrid libraries were established from pollen allergic donor-derived V_H regions complemented

with synthetic V_L regions. By selection, antibody fragments with specificity for the major birch pollen allergen Bet v 1 were obtained, reconverted to human IgG, IgA and IgE formats, and assessed for their characteristics.

3.2.1 ESTABLISHMENT AND SELECTION OF THE HYBRID IGE REPERTOIRE LIBRARIES

The IgE V_H repertoires of three allergic donors were amplified from cDNA and the IgE background of individual V_H was confirmed by sequence analysis.

These repertoires subsequently were combined with a human semisynthetic V_L repertoire [116]. Resulting hybrid libraries of more than 10^7 independent clones for each donor were pooled for selection. After three rounds of iterative panning against Bet v 1, ELISA analyses demonstrated significant enrichment of the library (Fig. 3.8 A), and reactive clones (Fig. 3.8 B) were subjected to sequence analysis (data not shown).

Amplification of the V_H of one clone within its epsilon context selectively from the cDNA of one donor (data not shown) verified identity and isotype. After prokaryotic expression (Fig. 3.8 C) the monovalent scFv demonstrated robust immunoreactivity (Fig. 3.8 D).

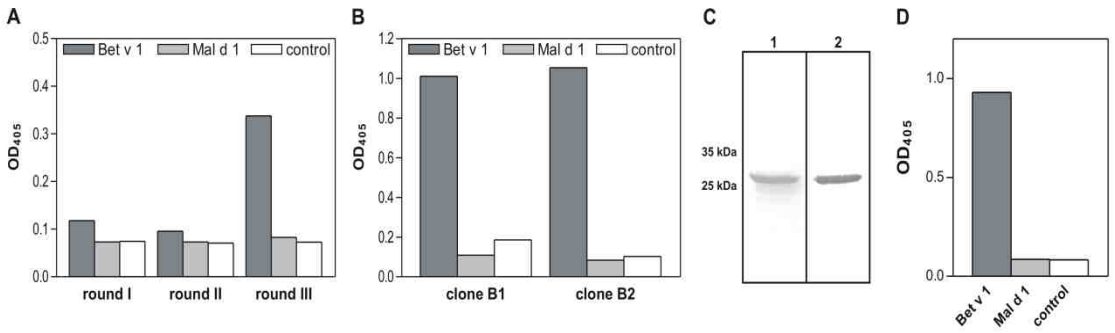


Fig. 3.8: Immunoreactivity and expression of selected antibody fragments
The immunoreactivity of the enriched library (A) as well as selected phage clones (B) against Bet v 1 (filled bars, controls by omission of antigen: open bars) was analysed by ELISA as described in methods. After expression, soluble, purified scFv was assessed by SDS-PAGE and immunoblot (C) analysis and reactivity verified in ELISA(D).

These data demonstrate that hybrid IgE repertoires allow for isolation of specific antibodies while circumventing donor-derived V_L repertoires.

3.2.2 GENERATION AND CHARACTERIZATION OF RECOMBINANT IMMUNOGLOBULINS

For subsequent analyses the antibody fragment was converted into allergy-related homodimeric IgG1, IgG4, IgE, IgA1, and IgA2 immunoglobulin formats. After expression in mammalian cells, immunoblotting analyses (data not shown) verified the molecular masses suggesting proper folding and glycosylation, in particular for the extensively glycosylated IgE and IgA formats.

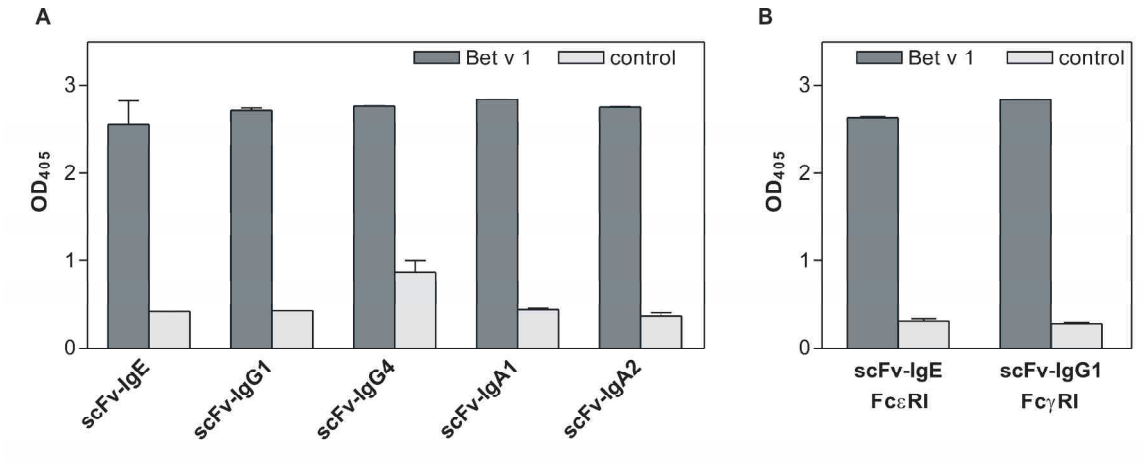


Fig. 3.9: Immunoreactivity in ELISA
Immunoreactivity with allergens and Fc receptors: (A) The immunoreactivity of the different recombinant human antibodies was assessed in ELISA using MBP-Bet v 1 and isotype specific antibodies conjugated to alkaline phosphatase. (B) Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high affinity Fc receptor and anti chicken IgG conjugated to alkaline phosphatase for detection.

All recombinant antibodies detected their particular target allergen in ELISA (Fig. 3.9 A) as well as simultaneous binding to the corresponding Fc receptor (Fig. 3.9 B). The use of commercial diagnostic lab analysers (Fig. 3.10, left panel) and AlaBLOT strips containing a plethora of extracted antigens (Fig. 3.10, right panel), suggested applicability of the different recombinant antibodies in diverse types of downstream approaches.

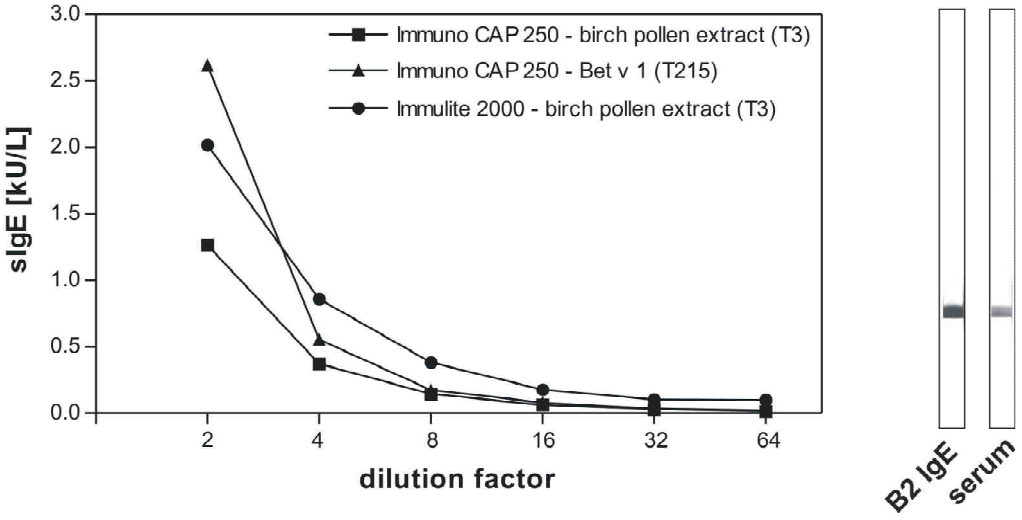


Fig. 3.10: Diagnostic application of recombinant IgE

A: Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE to immobilized birch pollen extract (T3) or Bet v 1 (T215) and detected using anti-human IgE AP conjugates. B: Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a pollen allergic donor to AlaBLOTs according to the manufacturer´ıs recommendations. Detection was performed with anti human IgE conjugated to alkaline phosphatase.

3.2.3 EPITOPE ANALYSIS OF THE BET V 1 SPECIFIC ANTIBODIES

In order to dissect the molecular interaction of the Bet v 1 specific IgE with its cognate target structure, the advantage of different Bet v 1 isoforms and other PR-10 allergens such as Mal d 1 was teken. Notably, Mal d 1 and Bet v 1 iso, the isoform obtained from birch genomic DNA, did not show any reactivity. N-terminally truncated Bet v 1 fragments and chimeras fused with MBP were generated (Fig. 3.11 A).

Immunoblot analyses demonstrated rIgE reactivities with Bet v 1a and the fragments d58, d91, d110, and d124 (Fig. 3.11 C). The d124 fragment comprises the C-terminal helical secondary structure, which apparently is sufficient for IgE epitope establishment.

As compared to the non-reactive isoform 1.02B only the amino acid residue glutamate 149 is substituted. Introduction of the corresponding substitution E149G abolished the rIgE reactivity of the fragment d124 E149G, a finding also observed for the full length Bet v 1 (Fig. 3.11 C). This inducible loss of IgE reactivity in the fragment and in the full length protein proves the critical relevance of this particular residue for the antibody/allergen interaction and strongly suggests that the terminal helix of Bet v 1 constitutes the epitope of the rIgE.

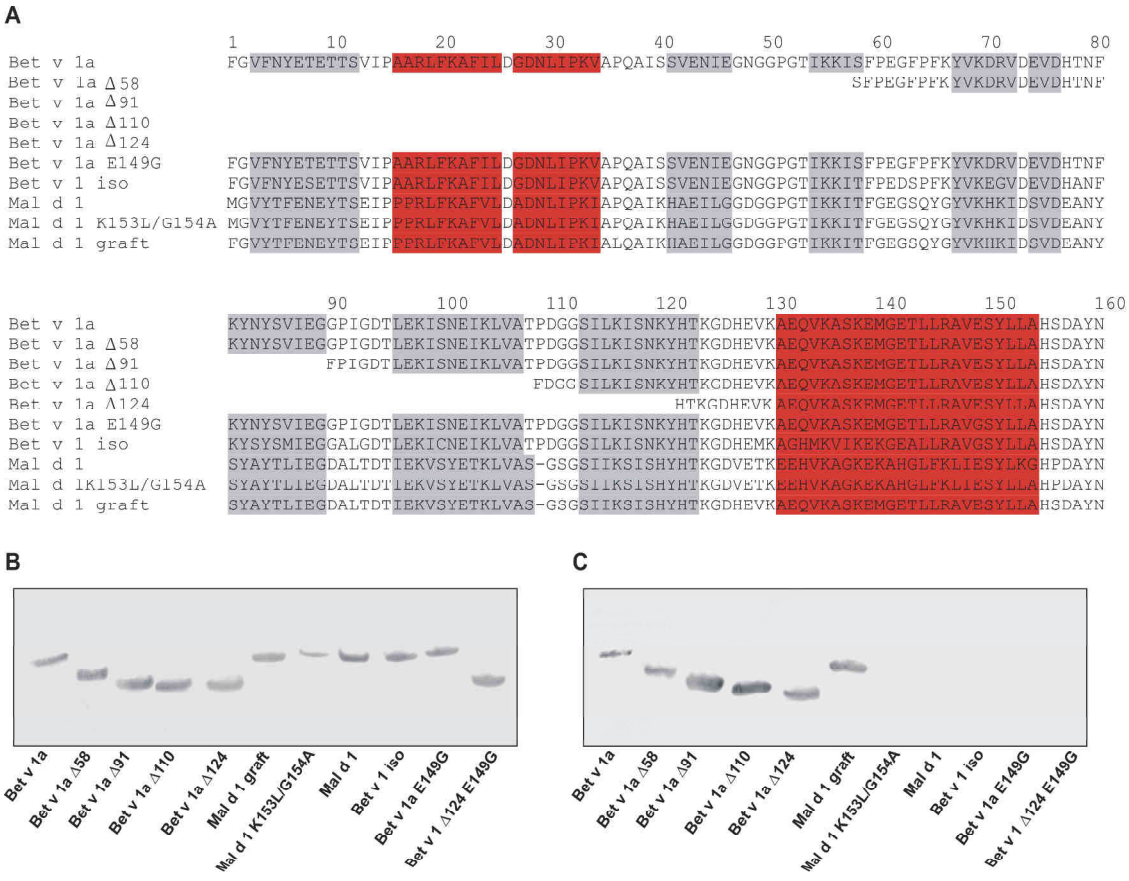


Fig. 3.11: Epitope analyses using fragments and mutants of Bet v 1 in immunoblot

A: Alignment of the Bet v 1a, isoforms, homologous and derived fragments developed using ClustalW2. Sequences forming predicted beta-sheets or helices are highlighted in grey and red, respectively. B,C: Immunoreactivities of different fusion proteins shown in A in immunoblotting using and anti-fusion protein antibodies as loading controls (B), and the hIgE (C). Detection was performed using the anti-human IgE conjugated to alkaline phosphatase.

To further elucidate the molecular basis of the selective specificity, the amino acids 128-156 of Bet v 1A were grafted into the per se non-reactive framework of Mal d 1. This grafting resulted in de novo establishment of pronounced IgE reactivity of Mal d 1 (Fig. 3.11 C) again emphasising that the terminal helix is sufficient and crucial for epitope formation.

By modelling approaches the impact of the helical C-terminus on the surface of Bet v 1, Mal d 1 and the epitope graft were assessed. Intriguingly, the residue E149 is conserved in Mal d 1 (E148) and folding and overall structure of Bet v 1 and Mal d 1 are highly similar (Fig. 3.12). The Mal d 1 E149 nevertheless appears less accessible. Analyses of electrostatic potential and hydrophobicity revealed striking differences of the surface composition of Bet v 1 and Mal d 1 (Fig. 3.12 B, C).

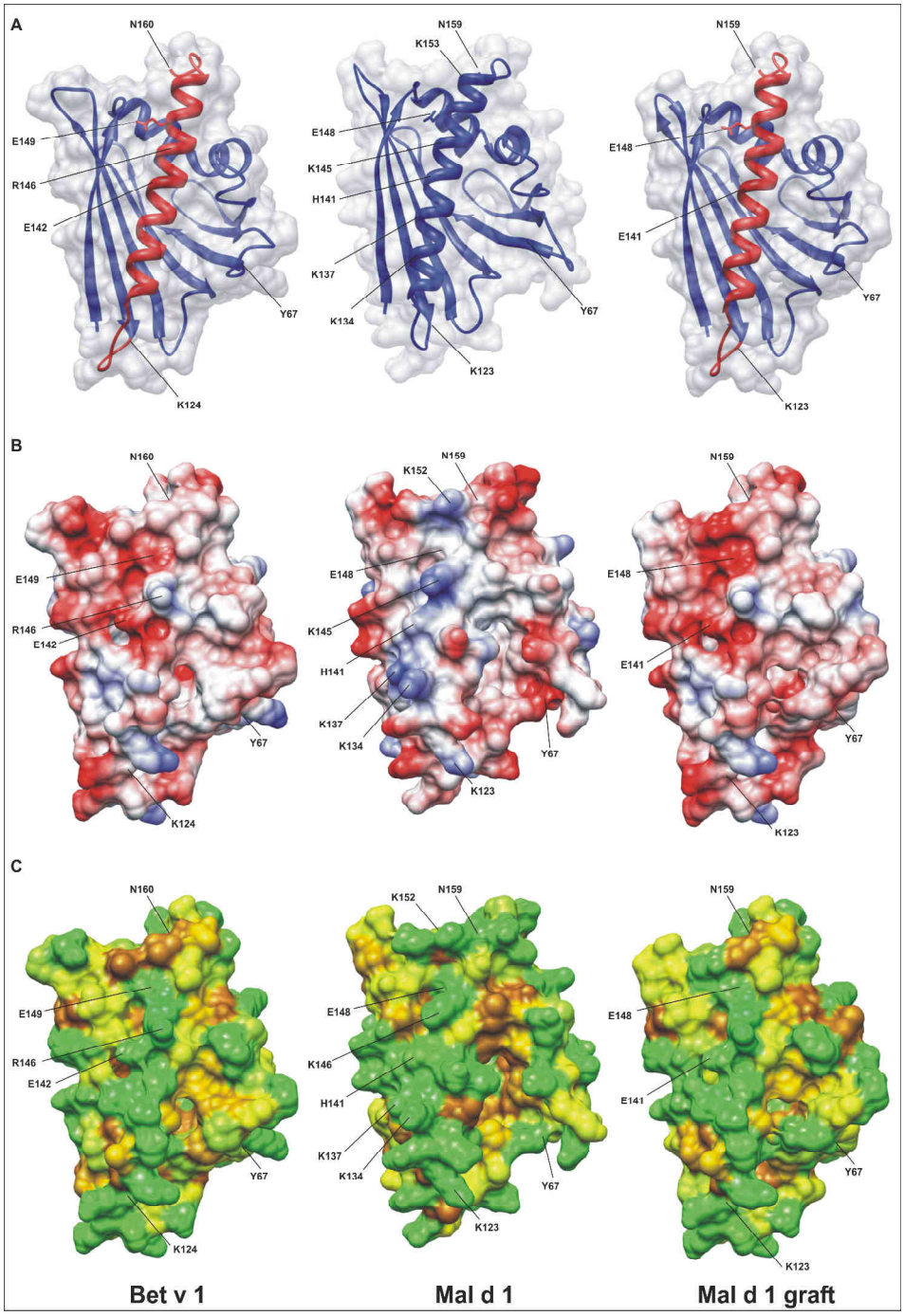


Fig. 3.12: Structure of Bet v 1 and the IgE epitope

Molecular modelling and IgE epitope mapping on the surface of Bet v 1 (left), Mald d 1 (middle), and the Mal d 1 graft (right). The modelling was performed using the structure of Bet v 1 as template. Amino acids that correspond to the identified epitope stretch are highlighted in red (A). The secondary structures and the surface representations according to electrostatic potential (B) as well as hydrophobicity (C) visualise the variations of the molecular topology.

As evident from the models, the helix of Mal d 1 as well as a number of residues in proximity is dominated by positively charged residues (H142; K140; K135; K138; K153) which form a linear entity over the length of the Mal d 1 molecule and render the

surface significantly different from that of Bet v 1. The surface clustered residues K135 and K138 are also present in Bet v 1, but apparently are spatially separated by S137.

3.2.4 EPITOPE EVALUATION OF BET V 1 ISOFORMS AND OTHER PR-10 PROTEINS

Primary structure analyses of different Bet v 1 isoforms and PR-10 proteins were performed to evaluate the presence of the epitope motif. Expectedly, the 30 Bet v 1 isoforms available from databases show high sequence conservation within the last 37 amino acid residues (data not shown). Notably, the residues 144-160 as well as the residues 125-135 show almost full identity in >95 % of Bet v 1 isoforms. Non-conservatively replaced residues however include position 137 (S or I), 140 (K or M), and 141 (A, G, or R).

Whereas this motif appears well conserved throughout Bet v 1 isoforms, only particular homologous PR-10 proteins show sequence identity rendering a formation of the epitope likely (Fig. 3.13). Roughly in accordance with overall sequence identities, the C-terminal 37 amino acids of Bet v 1 show an elevated identity to the *Fagales*PR-10 proteins Aln g 1 of 72 % and to Cor a 1 and Carb b 1 of 70 %, to other members such as Fag s 1 (54 %), Que a 1 (51 %), and Cas s 1 (51 %) a more reduced identity (Fig. 3.13).

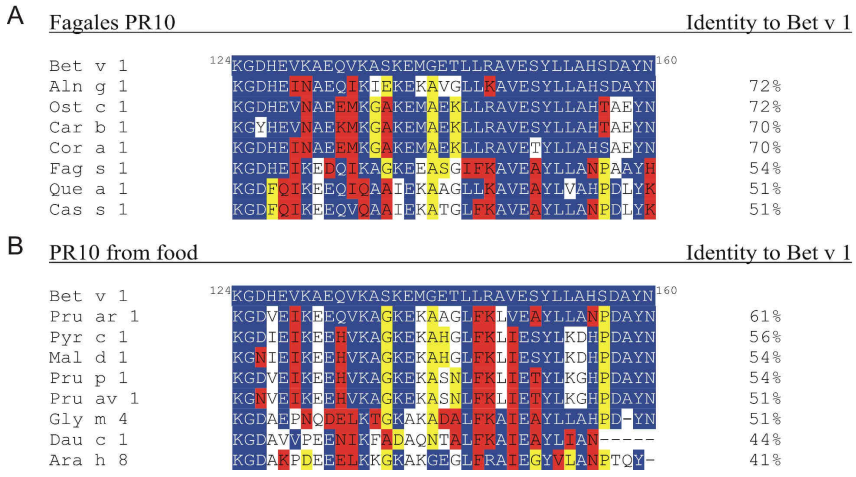


Fig. 3.13: Alignment of PR-10 protein C-termini from pollen and food
Alignment of *Fagales* (A) and food-derived (B) PR-10 protein C-termini. Residues identical to Bet v 1 are highlighted in blue, conservative amino acid substitutions in red and semi-conservative substitutions in yellow.

In PR-10 allergens from food, however, the epitope appears to be absent. Sequence identities within the terminal 37 residues are in the range of 41% (Dau c 1) to 56 % (Pyr c 1). Somewhat outstanding in this context is Pru ar 1 having 61% identity (Fig.

3.13). Interestingly, the only helix residues conserved throughout all proteins included here are the E149 and the Y151 emphasising their relevance for PR-10 proteins.

These findings conclusively emphasises that amino acid substitutions throughout PR-10 family members are crucial for IgE cross-reactivity.

3.3 EVALUATION OF DIFFERENT GLYCOFORMS OF HONEYBEE VENOM MAJOR ALLERGEN PHOSPHOLIPASE A2 (API M 1) PRODUCED IN INSECT CELLS

Anaphylaxis due to honeybee (*Apis mellifera*) venom is one of the most severe clinical outcomes of IgE-mediated hypersensitivity reactions. Although venom immunotherapy is effective in the majority of patients the occurrence of systemic side effects in 20-40 % of treated individuals [139] and the failure of therapy in 10-20% of cases [140] demands a component-resolved approach to HBV allergy. Since the use of native allergens is often hampered by means of quantity and purity recombinant allergens are increasingly introduced into diagnostic and therapeutic applications [141]. Moreover, the recombinant availability is a prerequisite for the rational design of hypoallergenic variants and molecules with defined characteristics such as proper folding and glycosylation and concurrent lack of cross-reactive carbohydrate determinants which still represent a challenge for adequate allergy diagnosis. Defining glycosylation of allergens can have intriguing consequences for our understanding of IgE reactivities reported previously for the hyaluronidases [142].

HBV is a complex mixture of biogenic amines, peptides, toxins and proteins, with most of them being enzymes. Many of the proteins and polypeptides in HBV have been reported to be allergens including phospholipase A2 (Api m 1)[143], hyaluronidase (Api m 2) [144], acid phosphatase (Api m 3) [145], melittin (Api m 4) [146], dipeptidylpeptidase IV (Api m 5) [147], Api m 6 polypeptides (4 isoforms) [148], a CUB-serine protease (Api m 7) [149], a carboxylesterase (Api m 8), a carboxypeptidase (Api m 9) [150], and the Carbohydrate-rich protein (Api m 10), a component of unknown function [151].

HBV phospholipase A2 (Api m 1) was early identified as the major venom allergen [143]. Based on the fact that 97 % of HBV allergic patients have Api m 1 specific IgE

antibodies Api m 1 was extensively studied as model allergen for genetic engineering and specific immunotherapy [152]. Moreover, Api m 1 is considered an ideal marker allergen since it is structurally unrelated to the phospholipase A1 (Ves v 1) in vespid venom [153].

In general, phospholipases constitute a major component of various venoms, e.g. from snakes, bees, wasps and spiders. The PLA2 of *A. mellifera* has been sequenced from a venom gland cDNA library [154] and encodes a signal peptide of 18 amino acids, a propeptide of 15 residues, and a major peptide of 134 residues [155], the catalytic activity and crystal structure of which is well documented [156-158]. The mature peptide contains 10 cysteine residues forming 5 disulfide bonds [159] and an oligosaccharide attached to asparagin 13 [160].

PLA2 (EC 3.1.1.4) from HBV is a typical group III secreted PLA2 [161], representing about 12 % of the dry weight of the venom [162]. The cytotoxic effects of PLA2 rely on its catalytic activity which leads to cleavage of phospholipids at the SN2 acyl chain and damage of structural membranes [162]. The enzyme in HBV is structurally not related to the phospholipases A1 in vespid venoms, and therefore, not cross-reactive. In contrast to unglycosylated vespid PLA1, PLA2 of honeybees exhibits CCD (cross-reactive carbohydrate determinant)-reactivity [163] which is essentially based on the presence of α-1,3-core fucose and can lead to results with no or only low clinical relevance in diagnostic tests and therefore represents a challenge for diagnostic approaches [164].

The aim of this study was an improved production of soluble HBV PLA2 in glycosylation-competent insect cells. Therefore, an inactive mutant form of PLA2 by site-directed mutagenesis, which apart from an improved expression behavior should exhibit a reduced cytotoxicity in cellular assays, was generated. It was possible to produce both allergens as soluble and secreted proteins in high yields by baculovirus-based expression in Sf9 (*S. frugiperda*) as well as HighFive (*T. ni*) insect cells. By using cell lines with a varying capacity for α-1,3-core fucosylation, and, additionally, by generation of a non-glycosylated mutant form, the evidence for reduced IgE cross-reactivity and improved performance of the resulting PLA2 molecules was provided in diagnostic approaches to hymenoptera venom allergy.

3.3.1 cDNA CLONING AND RECOMBINANT EXPRESSION IN INSECT CELLS

For recombinant production the cDNA of the HBV allergen Api m 1 was amplified from whole *A. mellifera* venom gland cDNA. To avoid potentially detrimental effects of phospholipase activity on expression yields or on mediator release in cellular assays the inactive mutant Api m 1 H34Q was generated by site directed mutagenesis. To address the impact of glycosylation of the recombinant allergen on the IgE reactivity of sera derived from hymenoptera venom allergic patients an additional mutant version of Api m 1, Api m 1 H34Q N13Q, devoid of the singular glycosylation site was generated.

Subsequently, both epitope tagged proteins were produced by baculovirus-based infection of insect cells and secretion of the proteins into the cellular supernatant. The glycosylated version Api m 1 H34Q was expressed in Sf9 (*S. frugiperda*) cells and additionally in HighFive (*T. ni*) cells, which in contrast to Sf9 cells provide α-1,3-core-fucosylation of N-glycans, the structure responsible for CCD-based cross-reactivity. The mutant version Api m 1 H34Q N13Q lacking the relevant asparagine and, therefore, any glycosylation was produced in HighFive cells.

The rApi m 1 versions were obtained with yields of approx. 5-10 µg/ml of culture supernatant without further optimization of culture conditions. SDS-PAGE analysis and Coomassie staining (Fig. 3.14 A) of purified rApi m 1 H34Q produced in Sf9 as well as in HighFive insect cells showed three distinct bands in the range of approx. 18 to 22 kDa, a molecular mass to which the C-terminally fused V5 epitope and 6 fold His tag contribute 3 kDa.

The Sf9-expressed purified Api m 1 H34Q showed a double band of comparable intensity and a third, less intense band of lower molecular weight. In contrast, for the HighFive-produced Api m 1 H34Q the protein with the highest molecular weight represents the predominant product, while the product with the lowest molecular weight is only barely visible. Applying an antibody directed against the V5 epitope (Fig. 3.14 B) all bands of the Sf9-produced rApi m 1 H34Q were detected, a finding which is in good accordance with the distribution observed in Coomassie staining. The lowest molecular weight band of the HighFive-expressed rApi m 1 was not visible, most likely due to its reduced quantity. Native purified Api m 1, however, shows a similar distribution in Coomassie staining, whereas in HBV only the two higher molecular weight bands were visible. Thereby, the highest molecular weight versions represent the

predominating forms. Non-glycosylated rApi m 1 H34Q N13Q appears as a single band of approx. 19 kDa (Fig. 3.14 A). Application of the sensitive V5 epitope antibody, however, enabled detection of a faint double-band (Fig. 3.14 B).

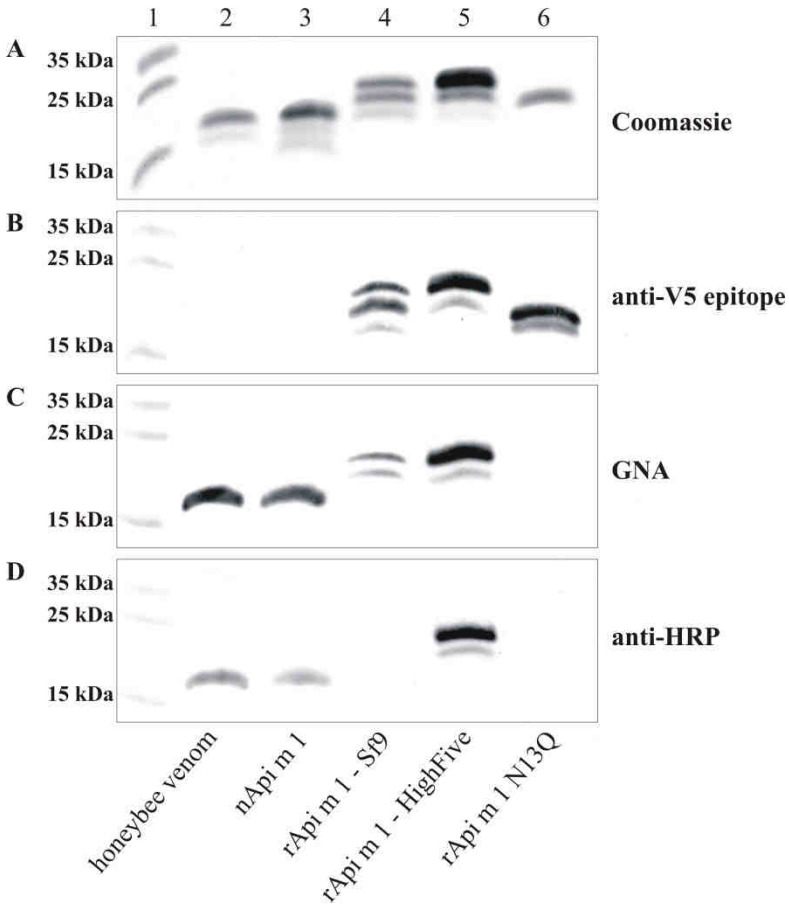


Fig. 3.14: Characterization of native and recombinant HBV phospholipase A2
Honeybee venom, native purified PLA2, recombinant PLA2 H34Q from Sf9 cells, recombinant PLA2 H34Q from HighFive cells, and recombinant PLA2 H34Q N13Q from HighFive cells were subjected to SDS-PAGE (10 % acrylamide) and analyzed by Coomassie staining and Western blot. A: Coomassie brilliant blue staining, B-D: Western blot analysis applying a monoclonal antibody directed against the V5 epitope C-terminally fused to the recombinant PLA2 versions (B), *Galanthus nivalis* agglutinin recognizing terminal mannose, 1,2-, 1,3- and 1,6-linked to mannose (C), and polyclonal HRP-antiserum produced in rabbit with specificity for α-1,3-core fucose (D).

Galanthus nivalis agglutinin (GNA) recognizes terminal mannose, 1,2-, 1,3- or 1,6-linked to mannose, a structure which is present in native Api m 1 as well as in glycans derived from lepidopteran insect cells. Thus, GNA reactivity indicates the presence of N-linked glycans. In immunoblot analysis of rApi m 1 H34Q (Fig. 3.14 C) GNA reacted with the two higher molecular weight bands proving the lowest molecular weight form to be non-glycosylated, whereas in HBV and nApi m 1 only the highest molecular

weight product is recognized. Thereby, the missing reactivity with rApi m 1 H34Q N13Q verified the successful substitution of the N-glycosylation site.

Apart from proteinic epitopes anti-HRP rabbit serum (Fig. 3.14 D) is specific for plant-derived glycostructures including α-1,3-core fucose and β-1,2-xylose, the causative structures for CCD-based cross-reactivities. Its use in immunoblot revealed pronounced α-1,3-core fucosylation for rApi m 1 H34Q produced in HighFive cells as well as for native Api m 1. In contrast, rApi m 1 H34Q produced in Sf9 cells did not exhibit any CCD-based reactivity.

3.3.2 IMMUNOREACTIVITY OF RAPI M 1

Reactivity of sera from patients with a clinical history of insect venom allergy was assessed by ELISA for specific IgE antibodies to the recombinant and native versions of Api m 1 (Fig. 3.15).

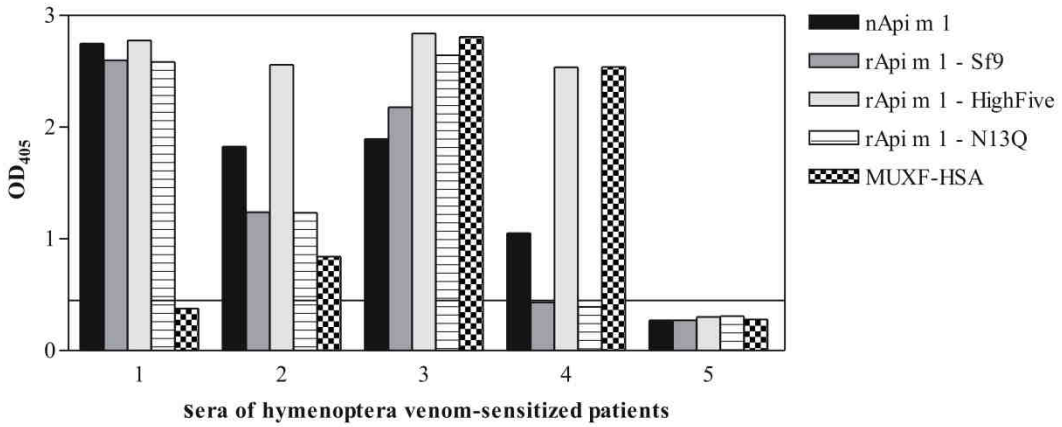


Fig. 3.15: IgE reactivity of individual patient sera with native and recombinant PLA2
The IgE reactivity of individual sera from hymenoptera venom-sensitized patients with native and recombinant PLA2 versions, as well as with the MUXF-HSA conjugate was assessed in ELISA as described in the Methods section. The lower end functional cut-off is represented by a solid line.

MUXF-HSA, a conjugate that provides the core-fucosylated glycotope from pineapple stem bromelain was included as a marker for sIgE directed against CCDs. To illustrate the distinct recognition patterns observed in insect venom sensitized patients the sIgE-reactivity of selected sera is shown. Serum 1 specifically recognized proteinic epitopes of Api m 1 as indicated by the missing reactivity with the CCD marker MUXF-HSA. All recombinant versions of Api m 1 and native Api m 1 showed comparable reactivity.

In contrast, sera 2 and 3 exhibit reactivity with Api m 1 but apparently CCDs contribute to this binding as suggested by the reactivity observed with MUXF-HSA, and this contribution is at varying degrees according to the more pronounced signal with the fucosylated allergen in the case of serum 2. Serum 4 was reactive with native Api m 1 und HighFive-derived Api m 1 H34Q, but neither with the counterpart produced in Sf9 cells nor with non-glycosylated Api m 1, indicating exclusive CCD-reactivity, which is corroborated by the pronounced MUXF reactivity. Finally, serum 5 from a patient with a clinical history of YJV allergy neither recognizes proteinic epitopes of Api m 1 nor CCDs.

These data demonstrate that Sf9-derived glycosylated Api m 1 as well as non-glycosylated Api m 1 lack detectable α-1,3-core fucosylation which contributes to or is the exclusive reason for reactivity with sera having CCD-specific IgE antibodies.

3.3.3 DEGRANULATION OF RAT BASOPHILIC LEUKEMIA (RBL) CELLS

Since native Api m 1 is described to induce mediator release from rodent and human basophils in an IgE-independent manner as a result of its catalytic activity, the ability of nApi m 1 and Api m 1 H34Q to mediate degranulation of RBL cells, as a model system for effector cell degranulation was tested (Fig. 3.16).

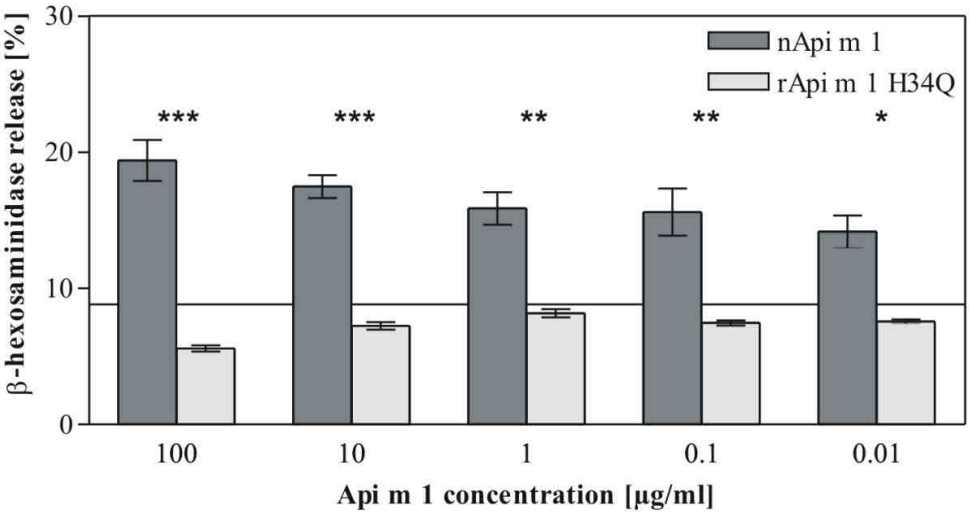


Fig. 3.16: IgE-independent activation of rodent basophils by active and inactive PLA2
Release of β-hexosaminidase from RBL-SX38 cells in response to native purified and recombinant inactive PLA2. The percent release is presented relative to total β-hexosaminidase content. All results were measured as triplicates and subjected to statistical analysis via student's *t*-test.

Upon incubation with nApi m 1 RBL cells showed significant β-hexosaminadase release over the entire concentration range from 0.01 to 100 µg/ml in a dose-dependent manner. In contrast, RBL cells incubated with inactive Api m 1 H34Q showed no degranulation when compared to negative controls. These results confirm that the enzymatic activity of Api m 1 promotes IgE-independent effector cell activation.

3.4 CLOSE-UP OF THE IMMUNOGENIC ALPHA-1,3-GAL EPITOPE AS DEFINED BY A MONOCLONAL CHIMERIC IGE AND HUMAN SERUM USING STD NMR

Circulating concentrations of IgE, the antibody class responsible for allergic hypersensitivity, are linked to the development of several immune-mediated diseases [98]. IgE antibodies bound to their high affinity receptor (FcεRI) on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. However, the exact interplay of different isotypes with their cognate allergens remains enigmatic.

Apart from the protein backbone, IgG and IgE may also be directed against xenobiotic and therefore immunogenic and cross-reactive carbohydrate determinants (CCDs) present on a plethora of proteins found in food, pollen and hymenoptera venom [165]. The hallmark of classical CCDs are alpha-1,3-linked core fucose residues found on insect venom allergens, and, additionally, beta-1,2-linked xylose on plant-derived CCDs. Antibody specificity for these spatially separated glycotopes represents the universal principle for reactivity of different proteins having CCDs [49]. The role of CCDs as a cause of allergic symptoms still is controversial [166]. IgE against classical CCDs has been shown clinically relevant [164, 167-169] but artificial or recombinant glycoproteins did not show clear cut effects in mediator release assays or skin prick tests [170, 171].

Recently, a novel type of CCD has entered the field and provided final evidence for the detrimental potential of glycans. Clearly IgE-mediated anaphylaxis via the well established Gal-alpha-1,3-Gal structure (alpha-Gal) as present on the chimeric therapeutic antibody cetuximab could be shown [68]. This epitope is also essential in

meat-induced allergy [65] and for cross-reactivity to other mammalian allergens [172]. Strong induction of alpha-Gal-specific IgE very recently was correlated with bites of tick species present within a restricted area of the United States [66].

Anti-Gal IgG antibodies especially of the IgG2 subclass constitute up to 3 % of serum immunoglobulins in man, are induced by commensal bacteria and putatively exert a natural barrier function [63]. Their clinical relevance is well documented for xenotrans-plan¬tation and blood group antigens providing alpha-1,3-linked galactose residues resulting in hyperacute xenograft rejection [64]. Scarce information however is available for alpha-Gal-specific IgE.

The interaction of polyclonal IgE with allergens has broadly been studied but detailed analyses of the particularities of IgE and its epitopes are hampered by two critical limitations, the low IgE levels in serum and the lack of specific human monoclonal antibodies. Murine monoclonals often used as substitute are neither compatible with human cellular assays nor recognize authentic IgE epitopes and thus can provide indirect evidence only.

This limitation would be obsolete if murine antibodies recognized B-cell epitopes identical to those of human antibodies, a scenario only true for small sized epitopes that obey identical immunological mechanisms in animals and man. Such a situation is given for IgE with specificity for CCDs, which are defined by their high immunogenicity in different species and their spatially extraordinarily well defined architecture.

Structural and molecular data on the interaction of antibodies with carbohydrates [173-176] still are scarce and for the alpha-Gal epitope in particular not available at all. Molecular analyses of biomolecules with ligands of limited size such as carbohydrates can be obtained using saturation transfer difference (STD) NMR [177]. Thereby, saturation is transferred from a receptor protein to ligands and leads to specific attenuation of resonance signals of ligands that bind to the receptor. This attenuation is made visible by difference spectroscopy and allows identification and characterization of the ligands and their interaction.

Thus, the aim of our work was to gain access to alpha-Gal-specific human antibody isotypes allowing insights into the molecular and functional basis of their interaction. IgE and IgG were generated and employed for cellular activation tests and

characterization of the IgE epitope by STD NMR. This work contributes to elucidation of the complex antibody carbohydrate interaction and molecular aspects in CCD-based anaphylaxis.

3.4.1 GENERATION OF ALPHA-GAL-SPECIFIC HUMAN ANTIBODY ISOTYPES

To establish chimeric mouse/human antibodies, the V_H and V_L sequences of the alpha-Gal-specific antibody M86 were used as templates for gene synthesis [122]. The variable regions were assembled in form of a single chain Fv allowing prokaryotic production (Fig. 3.17).

The antibody fragments were subsequently converted to human IgG1 and IgE isotypes by fusion with signal sequences and the particular heavy and light chain constant regions.

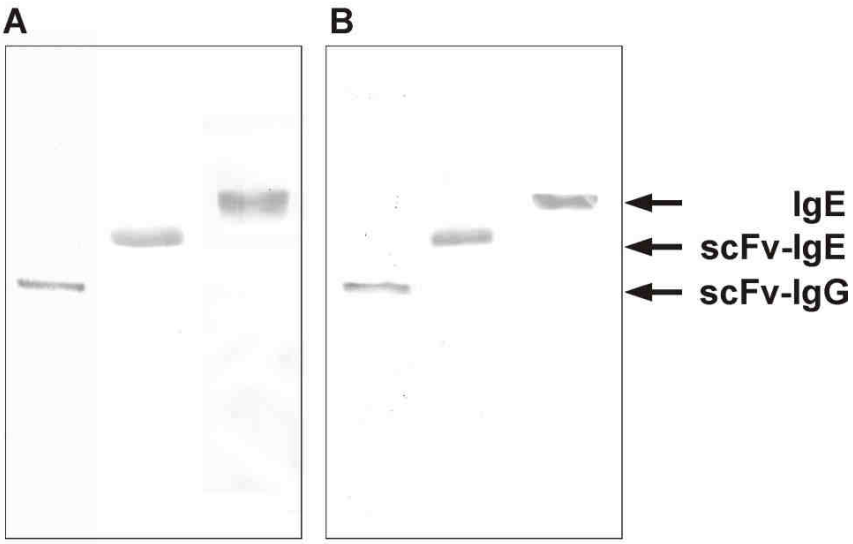


Fig. 3.17: SDS-PAGE and immunoblot analysis of IgG and IgE antibodies
Purified proteins were assessed under non-reducing conditions by Coomassie staining (A), or immunoblot (B); the different isotypes were visualized using anti human IgG and IgE antibodies conjugated to alkaline phosphatase.

SDS-PAGE and immunoblotting of proteins isolated from culture supernatants of stably transfected HEK-293 cells showed apparent molecular masses in the expected range of 120 kDa, 150 kDa and 200 kDa for the homodimeric and heterotetrameric IgG and IgE antibodies suggesting that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE (Fig. 3.17).

3.4.2 CHARACTERIZATION OF THE CARBOHYDRATE-SPECIFIC ANTIBODY ISOTYPES

Characterization of the recombinant proteins was pursued by different approaches to demonstrate both antigen and receptor binding. All antibodies detected alpha-Gal carrying thyroglobulin, cetuximab (n.d. for IgG), and HSA-conjugate in ELISA (Fig. 3.18 A). Notably, the biotinylated Gal-alpha-1,3-Gal disaccharide exhibited no interaction, a fact that might reflect the specific sterical requirements of antibody binding (data not shown).

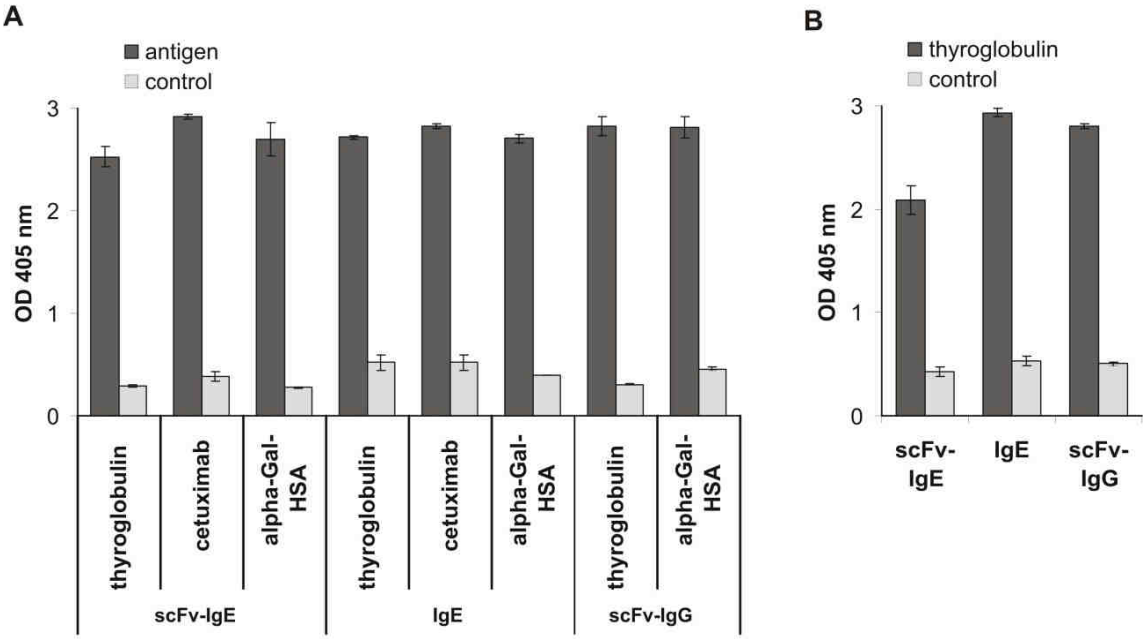


Fig. 3.18: Immunoreactivity with allergens and Fc receptor molecules
(A) The immunoreactivity of the recombinant human antibodies was assessed in ELISA using thyroglobulin, cetuximab and alpha-Gal-HSA for the scFv-IgE and IgE and thyroglobulin and alpha-Gal-HSA for the scFv-IgG, and isotype specific antibodies conjugated to alkaline phosphatase. (B) Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high affinity Fc receptors and anti chicken IgG conjugated to alkaline phosphatase for detection.

To confirm proper folding and glycosylation of the Fc domains, Ig Fc receptor extracellular domains of the ligand-binding alpha-chains of the human high affinity receptors FcεRI and FcγRI (CD64) fused with chicken IgG (IgY) Fc domains were used [90]. Recombinant IgE as well as IgG specifically bound to their soluble Fc receptors (Fig. 3.18 B). These data underline that the recombinant isotypes reflect the natural interaction with both the antigen and the cellular receptors and, therefore, should mediate comparable effects *in vivo*.

SPR-Analyses using immobilized IgE and cetuximab, alpha-Gal-HSA, and thyroglobulin as analytes resulted in dissociation constants in the nanomolar range (Fig. 3.19) which is in the range for both natural anti-Gal antibodies and carbohydrate-specific binders [178, 179].

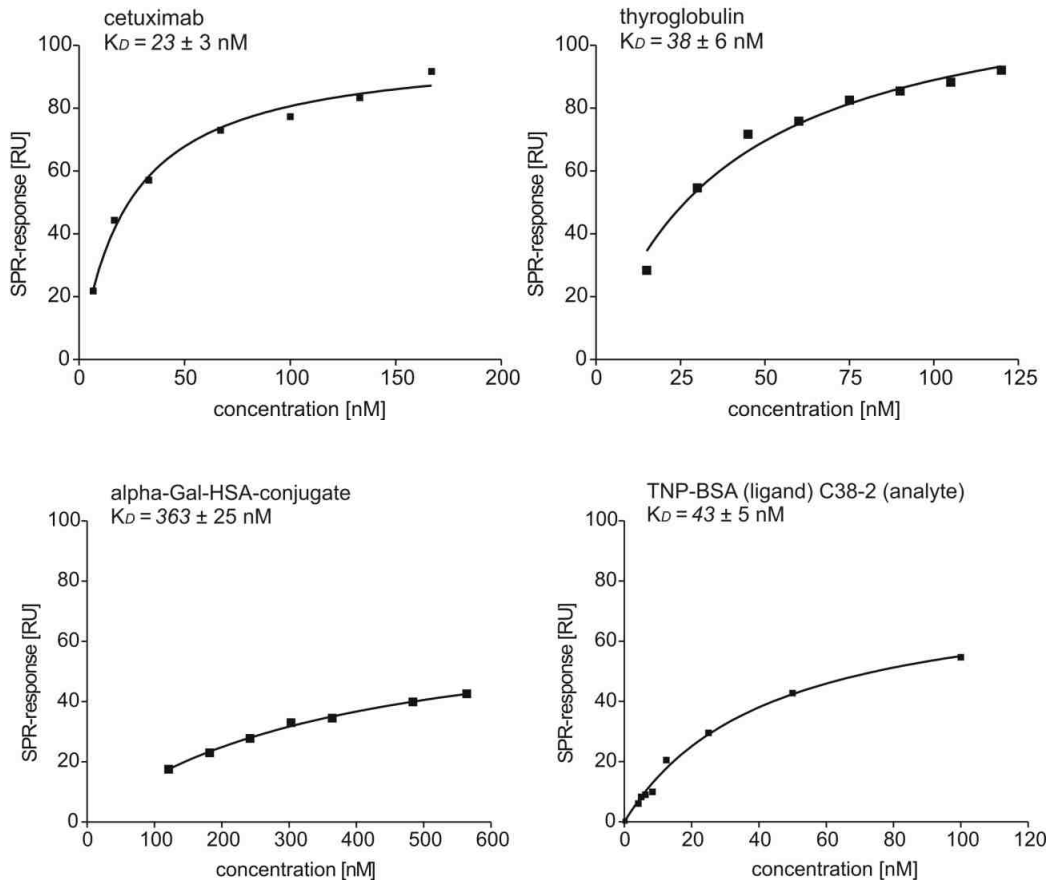


Fig. 3.19: SPR analyses of different alpha-Gal carriers

The dissociation constant KD was determined for alpha-Gal-HSA, thyroglobulin and cetuximab as analytes and the scFv-IgE as immobilized ligand as well as for the TNP-specific C38-2 as analyte and TNP-BSA as immobilized ligand. The concentration dependent curves were analyzed by the one-site-binding-model.

SPR analyses of the TNP-specific murine IgE C38-2 used below demonstrated affinities in a similar range. The HSA conjugate however showed a higher dissociation constant than the other alpha-Gal carrying proteins, a finding that might reflect the reduced sterical accessibility also seen for the biotinylated disaccharide and an impact of the missing third unit. This finding also suggests that the valency of the alpha-Gal (cetuximab<alpha-Gal-HSA <thyroglobulin) and the molecular architecture of bivalent cetuximab does not significantly favour interaction.

3.4.3 ASSESSMENT OF THE POTENTIAL FOR CELLULAR ACTIVATION

IgE-mediated cross-linking of the FcεRI and degranulation of RBL-SX38 cells was assessed by determination of β-hexosaminidase release (Fig. 3.20). Thereby, the medium affinity IgE C38-2 was used as activation control.

Both anti-Gal IgE formats bound to the FcεRI and induced mediator release in an antigen-independent manner using anti-IgE antibodies to an extent comparable to the murine reference (Fig. 3.20 A). Allergen-dependent cellular activation for monoclonal IgE usually is difficult to achieve, since in most cases only one epitope per allergen is available. Here, a multivalent molecule bearing at least two independent alpha-Gal epitopes is needed to bridge two identical IgE paratopes. This approach was addressed for the anti-Gal IgE using a broad panel of different alpha-Gal carrying proteins including alpha-Gal-HSA conjugate, thyroglobulin, and cetuximab and in parallel alpha-Gal microspheres generated by biotinylated thyroglobulin clustered on streptavidin-coated particles (Fig. 3.20 B). In contrast to the reference which also was cross-linked using a multimeric TNP-BSA-conjugate, neither the different alpha-Gal proteins nor alpha-Gal microspheres were able to induce significant mediator release (Fig. 3.20 B).

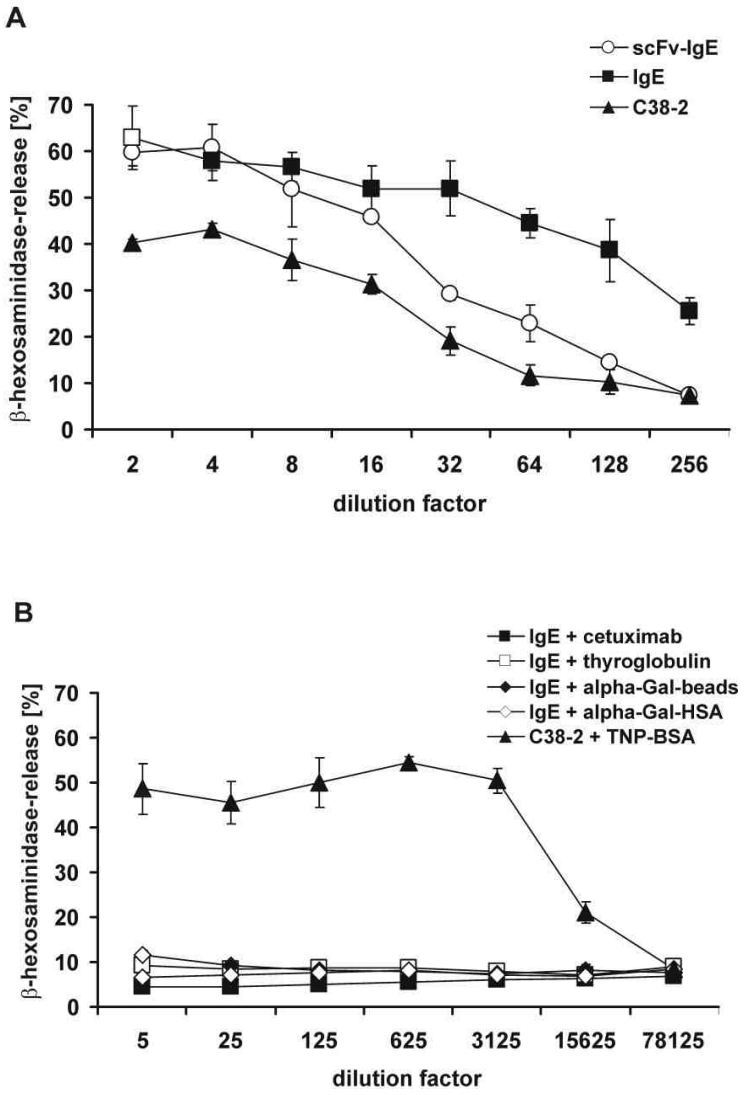


Fig. 3.20 :Mediator release of humanized RBL-SX38 by alpha-Gal

RBL-SX38 cells providing the human FcεRI were sensitized with the scFv-IgE, IgE and the TNP-specific mouse IgE C38-2 as control (A). Antigen-independent degranulation was induced by the addition of anti-huIgE and anti-muIgE, respectively (A). Antigen-dependent degranulation was induced by the addition of different alpha-Gal carrying proteins, including biotinylated thyroglobulin immobilized to streptavidin coated beads, and by the addition of TNP-labeled BSA for the control (B). Degranulation was monitored by β-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements.

These data suggest that the alpha-Gal IgE exhibits intrinsic potential to cross-link the FcεRI and activate effector cells, which is not deployed in an antigen-dependent manner suggesting an impact of affinity or spatial organization on alpha-Gal-mediated anaphylaxis.

3.4.4 EPITOPE ANALYSIS

Variant specificities for alpha-Gal and distinct modes of binding are reported for different lectins and antibodies [180], but structural data remain scarce. Here, it is aimed for a direct monitoring of the interplay of anti-Gal antibodies with the cognate antigen on a molecular level and comparability of the monoclonal IgE to polyclonal serum antibodies.

Interaction of the monoclonal chimeric IgE with the disaccharide Gal-1,3-Gal-OMe was followed by STD NMR (Fig. 3.21). Clear STD effects could be obtained for the carbohydrate ligand.

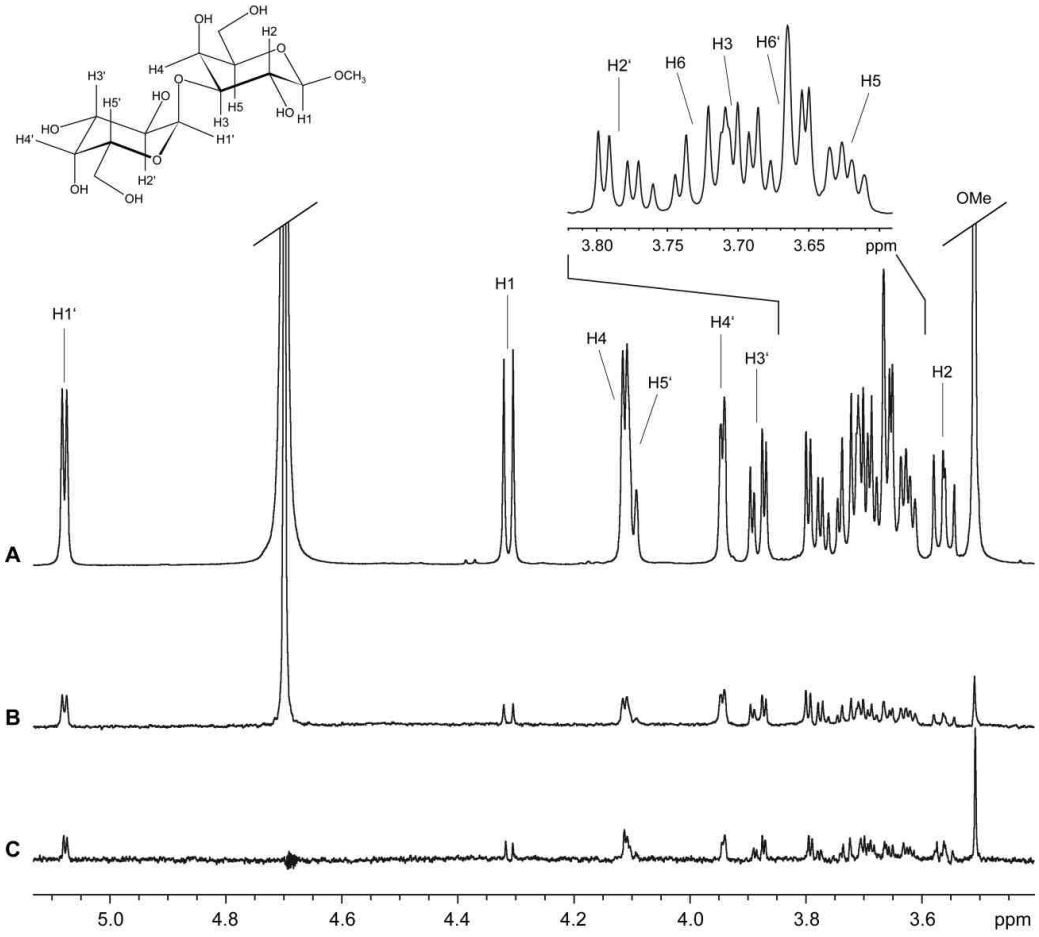


Fig. 3.21: Observation of STD signals for the alpha-Gal disaccharide.

(A) Reference 1H NMR spectrum and (B) STD NMR spectrum of Galα1,3GalβOMe in the presence of the M86-based IgE (molecular ratio = 260:1). (C) STD NMR spectrum of Galα1,3GalβOMe in the presence of polyclonal antibodies purified from human serum (molecular ratio = 200:1). Spectrum B has been scaled by factor 5 of its original intensity. All spectra were recorded at a temperature of 298 K and at 500 MHz with 1024 scans (A and B), and at 700 MHz with 2048 scans, respectively (C). STD spectra in the figure are not artifact referenced.

The interaction foot-print of the M86-based IgE on Gal-1,3-Gal-OMe reveals strong interaction with both galactose residues (Fig. 3.22 A).

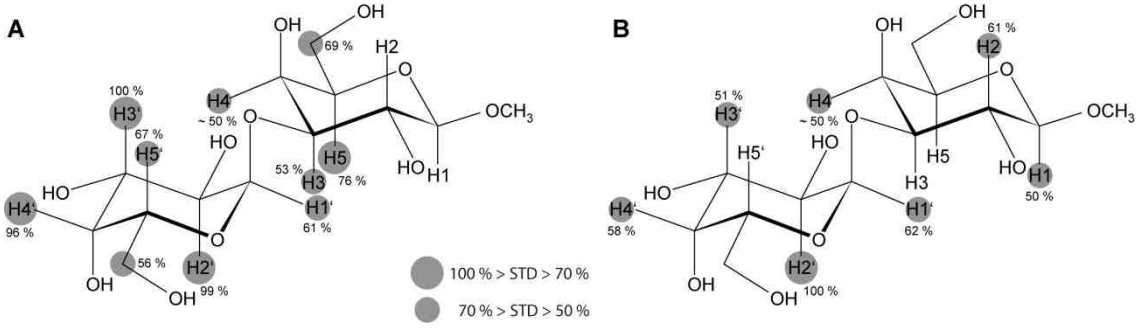


Fig. 3.22: Interaction footprint as assessed by STD NMR

Interaction footprint of Galα1,3GalαOMe binding to M86-based IgE (A) and polyclonal antibodies purified from human serum (B). Circles represent the relative size of the saturation transfer and reflect the vicinity of individual protons to protons in the binding pocket of the immunoglobulin as macromolecular binding partner. The closer the ligand protons are to protons of the binding pocket, the larger is the saturation transfer. Proton H4 could not be determined with high accuracy due to resonance overlap.

Major contacts of the terminal galactose are defined by the C3 proton having the highest STD signal and to a high extent the C2, and C4 protons. The C5, C1 and C6 protons also show medium STD effects. Although evident for nearly protons effects lower than 50 % were not included for reasons of reliability. For the adjacent galactose the major contacts are the C5 proton and the C6, C3, and C4 with all other protons showing medium STD effects. The methyl glycoside contributes weakly only (Fig. 3.22 A).

In parallel, polyclonal immunoglobulins were purified from serum of a donor having elevated serum IgE and IgG with specificity for alpha-Gal using thyroglobulin affinity chromatography. Enrichment of immuno-globulins specific for thyroglobulin was documented by ELISA (Fig. 3.23).

Observed enrichment of anti-Gal antibodies with specificity for cetuximab suggests that the recognized moiety in the immunoglobulin preparation corresponds to those responsable for IgE-based alpha-Gal interaction. These antibodies then were subjected to STD NMR using the disaccharide Gal-1,3-Gal-OMe (Fig. 3.21).

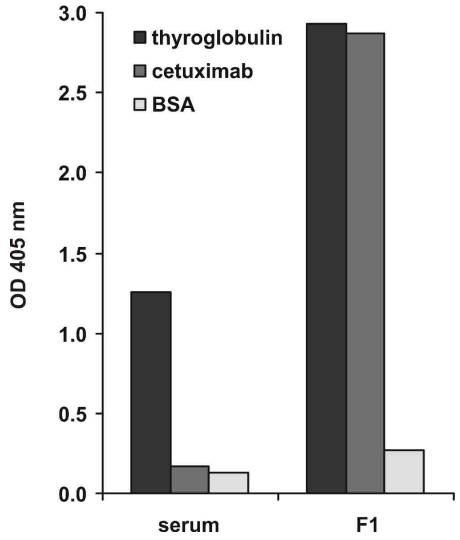


Fig. 3.23 : Anti-Gal purification by affinity chromatography
The anti-Gal immunoreactivity of human serum and immunoglobulins purified by thyroglobulin affinity chromatography was assessed by ELISA using thyroglobulin or cetuximab and anti-human IgE antibodies conjugated to alkaline phosphatase.

Intriguingly, the interaction foot print of the polyclonal immunoglobulins reveals strong interaction with both galactose residues in a manner highly similar to that of the monoclonal antibody (Fig. 3.22 A, B). Major contacts of the terminal galactose are defined by the C2 proton having the highest STD signal and the C3 and C4 protons. For the adjacent galactose major contact is the C2 proton followed by C1 showing medium STD effects. As above, weaker effects seen for most protons but lower than 50 % were not included for reasons of reliability. As for the monoclonal antibody the methyl glycoside contributes to a slightly increased extent only.

These findings verify that the Gal-Gal disaccharide block is crucial and sufficient for the epitope of the monoclonal M86 based IgE as well as human polyclonal antibodies present in the circulation. The glycosidic methyl group representing the downstream residue (GlcNAc or Glc) seems to contribute weakly.

3.5 COMPARISON OF TUMORICIDAL ACTIVITIES MEDIATED BY HUMAN ANTI-EGFR IGG VERSUS IGE ISOTYPES

Monoclonal antibodies like the chimeric anti-EGFR antibody cetuximab (IMC-225, Erbitux®) have become a mainstay for the specific treatment of cancer today. Prompted

by studies indicating that IgE effector functions potentially eradicate malignant cells, the tumoricidal effects of 225-IgG1 with 225-derived IgE *in vitro* were compared.

The presence of IgE vs IgG1, and IgG4 antibodies in allergy reflects the T helper cell type 2 (T_H2) versus type 1 immune milieu. A set of recombinant monoclonal tumor-specific IgE and IgG antibodies, which exhibit comparable affinities by means of identical binding moieties, allows the analyses of their behaviour in pathophysiology as well as their molecular interplay with immune cells in detail.

3.5.1 GENERATION AND CHARACTERISATION OF RECOMBINANT IMMUNOGLOBULINS

For recombinant production, transfection of HEK-293 cells with the particular expression plasmids yielded heterotetrameric Erbitux®-derived 225-IgE and 225-IgG1 antibodies. Both antibodies were purified from the cell culture supernatants using a kappa constant light chain specific affinity matrix (KappaSelect, GE Healthcare). Homogeneity was confirmed by SDS-PAGE and immunoblotting (Fig. 3.24).

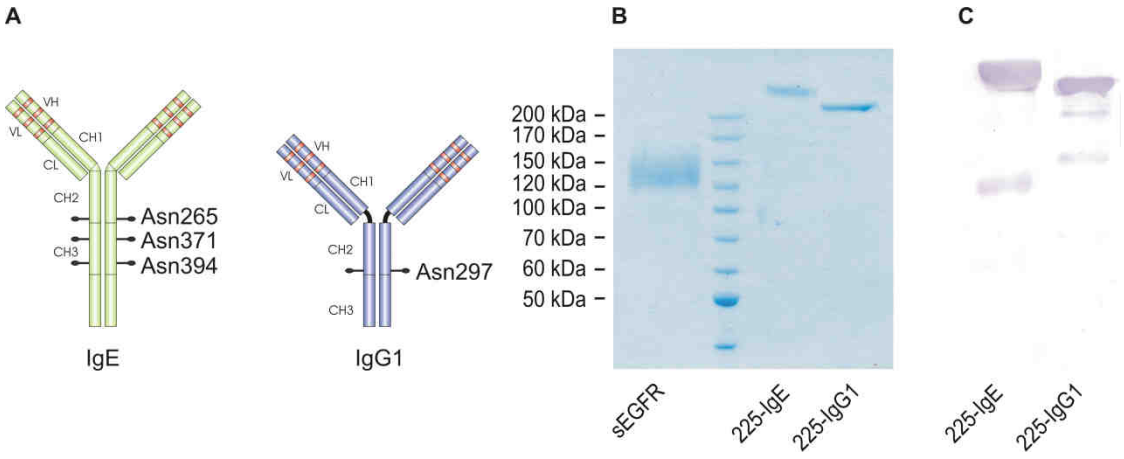


Fig. 3.24: Schematic representation and analysis of purified 225-IgE and 225-IgG1 antibodies.
A, Indicated are the immunoglobulin domains (green, violet), the CDR regions (red), Fc N-glycosylation sites (black dots). B, C Purified sEGFR and 225 antibodies were assessed by SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining (B) or immunoblotting (C). 225-IgE and 225-IgG1 were visualized using an anti-kappa light chain-AP conjugate.

3.5.2 ASSESSMENT OF IMMUNOREACTIVITY

To confirm their functionality after exposure to the acidic environment used during preparation, their antigen binding capacity with the cognate antigenwas evaluated. A

soluble EGFR (sEGFR) splice variant was used for *in vitro* analyses comprising the extracellular ligand binding domain including a short C-terminal stretch of 79 amino acids which are distinct from the membrane-bound isoform. The sEGFR is naturally secreted by A431NS cells and was affinity-purified from the cell culture supernatant [97, 181]. Immunoreactivity of 225-IgE and 225-IgG1 was confirmed with immobilized sEGFR and Erbitux® as a reference in ELISA (Fig. 3.25 A, B, C).

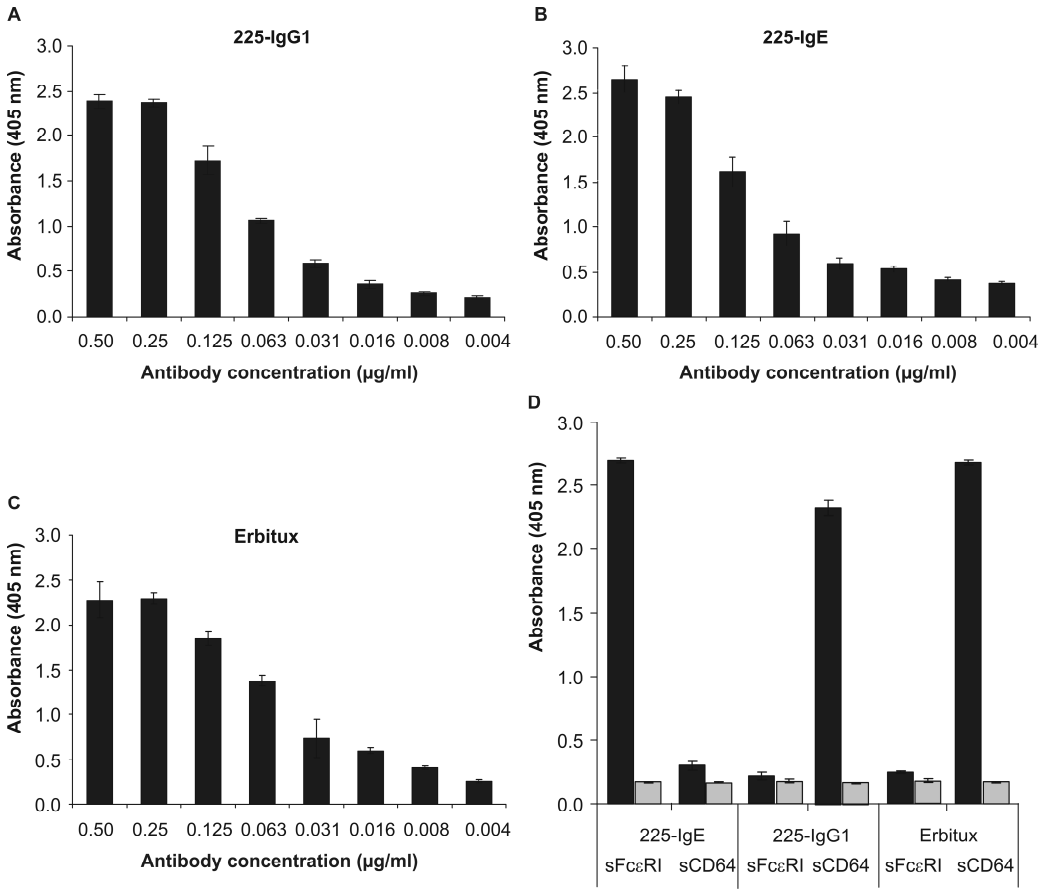


Fig. 3.25: Immunoreactivity of 225-IgE and 225-IgG1 with immobilized sEGFR in ELISA.
A-C The immunoreactivities of 225-IgE, 225-IgG1 and Erbitux® were assessed in ELISA with immobilized sEGFR and an anti-human kappa light chain-AP conjugate. D, The functionality of 225-IgE, 225-IgG1 and Erbitux® was assessed by binding to immobilized sEGFR and either soluble FcεRI-IgY Fc or CD64-IgY Fc and an anti-chicken IgY-AP conjugate. Controls were performed by omission of antigen. Data are mean +/- SD of triplicate measurements.

To affirm the interaction of 225-IgE with the IgE binding domain FcεRIα of the high affinity IgE receptor and the binding of 225-IgG1 to its high affinity IgG receptor FcγRI (CD64), soluble receptor-IgY chimeras (FcεRI-IgY Fc and CD64-IgY Fc) comprising the extracellular domains of FcεRIα [90]or CD64 [182]fused with chicken IgY Fc

regions were produced as a mimic for the natural membrane-bound receptor in ELISA (Fig. 3.25 D). Both 225-isotypes were captured by immobilized sEGFR and subsequently 225-IgE was incubated with FcεRI-IgY Fc, whereas 225-IgG1 and Erbitux® were incubated with CD64-IgY Fc. Detection of both receptor-IgY chimeras by using an anti-chicken IgG AP conjugate confirmed proper interaction with the particular Fc receptors.

3.5.3 AFFINITY MEASUREMENTS BY SPR

Furthermore, quantitative binding data derived from SPR analyses with antibodies injected over biotinylated sEGFR immobilized on a streptavidin coated biosensor surface led to apparent K_D values of 3.6 nM for 225-IgE, 4.7 nM for 225-IgG and 6.7 nM for Erbitux® (Fig. 3.26), which corroborates the functionality of all antibody preparations.

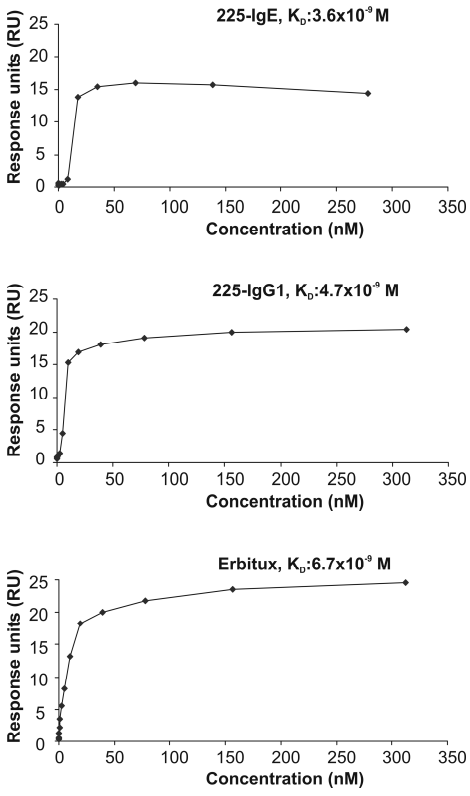


Fig. 3.26: Biosensor affinity measurement of 225-IgE, 225-IgG1 and Erbitux® with immobilized sEGFR.

A kinetic analysis of the interaction of immobilized biotinylated sEGFR on a streptavidin coated surface with 225-IgE, 225-IgG1 and Erbitux® was performed by injecting varying concentrations of the analyte. A streptavidin surface served as control. The apparent K_D values were calculated from the fits.

The values obtained were comparable to previously reported K_Ds of cetuximab binding to EGFR and EGFRvIII with K_Ds of 5 nM and 1.1 nM, respectively [183].

3.5.4 IMMUNOSTAINING ASSESSEDBYMICROSCOPY AND FLOW CYTOMETRY

The reactivity of 225-IgE and 225-IgG1 with EGFR expressing epidermoid carcinoma A431NS cells by fluorescence microscopy (Fig. 3.27 A) and flow cytometry (Fig. 3.27 B) were then analyzed. Both 225-IgE and 225-IgG1 stained the cell surface.

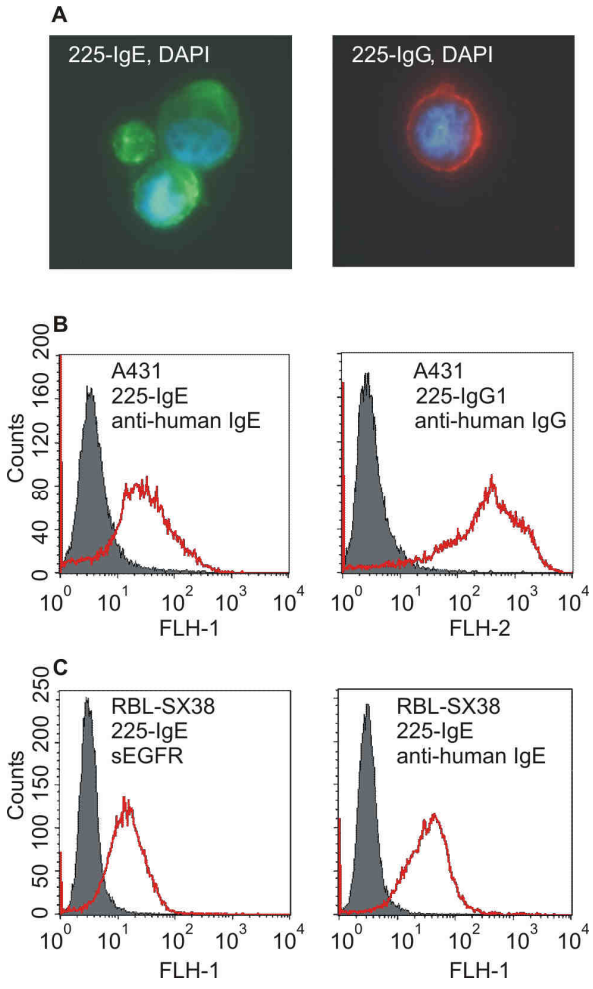


Fig. 3.27: Immunoreactivity of 225-IgE and 225-IgG1 with A431NS target cells and RBL-SX38 effector cells.

A, Cytospins were prepared from A431NS cells. The immunoreactivities of 225-IgE and 225-IgG1 were assessed by immunostaining and visualized using anti-human IgE-FITC conjugate (left panel) or anti-human IgG-TRITC conjugate (right panel) and DAPI to stain the nuclei. B, A431NS cells were analyzed by flow cytometry and stained with 225-IgE and anti-human IgE-FITC conjugate (left panel) or 225-IgG1 and anti-human IgG-TRITC conjugate (right panel). C, RBL-SX38 cells were analyzed by flow cytometry and stained with 225-IgE and FITC-conjugated sEGFR (left panel) or 225-IgE and anti-human IgE-FITC conjugate (right panel).

In order to demonstrate the interaction of 225-IgE with cell-bound FcεRIα rat basophilic leukemia cells (RBL-SX38) expressing the recombinant tetrameric human FcεRIαβγ$_2$ receptor, which is naturally exposed on the surface of human mast cells and basophils, were incubated with 225-IgE. Bound 225-IgE was visualized by flow cytometry using an anti-human IgE FITC conjugate or FITC-labeled sEGFR (Fig. 3.27 C).

3.5.5 CELL VIABILITY ASSAY

To examine the antiproliferative activity of 225-IgE and 225-IgG1, MTT assays were performed with A431NS as target cell population. Erbitux® as well as the recombinant antibodies decreased cell viability in a concentration-dependent manner, independently of the antibody isotype used (Fig. 3.28).

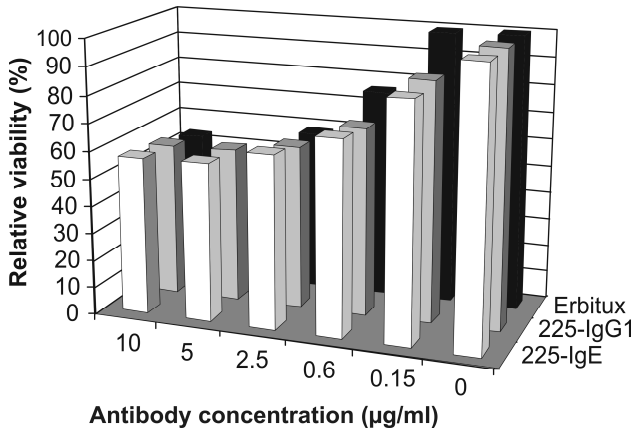


Fig. 3.28: Anti-proliferative effects of 225-IgE, 225-IgG1 in comparison with Erbitux® for A431NS cells.
A431NS cells were analyzed by the MTT assay in various dose concentrations of 225-IgE, 225-IgG1 and Erbitux®. The inhibition of proliferation was shown after 72 h as % viability referred to the control without antibody. Data are mean of triplicate measurements.

3.5.6 SENSITIZATION OF RAT BASOPHILIC LEUKEMIA CELLS (RBL-SX38)

In order to assess whether effector functions could potentially be induced *in vitro* RBL-SX38 sensitized with 225-IgE. Signaling through the IgE receptor was successfully induced by cross-linking mediated by anti-human IgE antibodies (Fig. 3.29 A). Biotinylated sEGFR complexed by tetrameric streptavidin forms microclusters

which mimic multivalent antigens. When applied to IgE-sensitized cells, activation was induced, thereby confirming direct antigen induced cellular activation (Fig. 3.29 B).

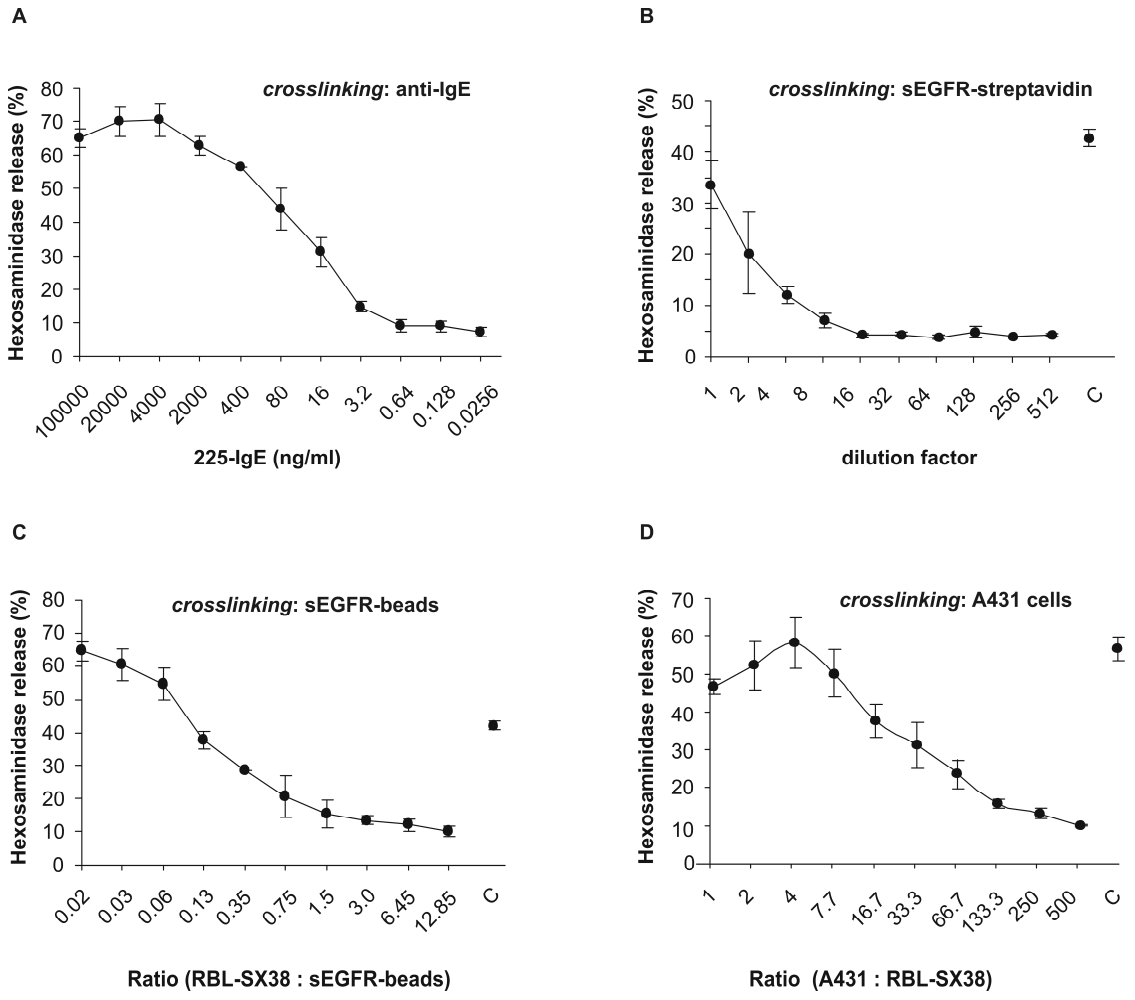


Fig. 3.29: Effects of 225-IgE cross-linking on effector cell degranulation.
RBL-SX38 cells were sensitized with 225-IgE (4 µg/ml) and degranulation was induced by addition of various concentrations of anti-IgE (A), a dilution series of sEGFR:streptavidin microclusters (stock solution: 50 mM biotinylated sEGFR complexed with 5 mM streptavidin) (B), biotinylated sEGFR immobilized on streptavidin beads (C) or A431NS tumor cells (D). Degranulation was monitored by β-hexosaminidase activity released into culture supernatants. C: positive control, 225-IgE is cross-linked by anti-human IgE antibodies. Data are mean +/- SD of triplicate measurements.

Coupled biotinylated sEGFR to streptavidin beads were applied to mimic a tumor cell by providing an artificial EGFR coated surface. Additionally, effector cell activation in a concentration-dependent manner was also observed (Fig. 3.29 C).

Microscopic pictures indicate that 225-IgE functions as a specific linker and that EGFR coated beads are able to attach firmly to the RBL-SX38 cells, whereas without IgE, they are randomly distributed (Fig. 3.30).

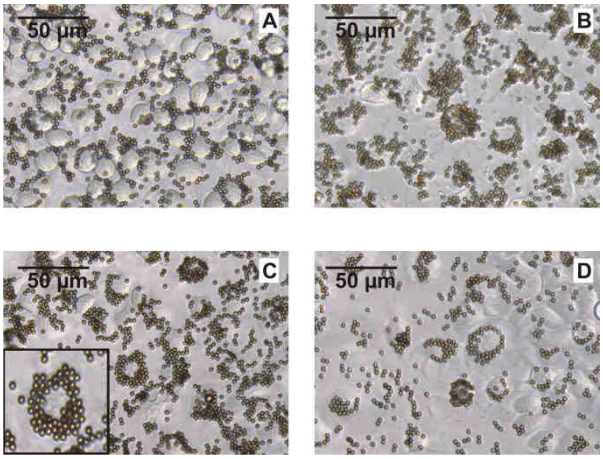


Fig. 3.30: Light microscopic images of RBL-SX38 cells sensitized with 225-IgE and sEGFR coated beads.
Light microscopic images of RBL-SX38 cells sensitized with 225-IgE and incubated with biotinylated sEGFR immobilized on streptavidin beads (round dots). A, negative control without 225-IgE; B, RBL-SX38:beads 1:50; C, RBL-SX38:beads 1:30; D, RBL-SX38:beads 1:15. Original magnification x 100. Scale bars 50 µm.

To assess the ability of 225-IgE to induce degranulation by EGFR naturally tethered onto a cell surface, A431NS tumor cells expressing EGFR were incubated with RBL-SX38 effector cells at different ratios. These results indicated that multivalent cell surfaces were indeed able to mediate effector cell activation (Fig. 3.30 D). Interestingly, no activation was observed when applying 225-IgE or sEGFR alone (data not shown). This finding indicated that any EGFR shed from cell membranes *in vivo* [184] would not induce anaphylactic reactions in response to soluble antigen in the bloodstream. Furthermore, anti-IgE mediated degranulation of 225-IgE sensitized RBL-SX38 did not show any deleterious effects on RBL-SX38 viability (data not shown).

3.5.7 ASSESSMANT OF ADCC/ADCP BY THREE-COLOR FLOW CYTOMETRY

Furthermore, the monocytic cell line U937 as a potential effector cell typewas tested for activation by antigen dependent cross-linking of FcεRI expressed by them. The experimental setting of the three-colour assay enablesthe discrimination between phagocytic and cytotoxic effector mechanisms, displayed by either 225-IgE or 225-IgG1 antibodies. ADCC and ADCP of A431NS cells cultured for 2.5 h with human U937 and either 225-IgE or human myeloma IGE as a control were determined.

Additionally,anti-tumoricidal properties of the IgE isotype with sEGFR-specific 225-IgG1, Erbitux® and Rituximab (RTX) as controls (Fig. 3.31, Tab. 3.1) werecompared.

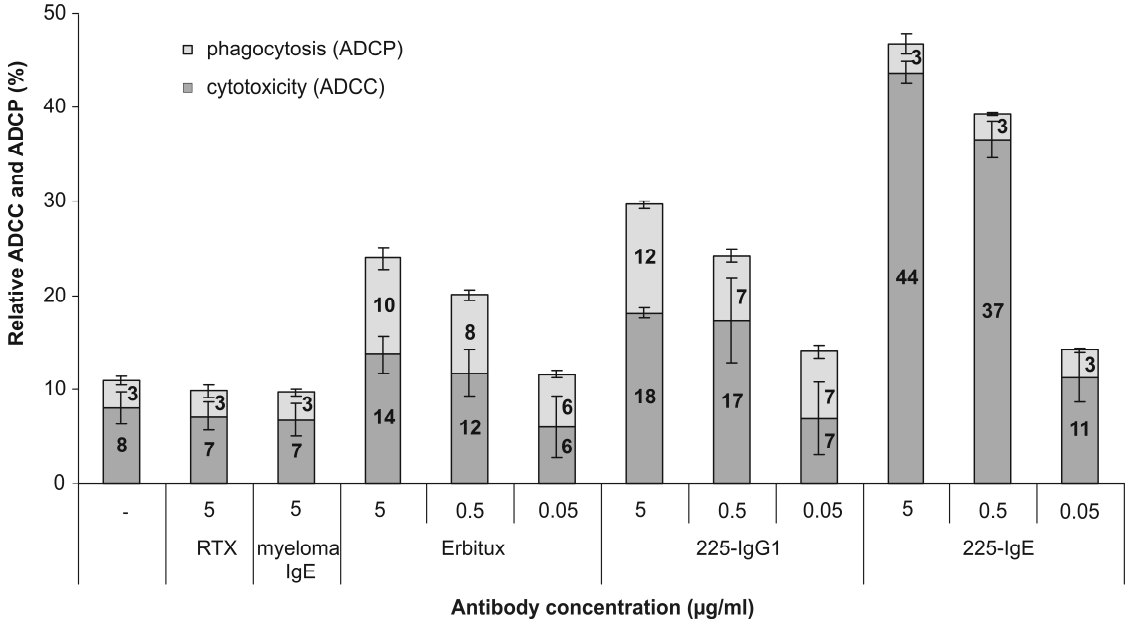


Fig. 3.31: Quantitation of 225-IgE, 225-IgG1 and Erbitux®-mediated tumor cell killing by U937 monocytes.

Different concentrations of 225-IgE, 225-IgG1 and Erbitux® were incubated with tumor cells (A431NS) and monocytic effector cells (U937) at a ratio of 1:3 using the ADCC/ADCP assay. Cytotoxicity: dark grey bars; phagocytosis: light grey bars. Results are means +/- SD of three independent experiments.

U937 cells alone mediated 8 % cytotoxicity, compared with 7 % for myeloma IgE and 7 % for Rituximab. Cytotoxicity was mediated by 225-IgE in a concentration-dependent manner ranging from 44 % to 11 % when applying 5, 0.5 or 0.05 µg/ml antibody. In contrast, 225-IgG1 induced dose dependent cytotoxicity was in the range of 18 % to 7 % and correlated well with the rate of cytotoxicity observed for the commercial product Erbitux® which ranged from 14 % to 6 %. IgE mediated phagocytosis was comparable with the non-specific controls or in the absence of antibody. Both 225-IgG1 and Erbitux® showed slightly increased phagocytic activity, ranging from 12 % to 7 %, and from 10 % to 6 %, respectively.

Tab. 3.1: Statistical analysis of the three-color ADCC/ADCP assay
Statistical analyses of *in vitro* ADCC/ADCP assays were performed by means of the unpaired two-tailed Student's *t* test, and significance was accepted at $P < 0.05$.

Pairs	P value	P value summary	Significance (P<0.05)
cytotoxicity IgG-IgE (1 μg)	0.0018	**	Yes
cytotoxicity IgG-IgE (0.1 μg)	0.0044	**	Yes
cytotoxicity IgG-IgE (0.0 μg)	0.0210	*	Yes
phagocytosis IgG-IgE (1 μg)	<0.0001	***	Yes
phagocytosis IgG-IgE (0.1 μg)	0.0016	**	Yes
phagocytosis IgG-IgE (0.01 μg)	0.0051	**	Yes

These data suggest that IgE mediated tumor cell killing is a mechanism that can render IgE a broadly relevant isotype in EGFR targeted therapeutic interventions.

In this study the tumoricidal and anti-proliferative properties of recombinant EGFR specific IgE were compared. IgE, in comparison to IgG1 may harness alternate effector cells at normally inaccessible tumor sites and improve tumor cell surveillance. This work points to the potential of IgE in tumor eradication strategies.

3.6 HUMAN MONOCLONAL IGE AND IGG ANTIBODIES AGAINST MBP AS TOOLS OF DEFINED SPECIFICITY FOR DIAGNOSTIC APPROACHES IN ALLERGY

Allergy can cause life-threatening anaphylactic reactions mediated by IgE in allergic individuals. Determining the susceptibility of an individual to develop clinical symptoms is based on the measurement of specific serum IgE levels. The ratio of allergen specific IgE and IgG4 serum concentrations of is thought to reflect the immune status of an allergic patient and the success of specific immunotherapy (SIT). This is a therapeutic intervention in which long-term exposure to higher concentrations of allergens results in a T helper cell type 1 (T_H1) shift in the immune response. That leads to an increased production of allergen specific IgG antibodies, particularly of the IgG4

subclass. These IgG antibodies are thought to exert their function by blocking the IgE allergen interaction. Concentrations of circulating IgE and IgG antibodies are routinely addressed in most diagnostic approaches in allergy. However, all available allergy immunoassays are essentially evaluated, established and standardized using polyclonal pool sera of allergic individuals, thereby not standardized or reproducible. For the development and standardization of reliable assay systems the availability of specific reagents is imperative, but specific IgE and IgG are available as polyclonal preparations only and pool sera of defined IgE reactivity face different problems. Usually they are difficult to obtain in substantial quantities especially those with reactivity for minor allergens or exotic allergen sources. As well the tendency towards component resolved diagnosis demands sera with so far unaddressed specificities potentially present in only a subset of individuals and the availability of individual sera showing outstanding sIgE reactivities is limited. In addition, sera within the context of pediatric allergy e.g. cow milk are scarce and the available amounts are even more limited. Finally, the costs of sera often exceed acceptable limits.

Thus, monoclonal antibodies of the IgG and IgE isotype with defined specificity have the potential to become unique tools for diagnostic, research and therapeutic purposes in allergy.

A strategy to circumvent the need for huge panels of antibodies reactive with singular allergens, might rely on antibodies with specificity for suitable, non-allergenic standard proteins. Standardization will likely become more important with the use of recombinant allergens for advanced diagnostics and intervention. Nevertheless, the complexity and individuality of the immune reactions in the field of allergy renders the diagnostic picture highly difficult. So far, more than 1000 different allergens from a plethora of sources have been identified and the number is still increasing. Additionally, a variety of isoforms makes the picture even more difficult. Against this background, the concept that each allergen could be detected by specific monoclonal antibodies in form of human IgG and IgE is far from reality.

Hence, tailor made reagents that could act as substitute for human sera have the potential to facilitate the development, standardization and commercialisation of allergy assays. Ideal reagents should be comprised by monoclonal IgE antibodies with defined specificity. However, the broad unavailability of monoclonal IgG and IgE antibodies

has thus far prohibited detailed analyses of their characteristics in pathophysiology as well as their molecular interplay.

Therefore, our aim was to establish the generation of reproducible allergen specific antibodies for the most relevant isotypes for allergy diagnostics and research. This development demands the generation of artificial ligands that are in addition easy to acquire, here a fusion protein broadly used for expression of recombinant allergens.

Therefore, a human synthetic scFv library to selection was subjected to the by far most often used fusion protein maltose binding protein (MBP). This constitutes the probably most often used protein fusion system MBPis the 43 kDa product of the *malE* gene from *E. coli*, resulting in the expression of an MBP fusion protein. The advantages of the MBP fusion systems are versatile, for instance it binds to amylose resins and elutes using 10 mM maltose making purification under mild conditions easy. This protein significantly increases solubility of the mostly insoluble allergens allowing proper folding and thus an improved use in subsequent immunoassays. Most systems additionally provide protease cleavage sites for removal of the fusion part and optional periplasmic secretion signals. Although allergen expression in *E. coli* appears a stringent approach problems may arise from the need for conformational IgE epitopes, the folding and solubility of proteins from difficult sources, and the codon usage. Most of these points can be circumvented by fusion to MBP.

These advantages, although also true for many other fusion systems led to the expression of a vast number of recombinant allergens as MBP fusion proteins.

Artificial scFv were selected against the MBP and one highly specific clone as human IgE, and IgG1 antibody was expressed. Expression of homodimeric or heterotetrameric recombinant proteins was performed in HEK-293 cells, and the antibodies were analyzed for immunoreactivity to different allergen/MBP fusion proteins.

In this study the generation of monoclonal IgE and IgG antibodies with specificity for MBP by conversion of selected antibody fragments into derivatives corresponding to the particular isotypes was reported.

3.6.1 SELECTION OF ANTIBODY FRAGMENTS WITH SPECIFICITY FOR MBP

To generate monoclonal antibody fragments with specificity for a defined and broadly applicable target structure, the human synthetic antibody library Griffin-1 was subjected to selection against the maltose binding protein immobilized on polystyrene tubes. After iterative panning, significant enrichment of the library in the 2nd and 3rd rounds of selection could be demonstrated by polyclonal ELISA (Fig. 3.32 A). Subsequently, individual phage clones exhibited signals 5-20 times background in the monoclonal ELISA analysis (Fig. 3.32 B). Individual reactive fragments were identified by sequence analysis demonstrating the predominance of one particular clone (data not shown).

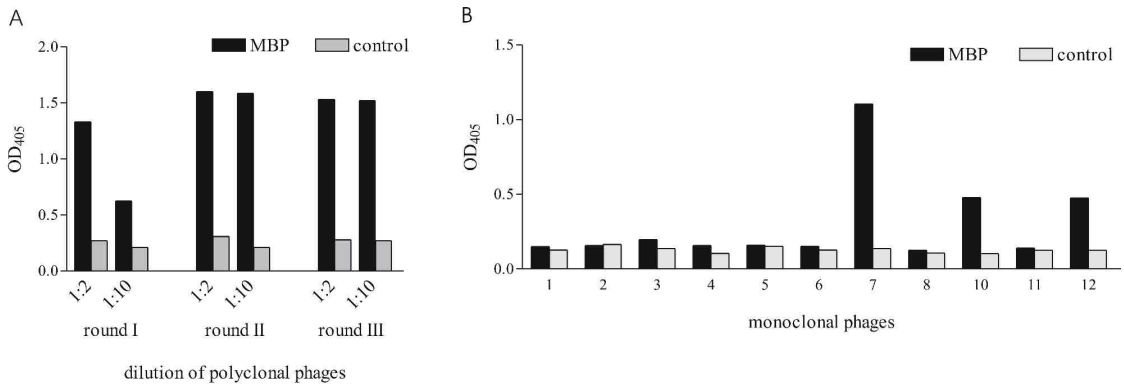


Fig. 3.32: Immunoreactivity of selected phages in polyclonal (A) and monoclonal (B) ELISA
The immunoreactivity of selected clones against MBP (filled bars, controls by omission of MBP, open bars) was analyzed by.

To verify the suitability for expression soluble antibody fragments were produced in prokaryotic cells and could be purified from the periplasmic space (Fig. 3.33).

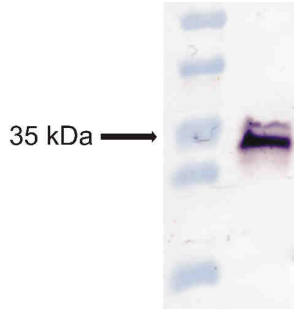


Fig. 3.33: Expression of soluble anti-MBP scFv
Soluble anti-MBP scFv was assessed by SDS-PAGE under reducing conditions followed by immunoblotting. Detection of the soluble scFv fragment was performed using an monoclonal anti-His antibody and anti-mouse IgG AP conjugate.

3.6.2 CONVERSION AND EXPRESSION OF IGE AND IGG ANTIBODIES

For expression of selected scFv as different Ig isotypes in mammalian cells, a set of modular cassettes containing leader sequences, constant Ig regions of human IgG1 and IgE, and restriction sites for the incorporation of scFv or separate variable regions into both homodimeric scFv C_H2-4 or scFv C_H2-3 as well as entire heterotetrameric (Fig. 3.34) antibodies were employed.

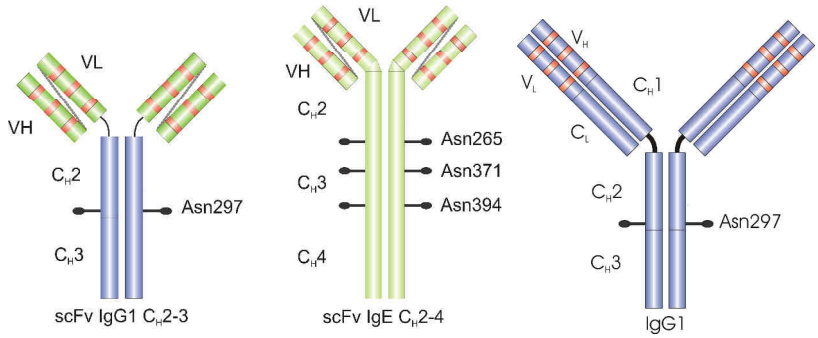


Fig. 3.34: Schematic representation of scFv and heterotetrameric antibody constructs
Indicated are the immunoglobulin domains (green, violet), the CDR regions (red), Fc N-glycosylation sites (black dots) of scFv IgG1 C_H2-3 and scFv IgE C_H2-4 as well as heterotetrameric IgG1.

Transfection resulted in establishment of stably transfected antibody secreting HEK-293 cells. After purification from culture supernatants, SDS-PAGE demonstrated homogeneity of the antibody molecules, and immunoblotting verified the presence of the particular Ig chains (Fig. 3.35).

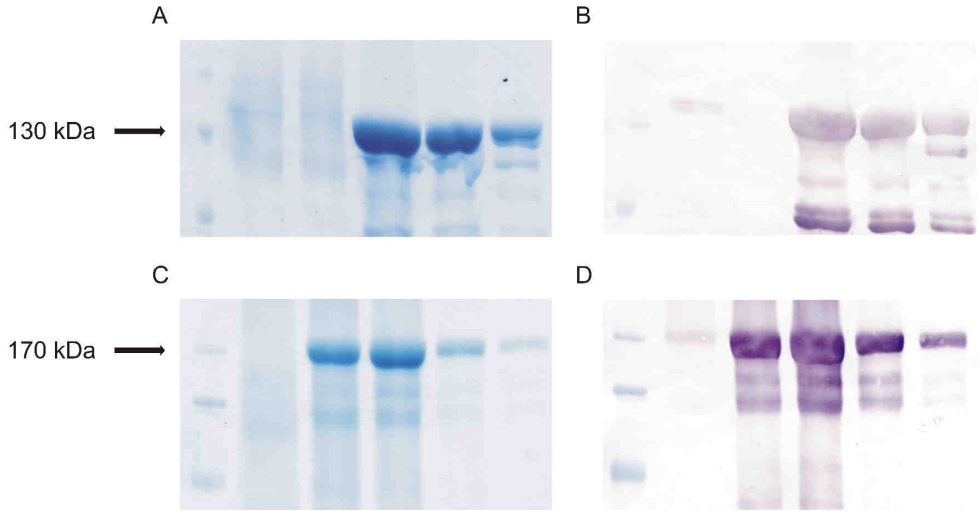


Fig. 3.35: Analysis of purified anti-MBP IgG1 C_H2-3 and anti-MBP IgE C_H2-4 antibodies.
A, B, C, D Anti-MBP IgG1 C_H2-3 and anti-MBP IgE C_H2-4 antibodies were assessed by SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining (A, C) or immunoblotting (B, D). Anti-MBP IgG1 C_H2-3 was visualized using an anti-human IgG AP conjugate and anti-MBP IgG1 C_H2-3 was visualized using an anti-human IgE AP conjugate.

Molecular masses were found to be 130 kDa and 170 kDa for the homodimeric scFv-based IgG- and IgE-analogous molecules, respectively, and 160 kDa for the heterotetrameric IgG (data not shown). The expected molecular masses in SDS-PAGE suggest that the majority of antibody secreted from the HEK-293 cells is properly folded and glycosylated, in particular the extensively glycosylated IgE.

3.6.3 ASSESSMENT OF IMMUNOREACTIVITY

Immunoreactivity of the resulting proteins was assessed by several approaches. In order to verify the broad suitablility of the recombinant antibodies, the specific immunoreactivity was demonstrated by detection of a panel of different allergens of varying molecular weight fused with MBP the recombinant proteins were assessed in ELISA (Fig. 3.36).

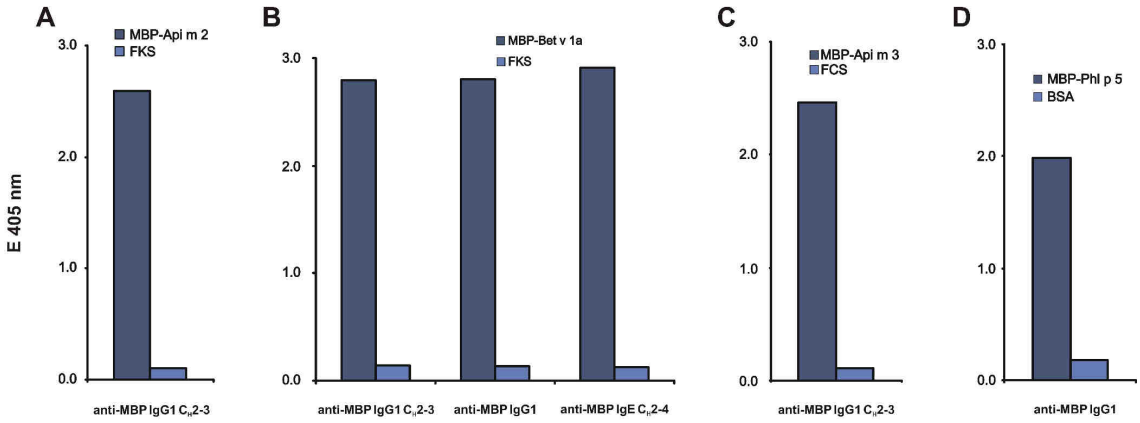


Fig. 3.36: Immunoreactivity of the recombiant anti-MBP antibody constructs
The immunoreactivity of the diverse anti-MBP antibody constructs were assessed in ELISA by the use of different MBP fusion proteins such as Api m 2 (A), Bet v 1a (B), Api m 3 (C), and Phl p 5 (D)

The data demonstrate that large fusion partners do not result in sterical hindrance of MBP recognition.

3.6.4 ACTIVITY OF THE ANTIBODIES IN CELLULAR ASSAY SYSTEMS

Finally, the suitabilty of the IgE-based antibodies to activated effector cells was assessed. Upon addition of anti-IgE pronounced degranulation followed by measuring the release of β-hexosaminidase was observed(Fig. 3.37).

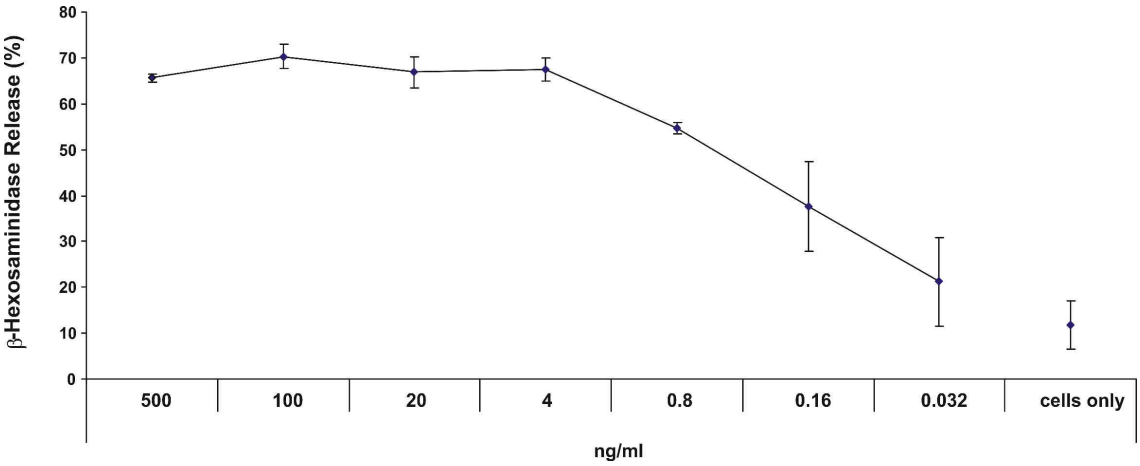


Fig. 3.37: Effects of anti-MBP IgE C_H2-4 cross-linking on effector cell degranulation
RBL-SX38 cells were sensitized with serial dilutions ofanti-MBP IgE C_H2-4and degranulation was induced by addition of anti-IgE.

Together these data demonstrate the reliability and stability of using recombinant IgE and IgG against a broadly used antigen as tools for all types of assay development, validation and control.

4 SYNOPSIS AND DISCUSSION

It is well known that IgE antibodies play a pivotal role in both allergy and host defense against parasites. Allergy can cause life-threatening anaphylactic reactions in allergic individuals. In such immune reactions allergen specific IgE (sIgE) that is bound to the high affinity IgE receptor (FcεRI) on mast cells and basophils, is cross-linked by minute amounts of allergen, and thereby, trigger degranulation which leads to the release of proinflammatory mediators. This hypersensitivity reaction type I affects more than 30 % of the population in Europe and its prevalences increasing.

This work highlights the relevance of the molecular understanding of IgE antibodies in the context of allergy as well as cancer and includes the characterization of their epitopes as well as their isotype specific effector functions. Therefore, IgE antibodies with specificities for a variety of antigens were generated and examined in diverse artificial and authentic systems. In order to cover a broad range of relevant allergens, on the one hand inhalative the major allergens Phl p 5 and Bet v 1 were investigated on a proteinic level. Furthermore, two of three highly relevant cross-reactive carbohydrate determinants (CCDs) were analyzed, namely the α1,3-core fucose on the injective major allergen Api m 1, and more detailled the alpha-Gal epitope. In addition to the epitopes of IgE antibodies their effector mechanisms were investigated by the use of the tumor-associated antigen (EGFR) wich exhibit exceedingly clinical importance.

Even though the structures of more than 50 allergens are available it remains a common pitfall in allergy research that the localization of IgE epitopes is hypothesized only based on indirect approaches. The generation of molecular mimetic epitopes, combined with molecular modeling [185, 186] is just able to give hints on antigen/antibody interactions, and therefore, their actual peculiarities remain enigmatic. A plethora of approaches that are based on direct interaction analyses of protein or peptide specific antibodies can be used to address the basis of allergen specific IgE reactivity [185, 187]. The most sophisticated strategy relies on the determination of crystal structures of antigen/antibody complexes. This approach is difficult to realize as illustrated by the fact that only five crystallographic structures of such complexes have been solved so

far, three of which have used only murine IgG antibodies [188-190]. In general, all approaches mentioned here are hampered by the fact that in all cases either polyclonal IgE from human donors or murine monoclonal antibodies were used. Only two epitopes have been crystallographically assessed by the use of authentic human IgE fragments, namely such of beta-lactoglobulin [191] and Phl p 2 [192]. Hence, apart from the phenomenological knowledge of IgE, molecular information about the antigen/antibody interaction is scarce due to the fact that the selection of monoclonal IgE derived antibodies from the immune repertoire of human donors is highly difficult [122, 123, 125, 126].

Generally, this scarcity of allergen specific human monoclonal IgE complicates the epitope characterization on a molecular level. This lack of information about their epitopes hampers the improvement of analyses of allergenicity, cross-reactivity, standardization of allergens, as well as allergy diagnostics and therapeutics. An epitope can be characterized by co-cristallization of allergens and human authentic monoclonal antibodies or antibody fragments [192], respectively, but primarily crystal structures of allergens in complex with murine fab fragments are available so far. This limitation would be obsolete if murine antibodies would recognize B-cell epitopes identical to those of human antibodies, a scenario only true for small sized epitopes that obey identical immunological mechanisms in animals and man. Such a situation is given for IgE with specificity for CCDs, which are defined by their high immunogenicity in different species and their spatially extraordinarily well defined architecture.

In this thesis, diverse methodologies for the establishment of a set of monoclonal antibodies as different isotypes were applied. These include human authentic antibodies. In the case of Phl p 5, these were generated by using the known sequence of a Phl p 5a-specific Fab fragment that was selected from an immune library as template for gene synthesis [122]. This recombinant antibody represents the first fully human monoclonal IgE antibody and the identification of its epitope defines the first authetic IgE epitope on Phl p 5. In parallel, a murine hybridoma line also specific for Phl p 5a was generated.

In contrast to the anti-Phl p 5a antibody, a monoclonal human antibody with specificity for the birch pollen allergen Bet v 1 was produced by the generation of hybrid scFv libraries. These hybrid libraries were generated by the use of the human synthetic

antibody library Griffin-1, in which the synthetic V_H were replaced by the previously obtained donor-derived IgE-specific V_H. A Bet v 1a-specific scFv was selected from the libraries via iterative panning on recombinant Bet v 1. The specificity and immunoreactivity to Bet v 1a were clearly verified and demonstrate that V_H IgE repertoires allow for the isolation of allergen specific antibody fragments, even though recombined with synthetic V_L repertoires. On the basis of the obtained recombinant human monoclonal IgE antibody, the first IgE epitope on Bet v 1 could be assigned.

In contrast to proteinic epitopes on allergens such as Phl p 5 or Bet v 1, polyclonal sera of atopic patients might be sufficient for the characterization of CCDs on allergens. The well defined α1,3-core fucose on the honeybee venom major allergen Api m 1 constitutes a singular epitope wich causes cross-reactivity of specific IgE from allergic patients to the same epitope but on different proteins.

To gain further insights into antibody/glycotope binding however, it is essential to generate monoclonal IgE antibodies. Thus, a chimeric mouse/human alpha-Gal (CCD) specific antibody was obtained by the use of the sequence information of M86 as template for gene synthesis of a scFv that was inserted into antibody formats of different isotypes. On the basis of the chimeric monoclonal IgE antibody as well as human sera, the first IgE CCD epitope could be assigned on a molecular level using STD NMR.

Additionally, a chimeric mouse/human anti-EGFR antibody was generated to immunologically address the full potential of IgE antibodies intrinsic to their Fc portion that may even be advantageous over IgG mediated immune responses.

The obtained antibodies were mainly expressed as homodimeric and heterodimeric constructs of different isotypes, primarily IgG1, IgG4, IgE, IgA1, and IgA2. These allow the analyses of their corresponding epitopes using fragmented or mutated proteins in immunoblots and ELISA approaches.

As the diverse isotypes provide defined immunological properties a variety of cellular assays was performed in order to characterize different effector functions. The ability of IgE to trigger mediator release of effector cells such as basophils, therefore may shift immune responses from T_H1-like to T_H2-like, hence, providing beneficial properties, for instance in therapeutical application of monoclonal antibodies in tumor therapy.

In addition to research and therapeutic purposes, monoclonal antibodies of the IgG and IgE isotype with defined specificity have the potential to become unique tools for allergy diagnostics because determining the susceptibiliy of an individual to develop clinical symptoms is based on the measurement of specific serum IgE levels. Hence, the availability of specific monoclonal reagents is imperative for standardization of reliable assay systems, nevertheless, specific IgE and IgG antibodies are available as polyclonal preparations only.

Thus, the aim of this work was the dissection of immunoreactivity, and interaction as well as fine epitope mapping of IgE antibodies to obtain insights into the general recognition of a plethora of epitopes and the immunological consequences on a molecular and cellular level. All results presented in this thesis were obtained in collaboration with different members of different research groups as evident from the published and submitted manuscripts listed in section 6.2.

4.1 IGE IN THE CONTEXT OF PROTEIN EPITOPE CHARACTERIZATION

4.1.1 IDENTIFICATION OF AN AUTHENTIC IGE EPITOPE ON PHL P 5 (*PHLEUM PRATENSE*)

In order to dissect IgE epitopes of Phl p 5a which is the major allergen of timothy grass *Phleum pratense* recombinant monoclonal IgE were applied in immunoblot and mediator release assays. Therefore, two Phl p 5a antibody fragments were converted into allergen specific immunoglobulins, namely IgE, IgG, and IgA. Immunoreactivity and binding to Fcε and Fcγ receptors (specific for IgE and IgG) verified their applicability.

The obtained Phl p 5 specific antibodies were applied in different assay systems to determine the corresponding human IgE (hIgE) epitopes on the surface of Phl p 5a. The converted antibodies represent the first full set of allergy related antibody isotypes and provide unique tools to study fundamental mechanisms and to gain structural information.

The major allergen Phl p 5 is a 29 kDa grass pollen protein that belongs to the superfamily of group 5 which is known to show high diversity [108]. They are also found in many members of the *Poodieae* (i.e. common grass) species. The isoallergens Phl p 5a and Phl p 5b show high amino acid sequence homology of 70-75 %, and presumably similar allergenic activity. There are two insertions/deletions in the N-terminal domains of Phl p 5a which explains the higher molecular mass and the more basic character [193].

To get information on the characteristics of IgE epitopes, a variety of approaches were pursued. Discontinuous epitopes were mimicked by phage displayed peptides [185, 194, 195] or bioinformatic modelling studies were performed [185]. Therefore, by the use of X-ray diffraction data of Phl p 6 two α-helix bundles of Phl p 5a were modeled [185]. Another approach to identify IgE epitopes on allergens constitutes the generation of allergen-specific monoclonal and polyclonal antibodies. These are then applied in immunoblot against synthetic allergen derived peptides [196] or in competitive ELISA with sera of allergic patients to search for sequences involved in IgE recognition [197].

These approaches are limited due to the lack of authentic human monoclonal IgE antibodies. Therefore, they provide only indirect data, and furthermore, distinct properties of an allergic patient serum pool due to the diverse total allergen specific IgE concentration, IgE affinity and clonality, and the spatial distribution of their epitopes. These indirect evidences of IgE epitopes imply that monoclonal IgE benefit over patient pool sera allowing a convergent interpretation of the obtained data.

To broaden the panel of antibodies with different properties, in addition to the Phl p 5 specific IgE and IgG isotypes, IgA were generated. These IgA antibodies are highly interesting mainly in the context of inhalative allergens that have first contact with the mucosa of an individual where IgA antibodies are induced as first defense antibody isotype. In general, IgA are able to act as blocking antibodies, but their affinities and their required cytokine milieu is less characterized and makes further investigation essential. In addition to the produced human antibodies a murine hybridoma line specific for Phl p 5 was established. The functionality of the human and murine derived IgE and IgG was demonstrated in receptor binding studies and mediator release assays which showed spatially separated epitopes of the applied two anti-Phl p 5 specific antibodies.

Affinities of allergen specific polyclonal IgE and IgG antibodies are controversely discussed. Based on mediator release assays in this work, apparently high affinities of the recombinant Phl p 5a specific IgE were supposed, because low affinity of IgE antibodies leads to significant decreased basophil activation [198].

The epitope of the authentic human IgE was analyzed by the use of Phl p 5 fusion proteins in immunoblotting and mediator release assays and was assigned to a looped stretch that is exclusively present in Phl p 5a. Loops that protrude from the surface are suggested typical epitopes for IgG [192]. In comparison to Phl p 5b, this loop seems to be formed by an insertion of 20 aminoacids that cause its protrusion. To further dissect the epitope, structural information of cytochrome c, Phl p 6, and the C-terminal domain of the isoform Phl p 5b were used as basis for more detailed analyses by modeling approaches. A formerly postulated IgE epitope on Phl p 5 includes the residues 58-76 [185], however, in this work it could be shown that this region apperently is not sufficient for IgE binding by immunoreactivity with the fragment F2a which lack this region. The obvious limitation of the epitope size contradicts the common hypothesis of large, structural more complex surface areas as IgE epitopes [189].

4.1.2 IDENTIFICATION OF AN AUTHENTIC IGE EPITOPE ON BET V 1 (*BETULA VERRUCOSA*)

The major birch pollen allergen Bet v 1 belongs to the pathogenesis-related proteins of class 10 (PR-10) and represents a major antigen in immunity and allergy, and thus, has been extensively characterized. Its crucial role in sensitization renders Bet v 1 an outstanding target for development of therapeutic strategies.

Birch pollen per se are the major cause of hypersensitivity type I and over 95 % of tree pollen sensitized patients display IgE binding to Bet v 1. Nearly 60 % of those allergic individuals react exclusively with Bet v 1 what makes it highly relevant. Besides, pollen of other *Fagales* species and several foods (nuts, vegetables, and rosaceae fruits) contain Bet v 1 homologues which cause reactions due to cross-reactivity. Cross-reactive IgE to food is responsible for the oral allergy syndrome (OAS), mostly the basis of food allergy in pollen allergic patients.

In this work the consecutive generation and epitope delineation of a human monoclonal IgE against the allergen Bet v 1 is reported.

For the generation of allergen specific human antibodies, an immune/synthetic hybrid library was established from pollen allergic patients derived V_H regions that were complemented with synthetic V_L regions. Hence, hybrid repertoires were established and their successful selection confirmed the general feasibility of this approach. The combinatorial technology itself poses inherent limitation and the *in vitro* combination of V_H and V_L domains is entirely randomly, and therefore, does not necessarily create an image of the original repertoire. The obtained antibody is the first authentic Bet v 1 specific IgE with verified origin and is highly reactive with Bet v 1 but not with PR-10 Mal d 1.

For the Bet v 1 specific antibody paratope, the corresponding epitope on the surface of Bet v 1 could be described. Bet v 1 consists of a seven stranded antiparallel beta-sheet that builds a cavity for a 25 residue long C-terminal alpha-helix [199] that comprises the amino acids 124-160. Formerly approaches on structural data identified three patches that are conserved in tree pollen major allergens of the order *Fagales*. The murine antibody BV16 was used to extrapolate human IgE epitopes and found to recognize one of these patches [189]. Furthermore, different monoclonal murine, polyclonal human antibodies or peptide-specific hybridoma derived antibodies has been used for the assessment of the structural basis of IgE reactivity to Bet v 1 [197, 200, 201]. To dissect the IgE epitope, advantage was taken of differential reactivities against Bet v 1 isoforms and Mal d 1. Truncation and mutagenesis approaches finally led to assignment of the terminal helix of Bet v 1 as IgE epitope. The helix sequence could also be found in tree pollen homologues but not in homologues from food such as Mal d 1 (apple) and Dau c 1 (carrot). A graft of this motif onto non-reactive Mal d 1 reestablished reactivity. These findings enable a more general view on allergen cross-reactivity due to the helix motif that is conserved in defined isoforms of different PR-10 proteins.

The recognition of the identified IgE epitope in this work again seems to contradict the hypothesis that IgE epitopes are preferably defined by large surface areas comprising several structural elements [192, 202].

Library derived antibodies against their corresponding IgE epitope on the surface of Phl p 5 and Bet v 1 were assigned. Both, the anti-Phl p 5 and the anti-Bet v 1 antibody constitute the first (semi-) authentic monoclonal human IgE antibodies whose epitopes,

which are obviously distinct from formerly proposed hypothesis [189, 192, 202], on major allergens were identified.

In this work, a unique set of allergen specific (semi-) authentic human antibody isotypes was generated which enables detailed analyses of the molecular interactions of allergens with the human humoral immune response. Strategies that were utilized to generate these antibodies might be adapted to a variety of settings. This may contribute to a broader access to molecular components in order to dissect the complex network of allergic responses and to a deeper understanding of the nature of B cell epitopes. Furthermore, the production of hypoallergenic variants that provide reduced IgE reactivity but conserved T cell reactivity for efficient and safe allergen specific immunotherapy could be accelerated.

4.2 IGE IN THE CONTEXT OF CLASSICAL CCDS

CCDs are found as α1,3-core fucose on insect venom allergens, and additionally as β1,2-xylose on plant proteins, and patient derived antibodies specific for these glycotopes represent the universal principle of reactivity of different proteins bearing CCDs [49]. Recently, a novel type of CCD has entered the field of research of atopic diseases, namely the alpha-Gal (α1,3-galactose) epitope [60]. CCDs became clinical relevant due to their identity and occurrence on a variety of proteins which can cause cross-reactivity in atopic individuals. In this work, immunoreactivites of the major honeybee venom allergen Api m 1 and alpha-Gal were examined.

4.2.1 α1,3-CORE FUCOSE ON API M 1 (*APIS MELLIFERA*)

Allergic reactions, such as life-threatening anaphylaxis, to hymenoptera stings are one of the major reasons for IgE mediated hypersensitivity reactions.

The major venom allergen of honeybee venom (HBV) is phospholipase A2 (phosphatidylcholin-2-acylhydrase, Api m 1, PLA2) which represents 12 % of the dry weight of venom [162]. This phospholipase specifically recognizes the sn-2 acyl bond of phospholipids [162] and catalytically hydrolyzes this bond leading to damage of

structural membranes. The mature peptide contains 10 cysteine residues that form 5 disulfide bonds and one defined oligosaccharide attached to asparagine 13 (N13) [160].

Api m 1 is recognized by sIgE of 97 % of HBV allergic individuals [153] and represents a typical group III secreted PLA2 [161]. Interestingly, PLA2 of the honeybee exhibits CCD reactivity due to the α1,3-core fucose. Thus, proper diagnosis using venom extracts is severely affected by molecular cross-reactivity due to CCDs, namely the α1,3-core fucose, and the false diagnosis of an allergy due to CCDs complicates therapeutic outcomes To avoid CCD based cross-reactivities recombinant allergens lacking this moiety are essential tools for therapeutic and diagnostic approaches. However, the recombinant expression of HBV PLA2 in *E. coli* only led to an unfolded protein devoid of any allergenic activity in skin test [203] because the complete epitope spectrum of a protein is based on native folding which in turn in many cases is glycosylation dependent. Most notably, defined glycosylation patterns of allergens can have intriguing consequences for our understanding of IgE reactivities [50].

In this study Api m 1 was recombinantly produced as a soluble and secreted protein by a nearly homologous baculovirus mediated expression in insect cells. An inactive mutant (H34Q) which should exhibit reduced cytotoxicity in cellular assays was generated by site-directed mutagenesis and produced as soluble protein by the baculovirus-based expression in Sf9 (*Spodoptera frugiperda*) and HighFive (*Trichoplusia ni*) cells. These cells are found to be suitable hosts for the production of properly folded, and, important for this approach, accurately glycosylated hymenoptera venom enzymes [50, 204-207].

Thus, by the use of baculovirus infection of different insect cell lines allergen versions which provide a varying degree of cross-reactive carbohydrate determinants as well as a non-glycosylated variant were obtained. They were analyzed for their glycosylation pattern and proved to show advantageous properties regarding cross-reactivity in sIgE based assays. Interestingly, the inactivated allergen did not induce IgE independent effector cell activation in contrast to the native enzymatically active protein.

Due to this singular N-glycan, Api m 1 features a valuable tool for epitope analysis of classical CCDs on the basis of α1,3-core fucose. This minimal epitope showed specific binding of IgE from an individual polyclonal patient serum demonstrating the relevance and specificity of sIgE to glycan structures. Even though artificial or recombinant

glycoproteins did not show a clear cut effect in mediator release assays or skin prick tests [170, 171], IgE against CCDs are appreciated as a source of allergic reactions, and therefore, has been shown clinically relevant [164, 167-169].

4.2.2 DISSECTING ALPHA-GAL AS AN IGE EPITOPE

IgG as well as IgE antibodies might also be directed against xenobiotics which is reflected by high titers of anti-alpha-Gal IgG antibodies in human serum. Therefore, these biological agents are thought to be highly immunogenic. A novel type of cross-reactive carbohydrates has recently entered the field and provide final evidence for the immunogenic and detrimantal potential of glycans. This CCD constitutes the α1,3-gal epitope (alpha-Gal) that is expressed on proteins derived from non-primate mammals, prosimians, and New World monkeys [60]. Thus, the alpha-Gal became a focal point due to its association with meat induced delayed symptoms of anaphylaxis, and is reported as a novel type of carbohydrate epitope that causes severe food allergy related to IgE antibodies [65].

It is very controversially discussed [166] if anaphylaxis can be mediated by CCD specific IgE antibodies. An evidence for a clinical relevance might be IgE specific for the xenotransplantation antigen alpha-Gal that is associated with a delayed type of anaphylaxis. Alpha-Gal is also crucial for meat induced allergy [65] and for cross-reactivity to other mammalian allergens [172]. Recently, intense induction of IgE specific for the alpha-Gal epitope was correlated with tick bites by special species present within a restricted area of the U.S. [66]. Additionally, allergic reactions to the monoclonal antibody cetuximab that is applied in therapeutic intervention in cancer was observed. This chimeric antibody comprises two defined alpha-Gal epitopes in its V_H and is suggested to induce allergic reactions [68]. In order to dissect this CCD regarding to binding characteristics as well as effector mechanisms, human/mouse chimeric antibodies were generated on the basis of an alpha-Gal specific antibody fragment to obtain structural and moleculare insights into its interaction with alpha-Gal. The monoclonal antibodies were recloned as IgE and IgG isotypes using exemplarily the alpha-Gal specific moiety of a well established murine IgM antibody (M86).

The functionalities of the alpha-Gal specific antibodies were assessed in ELISA as well as in receptor binding studies for both, the IgE and IgG. In general, the so shown

immunoreactivity of the antibodies and the functional binding with high affinities to the corresponding FcεRI and FcγRI verifies their applicability in various approaches such as immunological and allergy diagnostics. The interaction of the alpha-Gal specific IgE antibody was employed for cellular mediator release assays, and, in addition with human serum for the characterization of the IgE epitope by STD NMR.

The M86 based IgE antibody did not induce any mediator release as demonstrated by the disability to mediate basophil degranulation in the presence of multivalent conjugate, in contrast to a reference antibody, a fact that might reflect the delayed type of anaphylaxis. The mechanisms underlying this retardation remain enigmatic, even though that the murine C38-2 IgE, also of medium-affinity, exhibits clear cut cellular activation upon stimulation with alpha-Gal. This might be affected by the architecture of the alpha-Gal epitope and its availability on the surface of different carriers and may reflects the fact that limited affinities of allergen specific IgE can significantly decrease basophil sensitivity. These findings cannot explain that the class switching of preexisting IgM or IgG to IgE may cause anaphylactic reactions associated with alpha-Gal, a fact that remains to be elucidated as well as the affinity threshold that is needed for basophil activation.

For the generated alpha-Gal specific IgE antibodies affinities were assessed in SPR measurements using a variety of alpha-Gal bearing proteins with more or less defined N-glycans. In contrast to antibodies with specificity for protein derived epitopes, IgE is generally hypothesized as highly affinity matured isotype. However, antibodies with specificity for carbohydrate epitopes, in utmost cases exhibit low to medium affinities, for instance, alpha-Gal specific antibodies in humans are reported to have affinities in the range of 10^{-5} M to 10^{-6} M [179]. This finding holds true for most lectins often exhibiting affinities in the micromolar range which is compatible with the reasonable affinities of the generated antibodies in this work, although, the real affinity is difficult to assess due to the valencies of both the multivalent alpha-Gal antigen as well as the bivalent antibodies.

STD NMR provides a valuable technique to dissect epitopes by the use of antibodies and is fundamentally based on the dynamics of their interactions. Thus, the nuclear overhauser effect (NOE) is transferred to ligand residues which are essentially involved in antigen binding. An extraordinary richness in detail that is comparable to X-ray

structures only constitutes the resulting fine mapping of the antibody/antigen interaction. Additionally, this approach demonstrates that the characterization not only of monoclonal IgE but also of serum reactivities can be easily addressed to obtain information for individual patient sera. These information could be applied for the comparative analyses of patients sera to gain deeper insights into epitope recognition. As human serum consists of about 1 % of anti-Gal specific IgG antibodies, it is conceivable that different parts of the alpha-Gal are recognized. Due to Moreover, due to the enigmatic allergic response of some individuals to CCDs, it is highly interesting to assess such affinity purified antibodies from healthy and atopic donors to compare their binding characteristics.

The IgE epitope could be primarily assigned to the two galactose units and to a weaker extent to the residues downstream of the Gal-Gal unit by the paratope-inherent information of the M86 based IgE and the polyclonal antibodies wich were affinity purified using an alphal-Gal carrier protein coupled to a matrix. To go in detail, the more pronounced STD effects for the terminal galactose unit suggest a more direct implication in the carbohydrate/antibody interaction. This is in a good accordance with the relevance of the 1-3-linkage that is crucial for recognition. Additionally, the terminal galactose of the verified alpha-Gal epitope in STD resembled the binding moiety of the lectin agglutinin I from *Ricinus communis* with methyl-beta-galactosid. Both comprise a nearly identical distribution of STD effects, in particular with H2, H3, and H4 [208]. Furthermore, the galctose binding lectin VAA from *Viscum album*, whose epitope was reported as galactose [209, 210], and galectin-I seem to interact with the non-reducing end of digalactosides [211]. The complementary data of affinities and fine specificity in this settings might help to elucidate the recognition of carbohydrates and the molecular requirements of carbohydrate-based anaphylaxis.

These data enable valuable insights into the molecular interactions and cellular immune responses and may help to understand the nature of IgE B cell epitopes of cross-reactive carbohydrate determinants, here the alpha-Gal epitope that obviously can act as an authentic IgG epitope as well as an IgE epitope.

4.3 IGE ANTIBODIES IN THE CONTEXT OF CANCER

In this work the tumoricidial and anti-proliferative properties of a recombinant IgE with specificity for the epidermal growth factor receptor (EGFR) is described. Therefore, its possible potential to destruct tumor cells by the recruitment of adequate effector cells was tested in different assay systems in comparison to the well estblished IgG variant. Compared to IgG, IgE may recruit alternate immune competent cells to normally inaccessible tumor sites, particularly in tissues, and improve tumor cell surveillance.

4.3.1 COMPARATIVE ANALYSIS OF THE ANTI-EGFR IGE AND IGG

Monoclonal antibodies like the chimeric antibody cetuximab (IMC-225, Erbitux®) have become a mainstay for the specific treatment of cancer today. Cetuximab is a chimeric murine/human monoclonal antibody of IgG1 isotype and competes with endogenous ligands like epidermal growth factor (EGF) and transforming growth factor (TGF-α) [212]. It has been approved for use in combination with irinotecan in advanced stage EGFR expressing metastatic colorectal carcinoma and also for patients with advanced squamous cell carcinoma of the head and neck in 2004 and 2006 (US), respectively [213].

The ERBB receptor tyrosine kinases represent highly relevant tumor antigens that are targeted by many different antagonistic agents including humanized antibodies. EGFR is expressed by diverse cells, and plays a pivotal role in a variety of tumors upon abnormally activation or overexpression. This is associated with tumor progression and poor prognosis [80]. Inhibition of the EGFR pathways by the therapeutic antibody cetuximab showed relevant efficacy as monotherapy in a variety of human epithelial cancers and increased sensitivity to chemotherapy in clinical trials [214, 215]. The mechanism of anti-tumor activity of individual monoclonal antibodies directed to the different ERBBs is not yet entirely understood but reatment of EGFR based tumor disease using monoclonal antibodies seems to exert indirect effects that are mediated by Fc effector functions. Most of the observed tumoricidal activity is due to the recruitment of natural killer cells by an antibody dependent cell mediated cytotoxic mechanism (ADCC) [216]. Today, human IgG1 is the most widely used isotype for therapeutic

antibodies because it is capable of activating human complement, recruits effector cells for ADCC, and has an extended plasma half life.

Therefore, several groups have examined the question whether using IgE with specificity to tumor associated antigens may be beneficial in preventing disease progression [217-220]. It could be shown that upon cross-linking by allergens, IgE antibodies bound to FcεRI on mast cells and basophils are able to trigger degranulation and release of proinflammatory mediators which led to immediate reactions and initiation of biosynthetic pathways to produce prostaglandins and leukotrienes [221].

Compared to IgG, IgE attracts different effector cells and induces immediate cellular anti-tumor reactions by recruitment of mast cells and basophils that provide a distinct tissue distribution compared to FcγR-activated effector cells. Additionally, eosinophils have long been considered simply as effectors of adaptive immune responses during parasitic infections and inflammatory processes, but evidence is accumulating that FcεRI expressed by human eosinophils is also involved in IgE mediated cytotoxicity reactions [222].

Hence, there is a need to modify treatment modalities in order to increase the number of patients who benefit from therapy.

In this work, the effector mechanisms of EGFR specific human IgE and IgG1 (225-IgG1 and 225-IgE) antibodies were compared. To address this issue, mouse/human chimeric antibodies containing the cetuximab variable regions were produced and analyzed to figure out their potential to eradicate tumor cells that overexpress EGFR. Their specificity was demonstrated by a variety of different methods, all of them indicating similar binding properties compared with the commercial product Erbitux™. The 225-IgG1 and 225-IgE specifically inhibited tumor cell proliferation as determined by MTT cell viability assays and confirmed that receptor blockade in general is not affected by its isotype. Whether any differences regarding receptor internalization and turn-over rates of EGFR may exist and if this translates into differential activation of effector cells, needs to be further investigated.

The observed degranulation of rat basophilic leukemia cells transfected with human FcεRI subunits (RBL-SX38) indicated that the IgE Fc domains were able to trigger effector functions *in vitro*. Interestingly, monomeric soluble EGFR (sEGFR) that mimicked secreted splice variants or shed EGFR *in vivo* was not sufficient for cellular

activation. In contrast, membrane-bound EGFR or EGFR-coated microspheres such as sEGFR complexed with tetrameric steptavidin or macroclusters reflected by sEGFR coated on streptavidin beads efficiently activated degranulation, a finding which may help to elucidate the interplay of IgE loaded effector with its cognate target structures.

Further studies are needed to assess the impact of EGFR density and cluster size on formation of interactions with effector cells and the induction of downstream effects exerted by U937 effector cells when stimulated by the IgE and IgG1 antibody isotypes. IgE can induce infiltration of monocytes and ADCC by binding to the high affinity receptor FcεRI as well as antibody dependent phagocytosis (ADCP).

To recapitulate, monoclonal human IgE and IgG1 antibodies specific for EGFR were expressed and purified in order to evaluate their potential to differentially activate effector cells and target tumor cells. Both, 225-IgG and the 225-IgE possess comparable binding characteristics in terms of epitope recognition and affinity, but different effector functions. This suggest an advantage for the recruitment of different cells possessing distinct effector mechanisms.

It was found that EGFR specific IgE showed improved tumoricidal activity compared to IgG1 which was mediated by degranulation of basophils. ADCC effects could be clearly observed with human monocytic U937 cells and EGFR overexpressing cell line A431 as target cell, and confirmed the tumoricidal properties of the IgE isotype.

Since the monoclonal anti-EGFR antibody 225-IgE solely in the presence of multivalent antigen, i.e. cell bound EGFR, is able to trigger degranulation of effector cells, it is safe regarding to potentially anaphylactic reactions.

4.4 OUTLOOK

To gain first and detailed insights into epitopes in the context of IgE, namely epitopes on proteins as well as N-glycan derived CCDs, a variety of antibodies were generated in this work. These were obtained from authentic, hybrid, and synthetic libraries as well as hybridomacell lines. Thereby the obtained antibody fragments represent the antigen-specific moiety and were converted into diverse isotypes.

Overall this work points out that neither a difference in affinity nor in antigen recognition (alpha-Gal) associated with the isotype. This finding implies that epitopes of IgE are not necessarily different from other epitopes and consequently, that each kind of epitope is suited to be or become an authentic or artificial IgE epitopes. Particular epitopes such as CCDs impressingly represent examples for shared immune reactive minimal epitopes but appear to be not anaphylactoid in the context of an IgE response. The clinical relevance of CCD-specific IgE antibodies thus remains controversial, with only few data giving evidence for a delayed type of hypersensitivity reaction after consumption of red meat upon repeated tick bites [65].

The apparent consistency of IgE and IgG epitopes underlines that IgE antibodies could constitute an interesting tool for tumor therapy, exploiting an arm of the immune response different from that of routinely employed IgG antibodies. Moreover, monoclonal IgE antibodies may provide beneficial properties by inducing a local T_H2 immune response at defined tumor sites. Antibodies *per se* constitute valuable recombinant tools to unreval binding characteristics to specific epitopes, further dissecting into isotype specific epitopes, and, even into individual epitopes depending on the donor might become accessible. Such findings may help to understand, why IgE trigger degranulation in some individuals, but not in others, and therefore, may help to understand T_H2 mechanisms and immune responses in general.

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6 APPENDIX

6.1 CURRICULUM VITAE

Personal Information

Last Name:	Plum
First Name:	Melanie
Date of Birth:	07.12.1973
Country:	Germany
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Scholarships

12/2007 - 06/2008	Stipendium der Universität Hamburg
07/2008 - 12/2008	Stipendium der Universität Hamburg

Studies

Study of Biology at the University of Hamburg, Germany, 2001 - 2007

Diploma Thesis: "Generation and evaluation of recombinant IgE- and IgG-antibodies directed against tumor-associated target structures"; Group of Prof. Dr. R. Bredehorst, Department of Chemistry, Institute for Biochemistry and Molecular Biology II, University of Hamburg, Germany

Degree in Biology (Biochemistry, Genetics and Molecular Biology, Zoology) at the University of Hamburg, Germany, 2007

Doctoral Thesis

Doctoral thesis: (December 2007 until September 2011): "Dissection of the IgE Interactome on a Molecular Level"; Group of Prof. Dr. R. Bredehorst, Department of Chemistry, Institute for Biochemistry and Molecular Biology II, University of Hamburg

6.2 PUBLICATIONS

6.2.1 ORIGINAL ARTICLES

Melanie Plum, Yvonne Michel, Katharina Wallach, Tim Raiber, Simon Blank, Frank I. Bantleon, Andrea Diethers, Kerstin Greunke, Ingke Braren, Thomas Hackl, Bernd Meyer, and Edzard Spillner

Close-up of the immunogenic a1,3-Galactose epitope as defined by a monoclonal chimeric immunoglobulin E and human serum using saturation transfer difference (STD) NMR

J Biol Chem. 2011 Dec 16;286(50):43103-11. Epub 2011 Oct 11.

Julia Hecker, Andrea Diethers, Stefanie Etzold, Henning Seismann, Yvonne Michel, **Melanie Plum**, Reinhard Bredehorst, Simon Blank, Ingke Braren, and Edzard Spillner

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

Mol Immunol. 2011 May;48(9-10):1236-44. Epub 2011 Apr 6.

Simon Blank, Yvonne Michel, Henning Seismann, **Melanie Plum**, Kerstin Greunke, Thomas Grunwald, Reinhard Bredehorst, Markus Ollert, Ingke Braren, and Edzard Spillner

Evaluation of different glycoforms of honeybee venom major allergen phospholipase A2 (Api m 1) produced in insect cells

Protein Peptide Lett. 2011 Apr;18(4):415-22.

6.2.2 ABSTRACTS

Karagiannis, P; Braren, I; Singer, J; Mechtcheriakova,D; **Plum, M**; Knittelfelder, R; Spillner, E; Jensen-Jarolim, E

Characterizing the tumoricidic functions of an anti-EGFR antibody of the IgE class

Allergy 2010, 65 (Suppl. 92), 2014

Josef Singer, **Melanie Plum**, Ingke Braren, Panagiotis Karagiannis, Diana Mechtcheriakova, Regina Knittelfelder, Edzard Spillner, Erika Jensen-Jarolim.

Comparison of Tumoricidic Properties of Anti EGFR IgG and IgE Antibodies

J Immunotherapy 2011, Volume 34; 2

A. Diethers, J. Hecker, H. Seismann, S. Etzold, Y. Michel, **M. Plum**, S. Blank, R. Bredehorst, I. Braren, E. Spillner

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

Allergo J 2011, 20; 1

Y. Michel, **M. Plum**, F. Bantleon, S. Blank, I. Braren, E. Spillner

Human monoclonal anti-Gal IgE for diagnostic and mechanistic analyses of alpha-Gal associated reactivities

Allergo J 2011, 20; 1

M. Plum

Comparative analyses of IgE and IgG1 antibodies with specificity for the EGFR

Allergo J 2011, 20; 1

Blank, S; Bantleon, F; Seismann, H; Michel, Y; **Plum, M**; McIntyre, M; Bredehorst, R; Ollert, M; Braren, I;Spillner, E

Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies

Allergy 2011, 66 (Suppl. 94), 195

Hecker, J; Diethers, A; Etzold, S; Seismann, H;Michel, Y; **Plum, M**; Bredehorst, R; Blank, S; Braren, I; Spillner, E

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

Allergy 2011, 66 (Suppl. 94), 1385

Zennaro, D; Capalbo, C; Scala, E; Liso, M; Spillner,E; Penichet, M; Singer, J; Braren, I; Daniels, T; **Plum, M**; Santoro, M; Scoppola, A; Marchetti, P; Jensen-Jarolim, E; Mari, A

IgE, IgG4 and IgG response to tissue-specific and environmental antigens in patients affected by cancer

Allergy 2011, 66 (Suppl. 94), 209

6.2.3 CONFERENCE CONTRIBUTIONS AND ORAL PRESENTATIONS

Melanie Plum, Ingke Braren, Josef Singer, Panagiotis Karagiannis, Diana Mechtcheriakova, Regina Knittelfelder, Erika Jensen-Jarolim, and Edzard Spillner

IgE-Dependent Anti-Tumor Activity Is Not Dependent On Epitope Proximity

WAC, Buenos Aires, 2009,

Panagiotis Karagiannis, Ingke Braren, Josef Singer, Diana Mechtcheriakova, **Melanie Plum**, Regina Knittelfelder, Edzard Spillner, and Erika Jensen-Jarolim

Production and characterization of an anti-EGFR Antibody of the IgE subtype

YSA, Wien, 2010

Josef Singer, **Melanie Plum**, Ingke Braren, Panagiotis Karagiannis, Diana Mechtcheriakova, Regina Knittelfelder, Edzard Spillner, and Erika Jensen-Jarolim

Comparing antibody mediated-cytotoxicity and -phagocytosis of anti-EGFR IgG and IgE antibodies in a flow cytometric assay

EAACI (GA^2LEN), Davos, 2011

Simon Blank, Frank Bantleon, Henning Seismann, Yvonne Michel, **Melanie Plum**, Mareike McIntyre, Reinhard Bredehorst, Markus Ollert, Ingke Braren, Edzard Spillner

Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies

DGAKI, Mainz, 2011; EAACI, Istanbul, 2011

Andrea Diethers, Julia Hecker, Henning Seismann, Stefanie Etzold, Yvonne Michel, **Melanie Plum**, Simon Blank, Reinhard Bredehorst, Ingke Braren, Edzard Spillner

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

DGAKI, Mainz, 2011

Yvonne Michel, **Melanie Plum**, Frank Bantleon, Simon Blank, Ingke Braren, Edzard Spillner

Human monoclonal anti-Gal IgE for diagnostic and mechanistic analyses of alpha-Gal associated reactivities

DGAKI, Mainz, 2011

Melanie Plum, Ingke Braren, Josef Singer, Panagiotis Karagiannis, Diana Mechtcheriakova, Regina Knittelfelder, Erika Jensen-Jarolim, and Edzard Spillner

Comparative analyses of IgE and IgG1 antibodies with specificity for the EGFR

DGAKI, Mainz, 2011

Simon Blank, Frank Bantleon, Henning Seismann, Yvonne Michel, **Melanie Plum**, Mareike McIntyre, Reinhard Bredehorst, Markus Ollert, Ingke Braren, Edzard Spillner

Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies

EAACI, Istanbul, 2011

Danila Zennaro, Carlo Capalbo, Enrico Scala, Marina Liso, Edzard Spillner, Manuel Penichet, Josef Singer, Ingke Braren, Tracy Daniels, **Melanie Plum**, Mario Santoro, Alessandro Scoppola, Paolo Marchetti, Erika Jensen-Jarolim, Adriano Mari

IgE, IgG4 and IgG response to tissue-specific and environmental antigens in patients affected by cancer

EAACI, Istanbul, 2011

6.2.4 POSTER

Ingke Braren, **Melanie Plum**, Simon Blank, and Edzard Spillner

Addressing erbb1 by human and murine IgE

AllergOncology, Vienna, 2008

Panagiotis Karagiannis, Ingke Braren, Josef Singer, Diana Mechtcheriakova, **Melanie Plum**, Regina Knittelfelder, Edzard Spillner, and Erika Jensen-Jarolim

Investigating the tumoricidic properties of an anti-EGFR IgE

WAC, Buenos Aires, 2009

Panagiotis Karagiannis, Ingke Braren, Josef Singer, Diana Mechtcheriakova, **Melanie Plum**, Regina Knittelfelder, Edzard Spillner, Erika Jensen-Jarolim

Characterizing the tumoricidic functions of an anti-EGFR antibody of the IgE class

EAACI, London, 2010

Josef Singer, **Melanie Plum**, Ingke Braren, Panagiotis Karagiannis, Diana Mechtcheriakova, Regina Knittelfelder, Edzard Spillner, and Erika Jensen-Jarolim

Comparison of tumoricidic properties of anti EGFR IgG and IgE antibodies

ISBTC, Washington, 2010

Julia Hecker, Andrea Diethers, Stefanie Etzold, Henning Seismann, Yvonne Michel, **Melanie Plum,** Reinhard Bredehorst, Simon Blank, Ingke Braren, Edzard Spillner

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

EAACI, Istanbul, 2011

6.2.5 GRANTS

Travel Grant, WAO, Buenos Aires, 2009; $ 900 USD

6.3 GEFAHRSTOFFE UND SICHERHEITSDATEN

Folgende Reagenzien und Lösungsmittel waren mit Gefahrenhinweisen und Sicherheitsratschlägen gemäß §6 der Gefahrstoffverordnung versehen:

Verbindung	**Gefahrensymbol**	**R-Sätze**	**S-Sätze**
ABTS	Xi	36/37/38	26-36
Acrylamid	T	45-46-20/21-25-36/38-43-48/23/24/25-62	53-45
Ammoniumpersulfat	O, Xn	8-22-36/37/38-42/43	22-24-26-37
Ampicillin	Xn	36/37/38-42/43	22-26-36/37
BCIP	-	-	22-24/25
Calciumchlorid Dihydrat	Xi	36	22-24
Chloroform	Xn	22-38-40-48/20/22	36/37
Diethylpyrocarbonat	Xn	20/22-36/37/38	26-36
Dimethylformamid	T	61-20/21-36	53-45
Dimethylsulfoxid	Xi	36/38	26
Dithiothreitol	Xi	36/37/38	36/37/39-22
EDTA-Dinatriumsalz-Dihydrat	Xn	22	-
Ethanol	F	11	7-16
Ethidiumbromid	T+	22-26-36/37/38-40	26-28.2-36/37-45
Glutardialdehyd, 25 %	T, N	22-23-34-42/43-50	26-26/37/39-45-61
Imidazol	C	22-34	22-26-36/37/39-45
Kanamycin Sulfat	T	61	26-36/37-39-45
Methanol	F, T	11-23/24/25-39/23/24/25	7-16-36/37-45
NaOH	C	35	26-37/39-45
Ni-NTA-Agarose	Xn	10-22-40-42/43	13-26-36-46
NBT	Xn	20/21-33	22-45
Phenol (Tris-gesättigt)	T	24/25-34	28.6-45
2-Propanol	F, Xi	11-36-67	7-16-24/25-26
Salzsäure, konz.	C	34-37	26-36/37/39-45
SDS	Xn	22-36/38	22-24/25
TEMED	C, F	11-20/22-34	16-26-36/37/39-45
Triethylamin	F, C	11-20/21/22-35	3-16-26-29-36/37/39-45
Tris	Xi	36/38	-
Wasserstoffperoxid	C	34	3-28-36/39-45

30 %			
Xylen Cyanol FF	Xi	36	24
Zitronensäure	Xi	36	24/25

Auflistung der verwendeten KMR-Substanzen, Kat. I und II:

Cas-Nummer	**Stoffname (IUPAC) und Cat.**	**Verfahren und eingesetzte Menge**	**Kategorie (I oder II)**
79-06-1	Acrylamid	PAGE	1B
68-12-2	N,N-Dimethylformamid	Detektion Immunprint	1B
1239-45-8	Ethidiumbromid	Agarosegel, DNA Interkalation	2
50-00-0	Formaldehyd	PAGE Silberfärbung	2
7786-81-4	Nickelsulfat	IMAC	1A
108-95-2	Phenol	Phenol / Chloroform Fällung	2
67-66-3	Trichlormethan	Phenol / Chloroform Fällung	1B

DANKSAGUNG

An dieser Stelle möchte ich mich bei all jenen bedanken, die mich bei der Durchführung dieser Arbeit und darüber hinaus unterstützt haben.

Mein Dank gilt Herrn Prof. Dr. Reinhard Bredehorst für die Überlassung des interessanten Themas. Herrn Prof. Dr. Ulrich Hahn danke ich für die Übernahme des Koreferats sowie Dr. Thomas Hackl für die Begutachtung der Disputation.

Mein ganz besonderer Dank gilt Edzard Spillner für das Fördern durch Fordern, die interessanten und auch witzigen Gespräche sowie für seine Verlässlichkeit und stete Unterstützung. Ein großes Dankeschön auch für die kritische Durchsicht dieser Arbeit und die konstruktiven Beiträge, die zum Gelingen beigetragen haben.

Simon Blank danke ich ebenfalls für die Durchsicht dieser Arbeit und die technische Unterstützung. Unverzichtbar sind die vielen lustigen Gespräche.

Des Weiteren möchte ich mich bei Kerstin Greunke und Thorsten Mix für die schöne Atmosphäre in unserem Labor und die manchmal recht exotische musikalische Untermalung danken.

Frank Ingo Bantleon möchte ich für die vielen Gelegenheiten zu lachen danken und für die vielen Brötchen.

Selbstverständlich gilt mein Dank auch allen aktuellen und ehemaligen Mitgliedern unserer Arbeitsgruppen.

Abschließend möchte ich meiner Schwester Tanja, Gabi Heitmann und Bianka Petersen danken, die, jeder auf seine wundervolle Art, am Gelingen dieser Arbeit beteiligt waren.

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Ergebnisse aus Diplom-, Bachelor- oder Schwerpunktarbeiten, die in diesem Arbeitskreis angefertigt wurden und die teilweise in diese Arbeit eingeflossen sind, wurden von mir initiiert und unter meiner Anleitung angefertigt.

Ebenfalls versichere ich, noch keinen weiteren Promotionsversuch an einer anderen Einrichtung unternommen zu haben

Hamburg, den 14.12.2011

_____

Melanie Plum