A New Molecular Approach for Monitoring Autoantibodies against FcεRIα in Healthy Donors and Urticaria Patients

Inauguraldissertation der
Philosophisch-Naturwissenschaftlichen Fakultät
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Michaela Fux
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Leiter der Arbeit:
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Prof. Paul Messerli

A. SUMMARY

The aim of my thesis was to better understand the pathophysiological role of autoantibodies against the α-chain of the high affinity IgE receptor (FcεRIα) in healthy individuals and chronic urticaria (CU) patients and to develop a new diagnostic tool to monitor such autoantibodies in the human antibody repertoire. Anti-FcεRIα autoantibodies have been claimed to play a pathological role in a subset of CU patients and were therefore proposed as a selective marker for CU. However we have recently shown the presence of such autoantibodies in multidonor IgG preparations and sera of healthy individuals. Furthermore we have reported the isolation of different anti-FcεRIα autoantibodies from phage display libraries constructed from B cells of healthy children's tonsils and urticaria patients, respectively. Strikingly the same antibody was isolated independently from both the tonsil and urticaria libraries, named LTMα15 and UMα16, respectively. Sequence analysis revealed germline configuration of heavy chains with slightly mutated light chains supporting their classification as natural autoantibodies.

Our data indicate the ubiquitous presence of anti-FcεRIα autoantibodies, which is in contradiction to other studies demonstrating anti-FcεRIα autoantibodies exclusively in CU patients. Therefore our goal was to further analyse the distribution of such anti-FcεRIα autoantibodies in healthy individuals and CU patients. Up to now the distribution of anti-FcεRIα autoantibodies was analysed by functional and serological tests. However no direct relation was found between these two test types. Thus a new test that allows to monitor such anti-FcεRIα autoantibodies in different donors was needed. As in previous studies the detection of on single immunoglobulin sequence in a high background of other immunoglobulin sequences was demonstrated by PCR approach, we developed a semi-nested RT-PCR and a real-time PCR to detect LTMα15 sequence. Because of the presence of V sequences highly similar to the LTMα15 variable heavy (V_H) and variable light (V_L) chain sequences in the V repertoire we have written a new bioinformatic program, which predicts potential primer annealing sites in any sequence. This program allowed us to determine a potential primer annealing site, which is present exclusively in the LTMα15V_H sequence. To this annealing site we have designed a 3' end primer for the semi-nested RT-PCR or a minor groove binder (MGB) probe to perform the real-time PCR.

The semi-nested RT-PCR demonstrated the presence of LTMα15V_H germline family in healthy individuals (see Dissertation Equivalent A). However in 80% of the tested donors different CDR3V_H than the LTMα15V_H CDR3 were amplified indicating that the reverse primer was not specific enough to exclusively amplify LTMα15V_H sequence. To increase specificity we developed a real-time PCR that enhances specificity because of the use of an additional probe. Instead of using a conventional TaqMan probe we favoured a MGB probe that reduces non specific probe hybridisation to the target (see Dissertation Equivalent A and B). Applying this real-time PCR approach one single anti-FcεRIα V_H that is in germline configuration was detected in healthy individuals and CU patients. These data demonstrate that this approach may serve as a model to monitor anti-FcεRIα autoantibody sequences in the human antibody repertoire at a monoclonal level.

B. OVERVIEW

1. The Issue

In a subset of chronic urticaria (CU) patients autoantibodies against the α-chain of the high affinity IgE receptor (FcεRIα) have been claimed to be pathogenic. However the detection of anti-FcεRIα autoantibodies in healthy individuals challenges the pathophysiological role of these antibodies in CU patients. Furthermore diagnosis of anti-FcεRIα autoantibodies in chronic urticaria yields conflicting data, depending which diagnostic tool was used to characterize these autoantibodies. For these reasons we aimed to develop a new molecular approach to characterize such anti-FcεRIα autoantibodies in healthy individuals and CU patients.

2. Urticaria

2.1 Clinical Appearance

Urticaria is characterised by the appearance of wheals. In 50% of urticaria patients this wheal reaction is followed by angioedema. The wheals appear as swellings that are linked with itching or sometimes burning enduring 1-25 hours. On the other hand the swelling of angioedema is painful rather than itching and has an elongated duration of 72 hours (1).

Dermal mast cells and basophils (2) release histamine, eicosanoids, cytokines and proteases which results in the mentioned wheals and itching. Mast cells are derived from progenitors present in the bone marrow, which migrate to the peripheral tissue and undergo differentiation *in situ*. Mature mast cells are located near blood vessels and nerves and beneath epithelia and in the lymphoid organs. However, they cannot be found in circulation. Although basophils show structural and functional similarities to mast cells they derive from a different cell lineage. In contrary to mast cells, basophils mature in the bone marrow and can be detected in their differentiated form in the circulation. Mast cells and basophils express the high affinity IgE receptor (FcεRI). Hence they contribute to the immediate hypersensitivity responses also known as type I allergy. In a type I allergy an allergen cross-links the sensitised IgE bound through its constant domain (Fc) to FcεRI. This cross-linking leads to the release of histamine resulting in tissue inflammation, oedema and smooth muscle contraction (2-4). The implication of type I allergy in the induction of urticarial symptoms is still controversial. In infants an association between food allergy and acute urticaria could be observed (5) whereas such a correlation was not proven in adult patients (6) indicating that only in infants type I allergy may contribute to urticaria.

2.2 Classification

The classification of the different subtypes of urticaria is complicated. Chronic urticaria for example is characterised by the spontaneous occurrence of wheals lasting less than 25 hours over a period of more than six weeks. Physical urticaria is also of chronic nature and therefore often described as chronic urticaria. However it is associated with eliciting factors such as cold air in cold contact urticaria or visible light in solar urticaria (1). Thus physical urticaria should be classified as such if physical eliciting factors can be diagnosed. Up to now urticaria is divided in spontaneous urticaria containing acute and chronic urticaria, respectively. Additionally chronic urticaria is subdivided into chronic continuous and recurrent urticaria,

respectively depending on the frequency of the occurrence of wheals. Next to acute and chronic urticaria as already mentioned the physical urticaria exists. In addition there exist different special types of urticaria as for example cholinergic urticaria. In the next section CU is focused. The other types of urticaria will be not further discussed here as they are nicely reviewed in (1).

2.3 Chronic Urticaria

Up to now there exist no dependable data regarding the prevalence of chronic urticaria due to uncertainty of the diagnosis (see chapter 5 for diagnosis of autoimmune urticaria). Furthermore it is possible that different forms of urticaria can be diagnosed in the same patient (1).

Chronic urticaria (CU) is characterized by transient cutaneous wheals occurring daily, or almost daily, for at least 6 weeks. Like in the other forms of urticaria in CU the weal and flare reaction is due to histamine release from mast cells. However the causative agents of this histamine release are not well defined and several studies showed that type I allergic reactions are infrequent in CU patients (7, 8). Nevertheless viral and bacterial infections have been associated with CU. Most conflicting data in literature exist about the association of *Helicobacter pylori* infection and CU (9, 10). Gaig et al. observed that 70% of CU patients showed an improvement after eradication of *Helicobacter pylori.* However the results were not of statistical significance (10). The World Health Organization (WHO) rates the prevalence of *Helicobacter pylori* about 50-60% in the population. Although the prevalence of CU is not well defined, up to now such a high prevalence was never observed indicating that an infection of *Helicobacter pylori* does not strikingly induces CU. Due to ethics it is not possible to re-infect a CU patient who has recovered after elimination of *Helicobacter pylori* and to analyse this patient afterwards for a relapse of CU. Hence further studies are needed which contain appropriate randomised, double-blind and placebo-controls. In addition to viral and bacterial infections, chronic inflammation like gastritis, reflux esophagitis have been identified to contribute to CU in some patients (8). However, in about 20% to 30% of CU patients no causative agent can be identified (9) and is therefore named chronic idiopathic urticaria.

In a subset of CU patients the involvement of autoantibodies against the α-chain of the high affinity IgE receptor or the IgE itself has been described (11-13). This subtype of CU is known as autoimmune urticaria and outlined in the next paragraph.

2.3.1 Autoimmune Urticaria

In 1981, Mathews et al. described the passive transfer of urticaria by injecting CU patients' serum in normal skin. Therefore they suggested that some soluble factors are presents in CU patients' sera which can cause urticaria (14). Later, Gruber et al. identified these soluble factors as autoantibodies of the IgG and IgM isoytpe, which recognise cell bound IgE and which are functional to induce histamine release of basophils (13). It was demonstrated that such anti-IgE autoantibodies are present in 5% to 10% of CU patients (13, 15). Autoantibodies against the α-chain of FcεRI were reported to induce histamine release, too (11, 16). The presence of anti-IgE and anti-FcεRIα autoantibodies in CU patients was further confirmed by western blot analysis (17-19), ELISA (12) and β-hexosaminidase release from rat basophils leukemia cells (19). The manner by which such anti-IgE and anti-FcεRIα-chain autoantibodies, respectively can induced histamine release was thought to be comparable to the cross-linking of the FcεRI taking place in a type I allergy (11). Aditionally Fiebiger et al. demonstrated that the anti-FcεRI autoantibodies in CU patients are predominantly IgG1 and

IgG3 (12). These isotypes are able to activate the classical pathway of complement (20). The possible involvement of the complement in CU was then reported by several studies (21, 22). Kikuchi et al. have analysed the capacity to induce basophil histamine release of purified IgG and whole sera of CU patients, respectively. They observed that the reactivity of purified IgG is less compared to whole sera. Hence they supposed that the reactivity of IgG is augmented by complement (21). This is in line with the study of Fagiolo et al. demonstrating that basophil histamine release capacity is decreased when the complement of CU patients' sera has been inactivated by heating (22).

3. The High Affinity IgE Receptor

The high affinity IgE receptor seems to be an important autoantigen implicated in some CU patients. For this reason this receptor is described in more detail in this chapter.

3.1 Expression of FcεRI Receptor

FcεRI is expressed on mast cells, basophils, neutrophils, eosinophils, monocytes, macrophages, Langerhans cells as well as platelets and megakaryocytes (3, 23-28). As already mentioned mast cells and basophils are key effector cells of type I allergy trough interaction of IgE and FcεRI receptor. The expression of FcεRI receptor on the other cell types may have other functions than the induction of type I allergy. These cells and their expression of FcεRI are shortly described in the next paragraphs.

Neutrophils are the most abundant circulating white blood cell. They are also named polymorphonuclear leukocytes and are generated from a myeloid progenitor in the bone marrow. In response to infections and allergens an inflammation process may occur which results in the early recruitment of neutrophils (29-31). The fact that neutrophils also express the FcεRI receptor was for a long time unclear. However Gounni et al. could recently demonstrate that neutrophils from asthmatic patients express functional FcεRI receptor. Therefore they concluded that the expression of FcεRI on the surface of neutrophils might be a potential mechanism by which these cells contribute to the manifestation of allergic disorder (27).

Like neutrophils, monocytes (32) develop in the bone marrow. They populate all tissue throughout the body and additionally they can be detected in the blood. During maturation in the tissue these cells become macrophages. They infiltrate sites of inflammation and infections shortly after the recruitment of neutrophils and reside these sites for a long time. As macrophages express the FcγRIII receptor, they may contribute to the antibody dependent cell mediated cytotoxicity (ADCC). The mechanism of ADCC is as follows: IgG recognises some epitopes on the surface of a microbe and acts therefore as opsonin. The interaction of this IgG with FcγRIII results then in lysis of the microbe. The macrophages are also able to phagocytose microbes and through the production of nitric oxide these microbes are destroyed. Additionally they produce IL-12 and interferon INF-γ which results in the activation of the adaptive immune systems (33). Monocytes express on their surface 60 times less FcεRI compared to basophils (34, 35) and the receptor on monocytes is even of an other isoform than the receptor found on basophils indicating that they might contribute to other effector mechanism as basophils (see chapter 3.2.4).

Like neutrophils and macrophages, eosinophils are also involved in inflammatory diseases. They are generated from granulocytes in the bone marrow. They circulate in the blood but are also present in peripheral tissue, especially in mucosal linings of the respiratory,

gastrointestinal and genitourinary tracts. Eosinophils are involved in immunity against helmintic parasites whereby the FcεRI receptor plays an important factor. Defense against helminths through phagocytosis is not possible as they are too large to be pagocytosed and in addition the microbiocidal products of neuthrophils and macrophages are not able to destroy the helminthic integuments. Thus helminthic infections can only be cleared through a special type of ADCC: Eosinophils interact through their FcεRI with specific IgE, which has previously bound to the surface of helminths. This ligand:receptor interaction induces intracellular signals resulting in the release of eosinophilc granule contents and destroying of the helminths (36).

As shown by the work of Sihra et al. the low expression of FcεRI receptor on the surface on monocytes and eosinophils is significantly increased in atopic patients compared to non-atopic control subjects. The expression level correlated with serum IgE concentration indicating a role of these cells in IgE mediated type I allergy (24). However an other study, which was published three years later could not confirm the correlation of IgE concentration and FcεRI expression on monocytes (37). Nevertheless that monocytes are involved in delayed type hypersensitivity is well accepted. Delayed type hypersensitivity, also known as type IV allergy is T cell mediated. Monocytes are thought to bind an antigen specific IgE with the FcεRI receptor resulting in antigen uptake, processing and presentation on a MHC molecule to T cells. These T cells are activated and produce cytokines that stimulate inflammation which leads to tissue injury (38).

Langerhans cells are immature dendritic cells and are located in the epidermis of the skin. They capture antigens entering the epidermis and transport them to the draining lymph nodes. During their migration to the lymph node, Langerhans cells mature into dendritic cells. Dendritic cells, also known as professional antigen presenting cells (APC) can efficiently present peptide antigens bound to their MHC to naive T cells. Through this interaction dendritic cells can decide whether an immune reaction or tolerance is induced (39). Like monocytes, dendritic cells contribute to the IgE-dependent antigen presentation process. As dendritic cells are professional APC, the IgE-dependent antigen presentation process is even more effective in dendritic cells than in monocytes (40). In mice, the APC do not express FcεRI on their surface (41), thus the IgE-dependent antigen-presentation is found only in humans.

Human platelets are derived from megakaryocytes in the bone marrow, circulate in the peripheral blood and play an important role in the coagulation of blood. Joseph et al. reported that on human platelets and megakaryocytes of patients infected with parasites the FεRI is expressed. Furthermore they demonstrated that *Schistosoma mansoni* larvae can be destroyed through the action of FcεRI and therefore they concluded that these cells are involved in immunity against parasites (26). Additionally it was demonstrated that the release of RANTES (**R**egulated on **A**ctivation; **N**ormal **T** expressed, and presumably **S**ecreted) which is a potent attractant for eosinophils and neutrophils (43) can be induced in human platelets by stimulation of the FcεRI (42). A strong indication that platelets are involved in type I allergy was given just recently by Hasegawa et al (44). They induced RANTES release of platelets isolated from allergic patients and healthy individual by stimulating them with anti-human IgE. Thereby they discovered that platelets of healthy individuals show less capacity to release RANTES. Additionally they observed that the expression of FcεRI receptor was in both groups equal. For these reasons they concluded that the activation of platelets through ligand: receptor interaction was stronger in allergic patients compared to healthy donors (44).

3.2 Structure of FcεRI Receptor

The tetrameric isoform of FcεRI receptor consists of an α-chain, a β-chain and two γ-chains. The γ-chains are linked through disulfide bridges. The IgE binding site is located in the extracellular domain of the α-chain whereas signal transduction is performed by the β-and the γ-chains, respectively. These sub-regions of the FcεRI receptor are discussed in this section.

3.2.1 The α-Chain of FcεRI

The FcεRIα, belonging to the immunoglobulin superfamily, comprises two extracellular immunoglobulin-related domains (α1 and α2), one transmembrane domain and a short cytoplasmic tail (45). It was demonstrated that the transmembrane and the cytoplasmic tail of the α-chain are not needed for ligand:receptor interaction. Hence the leader peptide and the extracellular part of this chain, even after deglycosylation, are functional to bind IgE (46). However, it was also shown that for a proper folding in the endoplasmatic reticulum of this α-chain consisting only of the leader peptide and the extracellular part, glycosylation is necessary as shown by Letourneur et al. (47) who mutated the seven N-glycosylation sites present in the extracellular part of the α-chain. Additionally they observed no difference in folding efficiency between wild-type α-chain and the mutated α-chain expressed in *E. coli,* which do not contain an endoplasmatic reticulum. Hence they concluded that the α-chain has to be glycosylated if folded in the endoplasmatic reticulum but not if folded in *E.coli*. Thus the glycosylation of the α-chain is not an intrinsic factor (47). The study of Albrecht et al. confirmed and extended these findings. They also observed the need of glycosylation of the α-chain to result in a proper folding in the endoplasmatic reticulum and they could even show that an improper glycosylated α-chain is retained in the endoplasmatic reticulum (48).

As the α-chain of FcεRI binds IgE, some groups were looking for polymorphism in the gene coding for the α-chain and if there are any correlation with atopic diseases. Up to now no single nucleotide polymorphism (SNP) was observed in the coding region of the α-chain gene. Shikanai et al. published three SNPs in the promotor region of the gene for the α-chain. They performed comparative analysis of the allelic distribution of these three SNPs in asthmatic patients and healthy individuals. However both groups were similar in allele frequency and genotypes (49). One year later an additional SNP (T/C) in the promotor region at position –66 was published. The authors clearly demonstrated that the T allele is more transcribed than the C allele and that the –66T/C genotype can be detected more often in healthy individuals. In contrast the –66T/T genotype is significantly higher present in allergic patients. Therefore they concluded that this polymorphism may be implicated in allergy (50). If such a polymorphism is also present in CU patients has not yet been studied.

3.2.2 The β-Chain of FcεRI

The FcεRIβ has four transmembrane domains and both the C- and the N-termini are located within the cells (51, 52). As the β-chain contains immunoreceptor tyrosin-based activation motifs (ITAM) in its cytoplasmic tail it contributes to signal transduction after receptor cross-linking (53). However it was published that the β-chain is not autonomous in signal transduction. It rather amplifies the signals yielding from the γ-chain, which contains ITAMs, too (54). Next to this intrinsic signal amplification function, Donnadieu et al. showed that the β-chain also contributes to the enhanced surface expression of FcεRI. They proposed that the β-chain is early connected with the α-chain resulting in enhanced maturation of the receptor complex (55).

As the gene for FcεRIβ is mapped to chromosome 11q13 and this segment was suggested to be linked with allergy, it was proposed that the β-chain may contribute to allergic diseases (56). For this reason many studies handle with polymorphisms connected with the gene for FcεRIβ. However one SNP discovered in 1996 in exon seven (+6960A/C) (57) was discussed most as some studies claimed positive association between this polymorphism and atopic disease (57-60). Others could not show such an effect (61, 62). In addition, by in vitro analysis it has been demonstrated that the +6960A/C mutation has no influence on the expression or function of FcεRI (63, 64). The publication of Nishiyama et al. has just recently clarified these conflicting data (65). They detected four new SNPs in the promotor region of the β-chain gene and demonstrated that two of them are tightly linked with +6960A/C mutation resulting in higher transcription activity of the β-chain. They could further confirm their data by showing that the expression of the β-chain in basophils from atopic individuals, having these two SNPs in the promotor region, is significantly increased suggesting that the +6960A/C mutation together with the two SNPs in the promotor region contribute to development of atopic diseases (65).

3.2.3 The γ-Chain of FcεRI

The γ-chain is assembled with the α- chain and β-chain as a disulfide-linked homodimer. It has a short extracellular domain, one transmembrane domain and a long intracellular domain, which contains the ITAMs as first reported by Reth et al. (66) and, which contributes to signal transduction by phosphorylation. The γ-chain facilitates the cell surface expression of the FcεRIα (46). Interestingly the FcγRIII, TCR, FcγRI and FcαR are also associated with the γ-chain (67-73). It has been shown that the duration of the receptor cross-linking influences the intracellular signal pathway contributed by the γ-chain (74, 75). High-affinity cross-linking showing a low off-rate may induce downstream phosphorylation, whereas low-affinity antigens with a high off-rate lack this feature.

3.2.4 Different Forms of the Receptor

There are two isoforms of the human FcεRI (41). On mast cells, basophils, neutrophils and human platelets the receptor consists of four subunits, the α-chain, the β-chain and two covalently linked γ-chains ($\alpha\beta\gamma_2$) (3, 27, 42). On macrophages, monocytes, eosinophils Langerhans cells and dendritic cells, the β-chain is lacking ($\alpha\gamma_2$) (37, 40). The β-chain has two amplification functions, intrinsic signal amplification and amplification of the expression of the α-chain on the surface (54, 55). The intrinsic amplification signal of the β-chain might be important in type I allergy. The cell types not expressing the β-chain are therefore thought to contribute to IgE-dependent antigen presentation but not to type I allergy. However the final prove has not yet been delivered. Furthermore cells expressing the $\alpha\gamma_2$ isoform show less receptor molecules on their surface (34, 35), possibly because the second amplification function of the β-chain is missing.

3.3 The IgE-FcεRIα interaction

The ligand of FcεRI is exclusively IgE. The binding of human IgE is limited to human FcεRI. In contrast rodent IgE binds to both rodent and human FcεRI (reviewed (76)). Recently the structures of FcεRI (77, 78), IgE-Fc (79) and the IgE:FcεRI complex (80) were solved by crystallisation.

The receptor contains two distinct binding sites for the antibody named D1 and D2 that refer to the two immunoglobulin-related domains of the α-chain. The concentration of IgE in

sera is about 0.05mg/ml. IgE is therefore the last abundant immunoglobulin class. In contrast to the heavy chain of IgG, which contains 3 constant domains in its Fc part, IgE bears 4 constant domains (Cε1-Cε4). In 1988 Helm et al. claimed that IgE binds through the Cε2-Cε3 domains to its receptor (81). However, almost ten years later it was suggested the Cε3-Cε4 domain contributes to antibody:receptor interaction (82). This is in contradiction with the study of Vangelista et al. who demonstrated that the Cε3 domain is sufficient for receptor binding (83). This was confirmed just recently by the publication of Garman et al. who could even demonstrate that the Cε4 domains does not contribute in receptor interaction (80). The same study also showed that the N-linked oligosaccharides present in the IgE-Fc and FcεRIα do not participate in the interactions between the two molecules (80). Figure 1 which was adapted from (80) shows the two distinct binding sites through which the Cε3 domains interact with the receptor. The binding site 1 for one Cε3 of the heavy chain dimer is located in the D2 domain of the receptor, namely the C-C' region of this domain. The D1-D2 interface of the FεRIα forms the binding site 2 for the second Cε3 (80). The observed binding stochiometry of IgE to FcεRI is 1:1 (84, 85). It was thought that binding of a second IgE to the receptor is sterically inhibited (80).

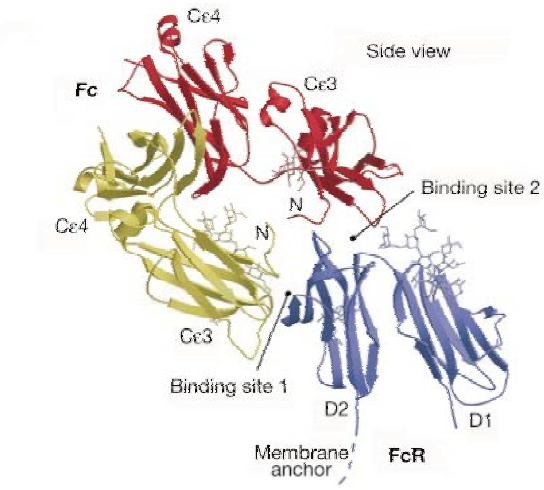

Fig. 1 The IgE/Fc-FcεRIα complex is shown in a side-view. In yellow and red are the two Fc chains of IgE, the blue chain represents the FcεRIα. Sticks represent the N-linked oligosaccharides and are coloured like the protein chains. Adapted from Garman et al. (80).

In contrast to the receptor which shows little conformational changes upon IgE binding (80), the Cε2 domains of IgE bend backward during ligand:receptor interaction as observed by Wen et al. (86). Due to these conformational changes the Cε2 of the first heavy chains comes into tight contact with the Cε3 of the second heavy chain and even touches the Cε4, whereas the Cε2 of the second heavy chain makes very few and no contacts with the Cε3 and Cε4, respectively of the first heavy chain (86). The most homologe to FcεRI is the FcγRIII known as a receptor for IgG. However, the affinity of IgE to FcεRI is $1x10^{-9}$M whereas IgG shows a lower affinity ($5x10^{-5}$M) to FcγRIII (87). Although Cε2 does not contribute to the receptor binding, it was observed that IgE Fc fragments, where the Cε2 are abolished, have an increased dissociation rate from the receptor (88). Novak et al. have now proposed that the mentioned conformational changes contributed by Cε2 led to the high affinity of IgE to its receptor (89).

4. Anti-FcεRIα Autoantibodies

According to the data mentioned above one may assume that anti-IgE autoantibodies and anti-FcεRIα autoantibodies can be used to characterize autoimmune urticaria. However, we have previously reported the presence of anti-FcεRIα autoantibodies in the serum of healthy donors (90) as well as in multidonor intravenous IgG (IVIg) preparations (91), which is a pool of IgG of over 20'000 healthy donors. This indicates the presence of anti-FcεRIα autoantibodies in healthy individuals. Therefore we aimed to compare anti-FcεRIα autoantibodies present in CU patients and healthy individuals. To characterize these autoantibodies at a monoclonal level, we constructed Fab phage display libraries.

4.1 Fab Phage Display Library

A possibility to generate human monoclonal antibodies would be the hybridoma technology. However, often a suitable fusion partner for human B cells is missing. Due to additional practical problems linked with the hybridoma technology, scientist have developed other technologies to generate human monoclonal antibodies (92). The phage display technology is a possibility to generate human monoclonal antibodies. This technology is mostly favoured as it allows the screening of large libraries whereby a specific ligand can be enriched and selected. This process may result in the isolation of high affinity human antibodies. Using this technology it is also possible to isolated antibodies which are only present at a low serum titer as for example natural autoantibodies (reviewed in (93)).

In phage display technology a Fab (fragment antigen binding) or a scFv (single chain variable fragment) instead of full length antibodies are used. A scFv bears the variable domains of heavy (V_H) and light chains (V_L), which are fused to each other by an amino acid linker. A Fab consists of one V_H joined with the first constant domain C_{H1} of a heavy chain and one complete light chain. A full length immunoglobulin however is composed of two identical heavy and light chains, respectively. This indicates that either scFv or Fab is smaller than a full length antibody and therefore easier to synthesize in *E. coli*. However as all of them the scFv, the Fab and the full length antibody contain paired V_H and V_L, which are primarily responsible for antigen binding, the scFv and the Fab retain the ability to bind antigen.

There are three subregions in the variable domain of V_H and V_L, which are the main antigen contact spots, termed complementarity determining regions (CDR1, CDR2 and CDR3) and they are separated by four framework regions (see figure 2). The CDR3s of both the V_H and V_L are the most variable of the CDRs and form the most extensive contact with bound antigen. However the CDR3 of V_H and V_L do not contribute equally to antigen recognition. Xu et al. have demonstrated that the CDR3 of V_H is the key determinant of specificity in antigen recognition and is the limiting factor to receive IgM antibodies which are specific to protein antigens (94).

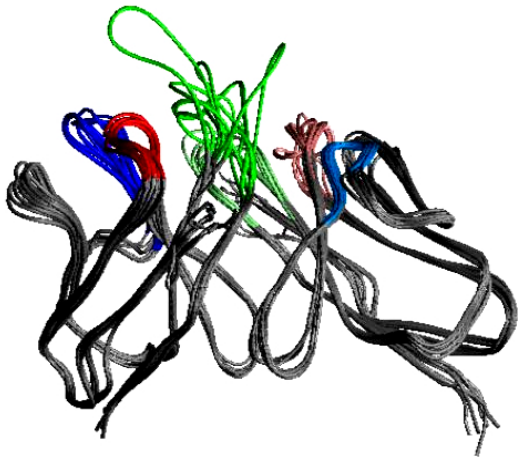

Fig. 2. Overlay of different variable regions of crystallized human antibodies. Left, heavy chains; right, light chains. Gray, framework regions; red, CDR1; blue, CDR2 and green CDR3. In the centre of the binding site are the CDR3 loops, which show the highest variability within the displayed antibodies, whereas the other regions fit between the antibodies. Figure provided by Dr. M.B. Stadler.

To construct Fab phage display library RNA is extracted from B cells, and the mRNA is transcribed into cDNA and PCR amplified using specific primers for each V_H and V_L family, respectively. Fab of different isotypes can be produced depending on the heavy chain constant region primer that is used. Therefore libraries of different immunoglobulin isotypes can be constructed as for example IgG, IgM and IgE. The heavy and light chains are subsequently cloned into a phagemid expression vector (95), resulting in libraries containing 10^6-10^9 clones. Co-transfection of these phagemids with M13 helper phage into *E. coli* results in phagemid particles that contain Fab DNA and carry Fab protein on the cell surface by fusion to a phage coat protein. In a process called panning, such Fab/phagemid display libraries are incubated with antigen to bind specific Fab/phagemid. Unbound Fab/phagemid are washed away and the bound Fab/phagemid are eluted and allowed to re-infect *E. coli* cells for further propagation. After two to four rounds of panning, enriched phagemids specific for a particular antigen can be obtained.

As mentioned above to construct a Fab phage display library, mRNA of rearranged IgM and IgG is used. The IgM isotype is generated during B cell ontogeny by rearrangement of immunoglobulin gene loci. The IgG isotype is made during a process called isotype switch. However to construct a scFv phage display library, DNA can also be used as the V_H and V_L consisting of V segments, D segments (in V_H only), J segments are already rearranged at the DNA level. The V(D)J rearrangement and isotype switch will be explained in the next section.

4.1.1 V(D)J Rearrangement and Isotype Switch

Three separate loci encode the κ light chain, the λ light chain and all the heavy chains. In humans the λ light chain locus is on chromosome 22, the κ light chain locus on chromosome 2 and the heavy chains locus on chromosome 14. Each locus is made up of several copies of V segments, D segments (in V_H only), J segments and the C segments. (The locus of V_H, Vκ and Vλ are discussed in more details in chapter 6). The V(D)J gene rearrangement is mainly regulated by two enzymes (RAG1, RAG2) which recognise two very conserved heptameric and a nonameric segments (96).

From stem cells in the bone marrow the pro B cell is generated. In pro B cell the D segment is genetically joined to a J_H segment by homologous recombination. The joining of the D and J_H segments is not very precise and additional bases are inserted by the terminal deoxynucleotidyl transferase (TdT), which increases the diversity of the B cell repertoire (97). If the recombination of D and J_H segments was successful the pro B cell differentiates into the pre-B cell. Subsequently, one of the V_H genes is recombined with the DJ_H. If the recombined V_HDJ_H encodes for a productive gene product further heavy chain gene rearrangements are blocked. If this first V_HDJ_H rearrangement is not productive, the second allele of the V_H locus will be rearranged. This mechanism is called allelic exclusion. The resulting V_HDJ_H is known as rearranged DNA. The rearranged DNA is afterwards transcribed into a primary RNA transcript. Afterwards the sequences located between the V_HDJ_H complex and the IgM isotype (Cμ) gene are spliced out resulting in an mRNA coding for the whole the μ heavy chain. This μ heavy chain is then expressed on the surface of a pre B cell together with a surrogate light chain which is generated from the two genes V*pre*B and λ5 (98). The rearrangement of the light chain happens in the same manner as in the immunoglobulin heavy chain locus except that the V_L segment is directly joined to a J_L segment due to the absence of D segments in the light chain loci. The κ locus rearranges first and a production of κ light chains inhibits rearrangement of the λ locus (V_L isotypic exclusion). In the primary RNA transcript an intron separates the V_LJ_L from the C_L. This intron is subsequently spliced out and a rearranged mRNA is generated which codes for a fully functional κ or λ light chain. In the endoplasmatic

reticulum the light chain and the previously synthesised heavy chain of the IgM isotype are assembled and expressed on the surface of immature B cells. The transition from immature to mature B cells is associated with the expression of both IgM and IgD on the cell surface.

Allelic exclusion and V_L isotypic exclusion are involved in the B cell tolerance induction and make sure that one B cell expresses only on specificity. These mechanisms were discovered by analysing the expressed V_H or V_L of a mature B cell which carries allotypically distinguishable V_H or V_L alleles (99). Skok et al. have tried to explain the mechanism underlying allelic exclusion by the differential nuclear localization of rearranged loci. They demonstrated that unrearranged V_H are associated with centromeric heterochromatin whereas rearranged V_H are not. Therefore they supposed that due to the recruitment of unrearranged V_H to centromeric heterochromatin, these V_H are protected against the recombinases and therefore the rearrangement of the second allele is prevented (100). Furthermore it was also demonstrated that allelic exclusion requires coexpression of a $V_HDJ_HC\mu$ and the surrogate L chain component $\lambda5$ in early pro B cells (101). However the precise mechanisms responsible for allelic exclusion remain elusive.

Whereas the IgM isoytpe is generated during B cell ontogeny, the IgG isotype is made upon activation of mature but still naive B cell, which results in isotype switch. During isotype switch the rearranged V_HDJ_H gene segment are joined to a C_H gene of a different isotype than IgM (γ, ε, α) and the intervening DNA is deleted. In principle it is the same mechanism, which combines the V_HDJ_H complex of a pre B cell with the $C\mu$ gene. Most importantly a B cell undergoing an isotype switch changes its effector function but not its specificity, as the V_HDJ_H complex remains the same.

4.2 Anti-FcεRIα Autoantibodies Isolated by Phage Display

Using the rearranged mRNA isolated from CU patients' B cells we have constructed an IgM and an IgG Fab library. Additionally we made an IgM Fab library using rearranged mRNA of B cells isolated from healthy children's tonsil. From these three libraries we isolated different anti-FcεRIα autoantibodies. From the IgM urticaria library we isolated one main clone, namely the UMα16. Two main clones, LTMα15 and LTMα35 were isolated from the IgM tonsil library, and one clone (UGα8) originated from the IgG urticaria library (90, 91, 102).

To perform functional tests, we have produced them as full length antibodies (91, 102). The affinity of the clones (LTMα15, UMα16; LTMα35) for the FcεRIα chain was evaluated by on line monitoring using the IAsys cuvette system (102). The affinities were $7.2x10^{-9}$M for both LTMα15 and UMα16 and $1.4x10^{-8}$M for LTMα35. Furthermore we assessed the anaphylactogenic potential of the LTMα15, LTMα35 and UMα16 in a histamine release assay using freshly isolated peripheral blood lymphocytes enriched for basophils. Interestingly under physiological conditions, meaning the FcεRIα chain is occupied by IgE (103), these anti-FcεRIα autoantibodies did not trigger the cells for histamine release. However upon stripping the cells with lactic acid in order to remove cell bound IgE from the FcεRIα chain, there was a clear induction of histamine release by these anti-FcεRIα autoantibodies (91, 104). This phenomenon we called conditional autoimmunity, which will be explained in the next section.

4.3 Autoimmunity versus Conditional Autoimmunity

An immune reaction which is accompanied by tissue injury and therefore resulting in a disease was for a long time mistakenly considered as autoimmunity although the self-antigen could not be characterised. The development of different animal models for autoimmunity as for example the experimental autoimmune encephalomyelitis in mice (105), delivered great opportunities to characterise the self-antigens. Using such models it was even possible to determine different genes, which may be involved in the development of autoimmunity. However the etiology of most human autoimmune diseases is confusing. Nevertheless it is generally accepted that such diseases develop due to a failure of induction or maintenance of self-tolerance. T cell tolerance is induced in the thymus. T cells are selected for capacity to recognize non-self peptides on self MHC molecules (reviewed in (106)). Here, we focus on the induction of B cell tolerance.

4.3.1 B cell Tolerance

B cell tolerance induction can be divided into central tolerance and peripheral tolerance. The central tolerance induction takes place in the bone marrow during B cell maturation. As mentioned immature B cells express on their surface a rearranged heavy chain of the IgM isotype in association with a rearranged Vκ or Vλ. It is clear that all the antigens, which an immature B cell encounters in the bone marrow, would be self-antigens and thus the B cell would be self-reactive. And indeed, it was mathematically proven that the majority of newly generated B cells are self-reactive (107). Furthermore Wardemann et al. could confirm these findings experimentally and demonstrated that 75.9% of antibodies isolated from immature B cells recognise self-antigens (108). Nevertheless in a healthy individual there exist three different mechanisms to eliminate self-reactive immature B cells, namely: receptor editing, apoptosis and anergy.

Self-reactive mature B cells, which encounter a self-antigen in the periphery in the absence of specific helper T cells may become anergic. This B cells are inhibited to migrate into lymphoid follicles and are therefore not activated by follicular dendritic cells resulting in inhibition of proliferation. However next to the T cell dependent antibody response exists the T cell independent antibody response. This is mainly the antibody response against polysaccharides and lipids. These antigens are termed thymus independent or T independent antigens. This raises the question why B cells recognising T cell independent antigens may become active and do not stay anergic. Nemazee et al. have recently proposed that self-antigens can be distinguished from foreign antigen by “self markers” like for example carbohydrate moieties. Therefore they suggested that B cells recognising T cell independent antigens identify these “self marker” and do not react. Whereas in a foreign antigen these “self marker” are missing resulting in activation of such B cells (109). This would be the same mechanism by which natural killer cells distinguish between self and non-self. Natural killer cells stay anergic if they recognise self-major histocombatibility complex (MHC) but are activated by antibody coated cells (110). Hence the MHC would represent a “self marker” whereas antibody coated cells do not.

4.3.2 Anti-FcεRIα Autoantibodies May Escape Tolerance Induction

The induction of tolerance in auto-reactive B cells implies that the auto-reactive B cell encountered the autoantigen during ontogeny or in the periphery. However the autoantigen of anti-FcεRIα autoantibodies is under physiological conditions occupied by IgE and therefore hidden (103). For this reason induction of B cell tolerance is not necessary. On the other hand anti-FcεRIα autoantibodies may be natural autoantibodies. Such antibodies may belong to a conserved set of self-reacting antibodies, which are present in all healthy donors and which

escape tolerance induction (see chapter 4.4.1). Such antibodies may only be pathogenic under certain conditions.

Based on our results showing that anti-FcεRIα autoantibodies can trigger histamine release only if the receptor is not occupied by IgE we proposed a hypothesis for the pathogenic role of anti-FcεRIα autoantibodies in urticaria. The FcεRI is occupied by IgE under physiological conditions (103). Hence the antigen for anti-FcεRIα autoantibodies is hidden. Furthermore the expression of FcεRI is mainly regulated by the IgE level (111). However several studies have also demonstrated that the expression of FcεRI is not exclusively regulated by IgE (112, 113). Therefore we claim that under conditions where the expression of FcεRI is increased and at the same time IgE levels are unaltered, the receptor may become available for anti-FcεRIα autoantibodies. These conditional anti-FcεRIα autoantibodies may then induce the symptoms of urticaria. Furthermore we suggest that if the amount of cells positive for FcεRI expression is increased locally, either through local proliferation or through an extravasation of chemotactically attracted cells, also represents a condition where free FcεRI is available for anti-FcεRIα autoantibodies. Therefore the equilibrium of occupied and free FcεRI may be the critical factor if conditional anti-FcεRIα autoantibodies are able to induce urticaria (91, 114, 115).

4.4. Anti-FcεRIα Autoantibodies are Natural Autoantibodies

Comparison with the known germline sequence of human variable heavy (V_H) and variable light (V_L) chain segments (116-118) indicated that LTMα15, UMα16 and UGα8 V_H chains showed 100% homology to the germline V_H3-23 sequence, whereas the λ light chains were slightly mutated and most similar to the V_λ germline λ6a. Sequence analysis of the heavy chain of LTMα35 revealed 100% homology to the germline V_H5-51. The slightly mutated light chain was most similar with the V_λ germline λ1. Furthermore, sequence analysis showed that LTMα15 and UMα16 are 100% identical. Due to the germline configuration of the heavy chains and the fact that the light chains are only slightly mutated, we suggest that LTMα15, UMα16 and UGα8 represent natural autoantibodies. In the next paragraph some general aspects of natural antibodies and autoantibodies are discussed.

Natural antibodies (NA) are defined as antibodies, which are generated independently of immunization or triggering with antigens. (119). De novo generation of naive mature B cells is the source of NA. To analyse the amount of B cells needed for maintenance of normal peripheral B cell pool Agenes et al. have used lethally irradiated RAG-deficient mice, which were reconstituted with different ratios of normal bone marrow (BM) cells and incompetent BM cells from B cell-deficient donors. In mice reconstituted with 30% of normal and 70% of incompetent BM cells, respectively a normal-sized peripheral B cell pool could be generated. Hence they concluded that one third of the normal BM cells fulfils the peripheral B cell pool size (120).

Natural autoantibodies (NAA) are also antibodies generated without triggering. In contrast to NA, which recognise foreign antigens, NAA may react with self-antigens. NAA can be detected in patients suffering from autoimmune disease but most interestingly also in healthy individuals. They belong to the IgM, IgG and IgA isotype (121-123). This is partly confirmed by our studies demonstrating the isolation of anti-FcεRIα autoantibodies of both the IgM and the IgG isotype (90, 91, 102). NAA were first detected in neonatal mice where IgM predominates. Thus it was initially debated that the IgM isotype represents most NAA. However up to now it is clear that the composition of the isotypes that are used in NAA can vary for different antigens. Cabiedes et al. for example have recently demonstrated that NAA

of the IgM isotype against phosphatidylcholine exist in sera of healthy individuals, whereas IgG could not be detected (122). In contrary Li et al. have showed that purified Ig fractions contain more natural anti-keratin autoantibodies of the IgG isotype than IgM (123). These examples may indicate that a B cell, producing a NAA against a T cell independent antigen (here phosphatidylcholine) does not make an isotype switch. In contrast a B cell producing NAA against an antigen, which has previously induced a specific auto-reactive T cells response (here keratin) may undergo isotype switch. However this would implicate the occurrence of self-reactive T cells in healthy individuals. And indeed a number of reports have described auto-reactive T cells in healthy individuals (124, 125). Thus self-reactive T cells would be available in normal individuals and might be able to partly control NAA repertoire and isotype. The fact that T cells are able to influence the antibody repertoire is in line with the publications of Freitas et al. who transferred mature T lymphocytes into athymic mice. Analysis of the V_H gene family usage before and after T cell transfer revealed significant difference in antibody repertoire (126). Furthermore, by measuring the serum level of IgM and IgG in athymic and normal mice, Malanchère et al. could observe that in athymic mice the IgG concentration was 40 fold lower compared to normal mice whereas the IgM concentration was not different in these two groups (127). However it is generally accepted that NAA are in germline configuration and do not show somatic mutations normally seen in antibodies which were generated upon B cell activation through T cells (128). The reason for this fact is up to now not solved. However Cuotinho et al. have proposed that this may be the key difference of normal and physiological autoimmunity (119). This hypothesis is supported by the works of Shlomchik et al. who have shown that autoantibodies undergo somatic mutations upon autoantigen triggering (129, 130). Furthermore Lacroix-Desmazes proposed that the well known cross-reactivity of NAA may be due to the germline configuration of NAA and the missing somatic mutations (131).

4.4.1 Functions of Natural Autoantibodies

The induction of tolerance should delete B cells producing self-reactive antibodies. Therefore the presence of natural autoantibodies (NAA) in healthy individuals has been for a long time neglected. However improvements of assays to detect NAA in the 1980s-1990s clearly demonstrated the presence of NAA in healthy individuals interacting with different self-antigens (reviewed in (131)). The work of Mouthon et al. (132), showing that the reactivity patterns of NAA is masked in whole sera of healthy individuals, might deliver one explanation why such NAA are not pathogenic. This group compared the reactivity pattern of whole serum and purified IgG. This comparison demonstrated that the reactivity pattern of purified IgG is homogenous among different healthy individuals whereas the reactivity of IgG in whole serum showed heterogeneity and limitation to a few self-antigens in the tested donors (132). Nevertheless several functions have been proposed for NAA under physiological conditions. These functions are outlined in the next paragraph.

NAA, which are mostly in germline configuration, exhibit polyspecificity or cross-reactivity between self and foreign epitopes and are therefore able to recognise pathogens as well as self-antigens. The ability of NAA to recognise pathogens may indicate that these antibodies may be an early support of the innate immune response till the acquired immune response develops to clear the pathogens (119). However contradictory results exist trying to explain if NAA found before antigen triggering and the induced specific antibodies upon antigen encounter are generated from the same B cell pool. Comparative analysis of hybridoma clones, generated from B cells before and after immunization, demonstrated that these two types of antibodies are produced by the same B cell clones (reviewed in (133)). However an other study could not confirm the connection of these two antibody types (134), which was confirmed later by a theoretical analysis (135). The studies of Baumgarth et al.

have now clarified the relationship between NAA and the induced antibodies upon infection. These studies demonstrated that B-1 B cells, which are considered to be an important source of natural autoantibodies, contribute to the early support of the innate immune response, whereas the induced specific antibodies are produced by the B-2 B cells (136, 137).

NAA may also contribute to the elimination of metabolic waste from the organism. For example there exist NAA recognising band 3 on red blood cells. These NAA are thought to opsonise senescent red blood cells by binding to band 3 resulting in activation of the alternative complement pathway. The complement afterwards clears these aged red blood cells (138). A role of NAA in immune surveillance against cancer has also been hypothesised (139).

Furthermore NAA have been proposed to be involved in idiotypic-anti-idiotypic reactions, first described by Jerne in 1974 and known as the network hypothesis (140). The idea is based on the fact that the variable region of an antibody (idiotype) may be recognised by other antibodies with complementary, or anti-idiotypic, specificities. It is thought that according to this network hypothesis the homeostasis of the immune system is regulated. Thus when a foreign antigen enters the system, B cells are activated and produce secretory Igs with particular idiotypes. This reaction is followed by production of anti-idiotypes, which complex the idiotypes. These complexes may then bind to CD32 (FcγRIIb) that is known to promote negative feedback signals and thereby anergising the activated B cells.

5. Assays to Detect Anti-IgE and Anti-FcεRIα Autoantibodies

As mentioned in chapter one autoantibodies against the FcεRIα or against IgE were detected in CU patients. Several groups tried to characterise these autoantibodies by different assays. However the data obtained up to now are conflicting. The main problem is the missing standardisation of the assays and that even in the same type of an assay different values for positivity were set by the authors (see table 2).

As shown in table 2 the percentage of CU patients positive in the histamine release assay vary from study to study. These confusing results may be due to the freshly isolated basophils from healthy donors that were used to perform the test. It is documented that basophils from different donors may differ in histamine release capacity. The same situation has also been observed in different blood samples taken from the same donor at different time points (11, 16). For these reasons it is not possible to standardise the histamine release assay for diagnostic purpose and it may only be applicable to answer scientific questions. Histamine release capacity of anti-FcεRIα autoantibodies can also be measured using FcεRI-transfectomas. Although the standardisation of such a test would be easier it may not be appropriate for routine use due to time which is used to cultivate such cell lines. A third possibility to analyse histamine realise capacity is the autologous serum skin test (ASST). However the injection of human serum that is not pre-tested for infectious agents implies obvious difficulties and in addition it is not allowed in certain countries for ethical reasons. Nevertheless it is also possible that some CU patients reveal a negative ASST but a positive result in a histamine release assay (see table 2 and (16)). Therefore it is difficult to determine which result is true negative and true positive, respectively.

According to the literature and our results it is clear that there must exist different types of anti-FcεRIα autoantibodies: i) autoantibodies which recognise the α-chain although the IgE occupies the receptor, ii) autoantibodies which compete with the IgE for binding to FcεRIα (see conditional autoimmunity). These facts may partly explain the results of Fiebiger et al. (see table 2 and (12)). They observed that a successful detection of anti-FcεRIα autoantibodies in an ELISA does not correlate with a positive result in the histamine release

assay, possibly because in the ELISA both types of anti-FcεRIα autoantibodies were detected whereas in the histamine release assay only type i) of anti-FcεRIα autoantibodies were measured. However it is also possible that not all anti-FcεRIα autoantibodies detected in the ELISA are able to cross-link the receptor and therefore are also not able to induce histamine release.

The detection of anti-FcεRIα autoantibodies in healthy individuals (90, 91) made the whole situation even more complicated. Hence it is not clear if such anti-FcεRIα autoantibodies can be used to characterise autoimmune urticaria. Furthermore we were able to isolated the same anti-FcεRIα autoantibody from healthy individuals (LTMα15) and CU patients (UMα16) which was even able to induce histamine release (90, 91, 102). A further analysis of the distribution of such anti-FcεRIα autoantibodies in healthy individuals and CU patients is therefore needed. Due to the problems connected to the detection assays explained above we wanted to develop a new approach to determine the distribution of anti-FcεRIα autoantibodies in healthy individuals and CU patients. One possibility to specify the distribution of rare antibodies would be a polyclonal B cell activation with limiting dilution assay which would then allow to detect specific antibodies (141, 142). However this approach is time consuming and needs intensive laboratory work. In recent years RT-PCR and real-time PCR have become important tools to predict the clinical outcome of minimal residual disease in leukaemia. Junctional regions of immunoglobulin and T cell receptor gene rearrangements can be regarded as leukaemia-specific DNA fingerprints. Using PCR analysis of these junctional regions it was possible to detect one leukemic cell in a background of approximately 10^4 to 10^6 normal cells (143-146). These studies indicated the feasibility of the detection of one single immunoglobulin sequence in a high background of other immunoglobulin sequences using PCR approaches. For these reasons we developed semi-nested RT-PCR and real-time PCR to monitor the distribution of anti-FcεRIα autoantibodies in healthy individuals and CU patients.

Table 2 Summery of studies for the detection of anti-IgE, anti-FcεRIα autoantibodies; SD=standard derivation, ASST=Autologous serum skin test

Study	Assay	Positivity represents:	Sample	% of positive patients
Gruber et al. (13)	ELISA to detect anti-IgE antibodies	binding greater than 2 SD above the mean of control group	Serum	55% IgG isotype, 22 % IgM isotype
	ASST	?	Serum	Patients with IgG anti-IgE: positive Patients with IgM anti-IgE: negative
Grattan et al. (15)	ASST	a mean wheal volumes exceeding 10mm^2	Serum	80%
	Histamine release form basophils	10% of total release	Serum	70% of patients with positive skin
Hide et al. (11)		a mean wheal volumes exceeding 9mm^2	Serum	100%
	Histamine release form basophils	10% of total release	Serum	65% of patients with positive skin test
Niimi et al. (16)	ASST	a mean wheal diameter 2mm greater than control injection		60%
	Histamine release form basophils	5% of total release		29% of patients with positive skin test 5% of patients with negative skin test
Fiebiger et al. (18)	Western Blot to detect anti-FcεRIα		IgG fraction	100%
	Immunoprecipitation of a cell line expressing FcεRIα		IgG fraction	100% of patients with positive western blot
	Histamine release form basophils	?	IgG fractions	100% (however not all patients tested in the western blot are included)
Tong et al. (19)	Histamine release form basophils	10% of total release	Serum	52%
	Western Blot to detect anti-FcεRIα		Serum	66%
	β-hexosaminidase release	10% of total release	Serum and IgG fraction	76% for serum 40% for IgG fraction
Fiebiger et al. (12)	ELISA to detect anti-FcεRIα	binding greater than 2.5 SD above the mean of control group	Serum	38%
	Histamine release form basophils	15% of total release	Serum	66% of patients with positive ELISA 17% of patients with negative ELISA
Kikuchi et al. (21)	Histamine release form basophils	16.9% of total release	Serum	69%

6. V_H and V_L Germline Families and Their Usage

The human V_H locus has been analysed mainly by two groups. One group reports that 39 segments of the V_H locus are mapped in regions which are present in most humans whereas 12 segments can be detected only in some individuals and therefore represent haplotype polymorphism (117, 147). Analysing a single human haplotype the other group demonstrated that it contains 39 expressed V_H segments. Of these, 38 are generated from regions common to all humans whereas one was mapped to polymorphic regions. The other polymorphic regions were absent in this haplotype (148). Nevertheless the V_H can be grouped in seven V_H families. The V_H3 family is most abundantly expressed in a human genome whereas the V_H7 is expressed least (see table 3 and (149)). The distribution of the different V_H families in general reflects their germline complexity, meaning a large family like V_H1 is more used than a small family like V_H7. However the V_H3 family is found significantly more often than expected in the productive rearranged repertoire. In contrast, the V_H4 family is less used than expected in the productive rearranged repertoire but was found at a higher frequency in the non-productive rearrangements (149). This indicates a negative selection for the V_H4 family. However in the B-1 B cell subset, which may be the major source of natural autoantibodies, such a negative selection of the V_H4 family could not be observed (149). Nevertheless it was demonstrated that the V_H usage may vary in different individuals and over time in the same individual (150). This is in line with our study, which demonstrated that the relative gene copy number of LTMα15V_H varies over time in one single individual (see Dissertation Equivalent A).

The human κ locus contains 76 Vκ segments. Of these, 32 are considered as functional, 16 are slightly defective and 25 are pseudogenes. Interestingly, the remaining 3 segments may be functional as well as slightly defective (151). The Vκ segments is divided into three main subgroups, Vκ1-Vκ3 and several smaller subgroups (Vκ4, Vκ5, Vκ6 and Vκ7) (151). The segments are clustered in four distinct regions: A, B, L and O. Vκ1-3 are scattered through the clusters A, L and O. Vκ4 and Vκ5 are mapped to cluster B and Vκ6 to cluster A. Like the V_H families also the Vκ families are differentially expressed in the human repertoire (see table 3 and reference (152)).

Table 3 Usage of V_H, Vκ and Vλ families in the human repertoire

V	1	2	3	4	5	6	7	8	9	10
V_H	13.1%	1.9%	53.9%	24.8%	2.9%	2.4%	1%			
Vκ	44%	19%	29%	5%	2%	< 1%				
Vλ	29.7%	43.6%	18.5%	1.5%	1.2%	1.9%	2%	0.8%	0.4%	0.6%

These percentage of V family usage was summarised from the data published in (149, 152, 153)

The human Vλ locus contains 37 Vλ segments located upstream of the seven Jλ-Cλ pairs. 30 of these are expressed, depending on the haplotype (118). The Vλ segments can be grouped into ten Vλ families (154) that are clustered on the genetic map: The cluster which is closest to the Jλ-Cλ region is named cluster A and contains Vλ2 and Vλ3 families and one member of the Vλ4 family, to the cluster B the Vλ1, Vλ5, Vλ7 and Vλ9 families are mapped and cluster C bears the other Vλ4 family members and the Vλ6, Vλ8 and Vλ10 families (118). Analysing the Vλ family usage in one individual over a time period of three months showed that it is not variable over time. In addition these families are used equally in different

individulas (153). However not every family is expressed at the same level (see table 3 and reference (153)).

From the data summarized in table 3 one can conclude that there exist a bias in V_H, Vκ and Vλ family usage. Different studies have tried to analyses the mechanisms by which such a bias in V usage can occur and it is generally accepted that these mechanism can be divided into two categories. The first category represents intrinsic genetic processes. Yancopoulos et al. have for example assumed that V_H segments located at certain chromosomal regions are favoured to be rearranged (155). In addition it was shown that sequences of the recombination signals (156), promoters (157) and the presence of enhancer elements (158) may contribute to the bias of V usage. The second category which may influence this bias contains mechanisms coupled with the Ig protein expression itself like the pairing of the V_H and V_L chains (159) and the receptor editing and receptor revision (160, 161). Ignatovich et al. have also analysed the V usage and concluded that intrinsic factors influence the Vλ repertoire (162). Most interestingly they hypothesised that V segments which recognise different antigens best, have evolved intrinsic mechanisms for guaranteeing their overrepresentation in the human repertoire (162).

6.1 Correlation of V_H and V_L Germline Usage and Diseases

In the previous section the usage of V_H and V_L genes in healthy individuals was discussed. However in some diseases the composition of expressed V_H and V_L genes is altered compared to healthy individuals.

In ankylosing spondylitis (AS) e.g. which is an inflammatory rheumatic disease involving the synovial membrane, an over-representation of V_H5 (11%) and an under-representation of V_H4 (10%) was detected (163). In contrast, in patients of rheumatoid arthritis (RA) and chronic reactive arthritis (ReA), where also the synovial membrane is the central site of inflammatory activity, an over-representation of V_H4 within the plasma cell subset could be observed (164). As the V_H4 family is normally negatively selected (149) the V_H4 over-representation may provoke these diseases. In systemic lupus erythematosus (SLE) which is an autoimmune disease and in primary amyloidosis which is a clonal plasma disorder, the expression of the λ6 family is ten fold increased (165-167). The unusual expression of V_H and V_L in these diseases may be an effect of an imbalance of the two categories, which contribute to the V usage mentioned above. Suzuki et al. have demonstrated that normal B cells edit the rearranged A30 segment of the Vκ locus with autoreactive potentials by receptor editing, whereas SLE patients may have a defect in this mechanisms (168). The V gene usage in the allergic response has also been investigated and was first reported by van der Stoep et al. They could observe a prominent usage of the V_H5 family in patients suffering from atopic dermatitis (169). In a study of 2 peanut allergic individuals, the V_H1 family was over-represented (170). Lastly a study where a Fab expression library was constructed from a patient allergic to egg allergens showed a biased usage of the V_H6 gene (171). The fact that the regulation of V gene usage in allergic reactions is regulated by antigen driven selective forces remains still controversial. Van der Stoep et al. (169) could not observe evidence for antigen driven selective forces, Janezic et al. (170) demonstrated for 35% of the analysed sequences an antigen driven selective force, whereas Davies et al. clearly demonstrated such a mechanism (172). By semi-nested RT-PCR and real-time PCR we have monitored the V_H3-23 segment in healthy individuals and CU patients. However we could not see any correlation between this segment usage and the state of health (see Dissertation Equivalent A).

7. Polymerase Chain Reaction: A possible Diagnostic Test?

Already in 1971, Kohrana and colleagues described the replication of a region of dsDNA using two primers so that their 3' ends pointed towards each other (173). However, additional 12 years were needed till the conventional polymerase chain reaction (PCR) was conceptualised by Kary Mullis and in 1985 the first paper describing the PCR was published by Saiki et al. (174).

PCR proceeds in three distinct steps depending on temperature: First the samples are heated resulting in separation of the complementary strands through denaturation of the DNA. Second the primers anneal as the reaction is cooled to the appropriate annealing temperature. Last the sample are heated again which allows the polymerase to elongate the target.

PCR involves two primers that are initiation sites for the DNA polymerase and therefore define the region of the template DNA that will be copied (175). To prevent mispriming, the primers have to be designed that they specifically bind to the DNA, which will be amplified. The guidelines for designing such specific primers are outlined in the next section.

7.1 Primer Design and Other Factors which Influence PCR Specificity

In the past, when computer programs did not support the primer design, people designed primers by following some simple rules. A primer should be 20-30 nucleotides long, which do not contain regions composed of the same nucleotide or repetitive sequences. To avoid mispriming at GC-rich regions the 3'end of the primer should not contain tree or more G or Cs. In addition to ensure primer annealing complemetarity within a primer sequence should be avoided. Last the primer pair should not anneal to each other, which would result in competition for the annealing to the target and in formation of primer-dimers. These guidelines were recommended by (176).

The primer specificity is checked by comparing the primer sequence to other sequences using any desired database. This comparison can be performed by applying a BLAST search (Basic Local Alignment Search Tool). The BLAST search reveals then different matches or mismatches of the primer sequence within the sequences that are contained in the chosen database. These alignment results have then to be analysed manually or as in our study by a bioinformatic program (see Dissertation Equivalent A). Such an analysis might give many matches of the primer sequences with other unspecific sequences. However to give a high primer specificity the first 3 nucleotides of the 3' end of the primer sequence should not be present in other sequences that are not desired to amplify. Ma et al. reported that even one mismatch at the 3' end resulted in no amplification (177). However although a reverse primer was designed that contains at its 3' end four nucleotides exclusively present in the target, we did not succeed in specific amplification (see Dissertation Equivalent A). This indicates that not only the primer sequence is important for a specific amplification. Other factors may influence specificity such as primer concentrations, annealing temperature (178, 179), dNTP concentrations (180), magnesium concentrations (180) and the kind of polymerase which is chosen. For an allele-specific PCR for example a non-proof reading polymerase is favoured instead of a proof reading polymerase. The reason for this is given by the fact that a non-proof reading polymerase does not contain a 3'-5' exonuclease activity and therefore does not repair mismatches formed between primers and template. Under this circumstance the primer with a 3' end mismatch should result in no amplification (181, 182). Hence, it is recommended to use a non-proof reading polymerase if the 3' end of the primer sequence contains only one nucleotide, which can discriminate between specific and unspecific amplification. However in our case using a non-proof reading polymerase did not rule out an unspecific amplification (see Dissertation Equivalent A).

7.2 RT-PCR versus Semi-Nested RT-PCR

The isolation of the same anti-FcεRIα autoantibody in healthy individuals (LTMα15) and in CU patients (UMα16) by phage display questioned the pathological role of such anti-FcεRIα autoantibodies in CU patients. Therefore we further analysed the distribution of such anti-FcεRIα autoantibodies by performing a semi-nested RT-PCR (see Dissertation Equivalent A). The immunoglobulin sequence to be detected belongs to the V_H3-23 family. As the V_H3 germline family is most abundant in a human repertoire (see table 3), semi-nested RT-PCR was favoured due to higher sensitivity compared to conventional RT-PCR: Semi-nested PCR allows the specific amplification of a target from a high background of unrelated templates. It generally uses one primer that hybridise internally to the product of the first PCR. The first PCR product is used as template for the second PCR yielding in a smaller PCR product than the PCR product of the first PCR. Although it is possible that the primer pair used in the first PCR reaction have amplified unspecific sequences, the probability that these unspecific products will also contain sites for nested specific primer is very low. It has been estimated that semi-nested RT-PCR leads to a 10^4 fold increase in sensitivity of detection of the correct product (176). However increased sensitivity presents the risk that any contamination will also be enhanced. Therefore precautions to avoid false positive PCR results should be made as described by Kwok et al. (183). However our semi-nested RT-PCR was not in all tested donors specific. To avoid time-consuming optimisation of the semi-nested RT-PCR we chose the real-time PCR approach, which should be more specific because of the use of an additional probe. This approach was successfully used to detect for example prostatic tumor cells (184), B cell tumor cells (143) and rhinovirus (185). This approach is explained in the next section

7.3 Real-Time PCR

Real-time PCR is based on the measurement of fluorescence signals. It has several advantages compared to a conventional PCR. First, the specificity of a real-time PCR is higher than in a conventional PCR. The high specificity of real-time PCR is due to the use of an additional probe as a fluorescence signal can only be detected if the probe anneals to the target sequence. Second, instead of analysing the end point of a PCR as in a conventional PCR, the reaction can be studied in real time. Third, there is no additional manipulation step needed as for example gel electrophoresis to analyse the PCR products. Fourth, it provides a reliable method for rapid quantification of specific gene copies. In the next section some major points of a real-time PCR are explained.

7.3.1 Principle of Real-Time PCR

To perform a real-time PCR two important requirements are needed. First the *Taq* polymerase which exhibits a 5’ to 3’ end exonuclease activity (186); second, a dual-labelled probe which acts according to the fluorescence resonance energy transfer (FRET) principle (187). The FRET principle is known as the transfer of fluorescence from a donor dye to an acceptor dye. The fluorescence of the donor dye can only be detected if it is separated from the acceptor dye (187). Using these two requirements in one assay Heid et al. reported for the first time the quantitative real-time PCR (188). As the *Taq* polymerase possesses a 5’ to 3’ end exonuclease activity, it is able to cleave the so-called TaqMan probe. The TaqMan probe bears at the 5’ end a reporter dye, e.g. FAM (6-carboxyfluorescein), which represents the donor dye. The acceptor dye, known as quencher is located at the 3’end of the probe. As the TaqMan probe acts according to FRET principle the reporter dye emission (e.g. for FAM at 518nm) can only be detected if cleavage of the probe occurs. This implies that the probe has

to have bound to the target together with the primer. During elongation of the primer sequence by the *Taq* polymerase the TaqMan probe is degraded and the reporter dye emission can be detected in real-time. However the amplification of unspecific products is also possible in a real-time PCR as the probe does not influence the actual amplification. Hence if one has to design a primer pair not 100% specific for the target, primer competition can be expected. Nevertheless most importantly is the specificity of the TaqMan probe as a failure of its annealing results in no signal.

In some real-time PCR approaches the design of a specific primer pair together with a specific probe is not possible due to the target that is partly in germline configuration. This was for example the case in the work of Verhagen et al. (146) and in our study (see Dissertation Equivalent A). Thus we designed a special probe with high specificity (see chapter 7.3.5) and a primer pair, which preferentially amplifies our target but not exclusively. We choose this strategy, as the semi-nested RT-PCR already demonstrated that our specific primer also amplifies unrelated sequences. In contrast, Verhagen et al. (146) preferentially designed a specific primer pair and a consensus probe which may anneal to unrelated target. This was possible as they could demonstrate that the primer pair exclusively amplifies the target. Using a consensus probe together with a specific primer pair results in higher sensitivity of the real-time PCR as under this circumstance no primer competition between the target and unrelated sequences occurs. However in our real-time PCR strategy primer competition was expected resulting in a lower sensitivity of the assay. To compensate the lower sensitivity of our assay we used DNA instead of cDNA. The usage of DNA was possible as at this stage the VDJ segments of an immunoglobulin sequences are already rearranged (see chapter 4.1.1). We supposed that the expected primer competition in our real-time PCR might be reduced in a genomic DNA sample, which contains a more homogenous composition of rearranged immunoglobulin sequences than a cDNA sample where some immunoglobulin sequences are overrepresented due to clonal expansion. This reduced primer competition might result in a higher sensitivity using DNA instead of cDNA.

7.3.3 Monitoring the PCR in Real-Time

The fluorescence emission of the reporter dye increases in every cycle if the target is amplified. A sequence detector measures this increase of emission in „real-time". During the amplification a computer software program calculates the ΔRn. ΔRn represents the value of Rn^+ - Rn^-. Rn^+ corresponds to the fluorescence emission generated during target amplification whereas Rn^- stands for the background of the fluorescence emission (baseline, see figure 3 and references (188, 189)). Using this equation ($\Delta Rn = Rn^+ - Rn^-$) the computer software generates amplification plots where one axis represents the ΔRn values and the other the cycle number, respectively (figure 3). Therefore, if a sample contains a high copy number the ΔRn values exceed the baseline during the early cycles whereas a low copy number is detected late during PCR amplification. To determine if a sample is positive for a target an arbitrary threshold is chosen which normally represents 10 times the standard deviation of Rn^- generated during cycles 3 to 15. The cycle where the Rn^+ exceeds this chosen threshold is then taken as threshold cycle (C_T) value. Therefore the C_T value is the minimum number of cycles needed before the product can be detected, meaning a sample containing high target copy numbers results in a low C_T value. Hence C_T values increase linearly with decreasing target copy numbers (190). The C_T values generated during a real-time PCR can then be used to perform a relative quantification, which is discussed in the next section.

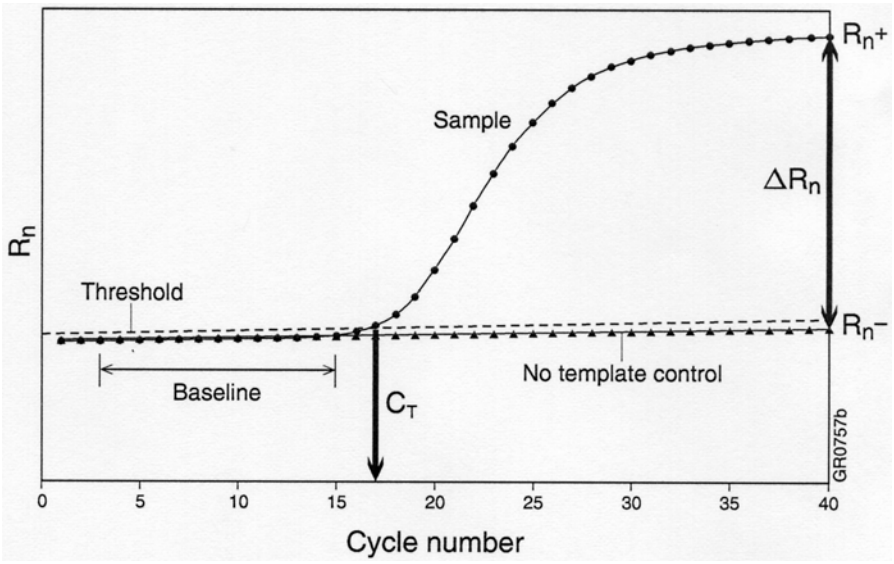

Fig. 3 Amplification plot of a real-time reaction.

- $\Delta Rn = Rn^+ - Rn^-$
- Rn^+ = fluorescence emission of the product
- Rn^- = fluorescence emission of the baseline
- C_T = minimum number of cycles needed before the product can be detected

Adapted from Applied Biosystems (190)

7.3.4 Relative Quantification of Gene Copy Numbers

Relative quantification, known as comparative C_T method, describes the differing gene copy number relative to an arbitrarily chosen calibrator. In our study a healthy donor showing the highest C_T value was chosen as calibrator (see dissertation equivalent A and B). It is also possible to set a sample at time zero as calibrator allowing the analysis of gene copy number changes over time (see dissertations equivalent A). However, in order to use the comparative C_T method for quantification analysis, the efficiencies of amplification of the target and a housekeeping gene, respectively should be approximately equal. This is important as the housekeeping gene is used for normalisation (see below). To determine these two efficiencies of amplification, efficiency plots are made. In these plots the measured average C_T values of the target and a housekeeping gene, respectively are drawn against corresponding amounts of input DNA. The resulting slopes for efficiency plots of the target and the housekeeping gene should not differ more than 0.1 (190).

Suitable housekeeping genes for real-time quantitative PCR include among others β-actin, β2-microglobulin (191) and porphobilinogen deaminase (PBGD) (192). The fact that the amount of a housekeeping gene remains constant, regardless of sample type, is the most important criteria such a gene has to fulfil. Therefore the housekeeping gene should be properly validated. In general the C_T values of the housekeeping gene should differ less than 1.1 between the different samples to guaranty that the quality and quantity of DNA sample per tube are comparable (190).

The arithmetic formula for the comparative C_T method is given by $2^{-\Delta\Delta CT}$, where ΔC_T is the C_T value of the target minus the C_T value of the housekeeping gene, which gives a normalized value. The normalised ΔC_T value of the target is then subtracted from the normalised ΔC_T value of the calibrator, which gives the $\Delta\Delta C_T$ value. The formula $2^{-\Delta\Delta CT}$ therefore gives a relative value when comparing the target to the calibrator. For each data point the standard deviation of the difference are calculated from the standard deviations of the target and the housekeeping gene values as follows: S (std dev of difference) = $\sqrt{S1^2 + S2^2}$ (190).

7.3.5 TaqMan Probe versus MGB Probe

Both the TaqMan probe and the MGB probe have at the 5’ end a reporter dye, for example FAM. However these probes differ at the 3’ end. The TaqMan probe contains at this end a

quencher dye as for example TAMRA (6-carboxytetramethylrhodamine) whereas the MGB probe bears a non-fluorescence quencher to which a minor groove binder (MGB) is attached. The MGB probe shows several advantages compared to the conventional TaqMan probe. MGB such as dihydrocylopyrroloinodole has the capacity to bind to the minor groove of DNA with a high affinity. This high affinity was made use of by conjugating the MGB with oligodeoxynucleotides, resulting in very stable hybrids with complementary DNA (193). Hence the melting temperature is increased and allows therefore the design of probes shorter than 20 nucleotides. As the probe is shorter, the mismatch discrimination is improved resulting in a higher specificity (194). For this reason such MGB probes have also been used for single nucleotide polymorphism (SNP) analysis (195) as one single mismatch results in no fluorescence signal. The additional advantage of a shorter probe is the enhancement of fluorescence quenching as the distance of the donor dye to the acceptor dye is smaller, giving increased sensitivity (194). Although TAMRA present in a conventional TaqMan probe quenches the reporter FAM dye efficiently, it also adds its own emission to the total fluorescence spectrum measured for a sample. Hence the calculation of the contribution of the donor dye and the acceptor dye to the total fluorescence is complicated. This complication is abolished in a MGB probe as it contains a non-fluorescence quencher and therefore the sensitivity of fluorescence measurement is increased.

Using this MGB probe in our real time PCR we were able to detect one single antibody heavy chain sequence that is in germline configuration in healthy individuals and CU patients. Therefore we propose that this new molecular approach can be used to monitor natural autoantibodies sequences against FcεRIα in healthy individuals and CU patients. Furthermore this new molecular approach may serve as model to monitor any desired antibody sequence in different donors.

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C. DISSERTATION EQUIVALENTS

Dissertation Equivalent A

Fux, M., Vogel, M., Stadler, M. B., Stadler, B. M. and Miescher, S. M.,2004 Detection of One V_H Antibody Sequence in Both Healthy Donors and Urticaria Patients. *submitted*

Dissertation Equivalent B

Vogel, M., Tschopp, C., Bobrzynski, T.; Fux, M., Stadler, M. B., Miescher, S. M. and Stadler, B. M. 2004 A Highly Conserved Interspecies V(H) in the Human Genome. *Journal of Molecular Biology 341: 477-489*

Dissertation Equivalent A

Fux, M., Vogel, M., Stadler, M. B., Stadler, B. M. and Miescher, S. M.,2004 Detection of One V_H Antibody Sequence in Both Healthy Donors and Urticaria Patients. *submitted*

Detection of one V_H antibody sequence in both healthy donors and urticaria patients

Michaela Fux[1]*, Monique Vogel[1], Michael B. Stadler[1], Beda M. Stadler[1] and Sylvia M. Miescher[1,2]

[1]*Institute of Immunology, University of Bern, CH-3010, Switzerland*

[2]*ZLB Behring, Bern, CH-3022, Switzerland*

*Corresponding author; Michaela Fux, Institute of Immunology, Inselspital, 3010 Bern, Switzerland Tel.: +41 31 632 13 07; Fax: +41 31 381 57 35.

e-mail: mfux@students.unibe.ch

Abstract

We have previously isolated anti-FcεRIα autoantibodies from phage libraries of healthy donors and urticaria patients. Strikingly the same antibody, LTMα15, was isolated from both libraries. Sequence analysis revealed germline configuration of the LTMα15 variable heavy (V_H) chain with a slightly mutated variable light (V_L) chain supporting its classification as a natural autoantibody. The distribution of LTMα15V_H in healthy donors and urticaria patients was analysed by real-time PCR. Our new bioinformatic program allowed the design of a minor groove binder (MGB) TaqMan probe that specifically detects the LTMα15V_H. We demonstrate a broad range of rearranged V_H gene copy number without any correlation to the state of health. Monitoring LTMα15V_H gene copy number in a single donor over a period of 70 days revealed a time related fluctuation of circulating B cells carrying LTMα15V_H. We propose that our real-time PCR may serve as a model for the quantification of natural antibody sequences at a monoclonal level.

Introduction

Chronic urticaria (CU) is defined by the occurrence of daily wheals and itching for at least 6 weeks. The wheals, redness and itching of CU are due to release of histamine and other mediators, including eicosanoids, cytokines and proteases, from dermal mast cells. In CU, the triggering stimulus causing the characteristically unpredictable dermal mast-cell activation has proved elusive. Prima facie evidence of the involvement of functional autoantibodies against the α-chain of the high-affinity IgE receptor (FcεRIα) of dermal mast cells and basophils was first reported in 1993 and 1994 by Hide et al.(1, 2). Such anti-FcεRIα autoantibodies were claimed to be a selective marker for a subset of urticaria patients (3). However, we have previously reported the presence of anti-FcεRIα autoantibodies in the serum of healthy donors (4) as well as in multidonor intravenous IgG (IVIg) preparations (5). Furthermore we have published the isolation of anti-FcεRIα autoantibodies (LTMα15, UMα16, UGα8) using phage display technology (6, 7). LTMα15 was isolated from an IgM library constructed from children's tonsils B cells. UMα16 and UGa8 originated from an IgM and IgG library, respectively that were constructed from urticaria patients' B cells. To perform functional tests, we have produced them as full length antibodies (6, 7). Both LTMα15 and UMα16 demonstrated a high affinity interaction ($7.2x10^{-9}$M) with the FcεRIα chain using the IAsys (7). Comparison with the known germline sequence of human variable heavy (V_H) and variable light (V_L) chain segments (8-10) indicated that LTMα15, UMα16 and UGα8 V_H chains showed 100% homology to the germline V_H3-23 sequence, whereas the λ light chains were slightly mutated and most similar to the V_λ germline λ6a. Furthermore, sequence analysis showed that LTMα15 and UMα16 are 100% identical. Due to the germline configuration of the heavy chains and the fact that the light chains are only slightly mutated, we suggest that LTMα15, UMα16 and UGα8 represent natural autoantibodies, which may be present in all humans. Thus evidence points to the ubiquitous presence of natural anti-FcεRIα autoantibodies but their role as a selective marker for urticaria patients remains unclear. Therefore our goal was to further investigate the distribution of anti-FcεRIα autoantibodies in healthy donors and urticaria patients.

Up to now the distribution of anti-FcεRIα autoantibodies was analysed by functional and serological tests. However no significant relation was found between autologous serum skin test positivity and serological detection of anti-FcεRIα autoantibodies by enzyme immunoassays (4, 11). This discrepancy may occur due to the sensitivity of the performed tests. Here we describe the analysis of the distribution of the LTMα15V_H sequence by semi-nested RT-PCR which was shown to be two times more sensitive than a serological test (12). Additionally we have performed a real-time PCR. Quantitative analysis of the real-time PCR results enabled us to compare the relative gene copy number of LTMα15V_H sequence in healthy donors and urticaria patients. Because of the presence of V sequences highly similar to the LTMα15 V_H and V_L sequence in the V repertoire we have written a new bioinformatic program, which predicts potential primer annealing sites in any sequence. Using this program we were able to determine a potential primer annealing site, which is present exclusively in the LTMα15V_H sequence. To this annealing site we have designed a 3' end primer for the RT- PCR or a minor groove binder (MGB) probe to perform the real-time PCR. To our knowledge, this is the first report describing the detection of a single natural immunoglobulin sequence in different donors. Our approach may therefore serve as a model for the detection and quantification of antibody sequences coding for natural antibodies.

Material and Methods

DNA, RNA extraction, cDNA synthesis and plasmid DNA

Mononuclear cells of peripheral blood from 26 unselected healthy donors, 11 urticaria patients and of 25 cord blood samples were purified by Ficoll density gradient centrifugation (Lymphoprep, Pharmacia, Dübendorf, Switzerland). Total RNA was extracted using RNeasy Mini Kit (Qiagen AG, Basel, Switzerland). RNA was transcribed into cDNA using a primer specific for IgM gene constant region (5' GCTCACACTAGTCTAGGCAATCACTGGAAG AGG 3') and the Superscript TM II reverse transcriptase system (Invitrogen, Life technology, Basel, Switzerland). Genomic DNA was isolated using QIAamp DNA blood Maxi kit (Qiagen AG, Basel, Switzerland). Plasmid DNA containing the anti-FcεRIα antibody LTMα15 heavy chain sequence was produced in our laboratory as described (7).

Primer design for semi-nested RT-PCR

Specific primers were designed in order to detect the LTMα15 sequence in healthy donors and urticaria patients using PCR. We intended to design a forward primer annealing either in the complementary determining regions (CDR) 1 or CDR2 and a reverse primer hybridising to the CDR3 of LTMα15 variable light (V_L) and variable heavy (V_H) chains. To identify specific primers, the CDR sequences of V_H and V_L of LTMα15 were independently compared to 82,566 different immunoglobulin sequences (ImMunoGeneTics database, University of Montpellier, France and French National Centre for Scientific Research: CNRS, France (13)) applying a BLAST search (Basic Local Alignment Search Tool, version 2.2.8). BLAST was used with gap open and extension penalties of 5 and 2, respectively. E-value threshold was set to 1000. For comparison sequences of CDRs of LTMα15V_H and V_L, which were shorter than the desired primer length of 23 nucleotides, were extended to 23 nucleotides by adding bases of the flanking frameworks. Immunoglobulin sequences were identified that contain LTMα15 like CDRs and were then checked for potential primer annealing sites. A potential primer annealing site was defined as a window of 20 or more nucleotides with at most two mismatches in total and no mismatch in the five nucleotides at the 3' end of the primer. The number of potential PCR products was then counted in the case that a forward and a reverse primer annealing site occurred simultaneously in sequences containing LTMα15 like CDRs. All scripts were written in Perl, using modules from Bioperl (14). Primers specific for the LTMα15 were designed using the PrimerExpress software (Applied Biosystems, Rotkreuz, Switzerland).

Semi-nested RT-PCR

The presence of LTMα15V_H in 26 different healthy donors was analysed by semi-nested RT-PCR. Cord blood cDNA known to be positive for LTMα15V_H, was used as a control, for testing primer specificity in RT-PCR. The first PCR was performed using 5µl of cDNA, CDR1V_H forward primer (5' CTGGATTCACCTTTAGCAGCTATGC 3') that anneals to the CDR1 and Cµ1 reverse primer (5' GGAGTCGGGAAGGAAGTCC 3') that matches the first constant domain of IgM. To perform the second PCR, the first PCR product was first gel purified from a 2% DNA agar gel (Axon Lab AG, Baden-Dättwil, Switzerland) using the MinElute gel extraction kit (Qiagen AG, Basel, Switzerland) and then amplified. The second PCR was carried out using 50ng of purified first PCR product, CDR1V_H forward primer and CDR3V_H reverse primer (5' TCCATGTAGTAGGACGGTAGC 3'), which hybridises in the CDR3. In parallel and in order to investigate its quality, cDNA was directly amplified using two primers that hybridise to the first constant region of IgM gene Cµ1for

(5'GGAGTCGGGAAGGAAGTCC 3') and Cμ1rev (5' GCATCCGCCCCAACCCTT 3'). PCR amplifications were performed according to manufacture's instructions (Invitrogen, Life technology; Basel, Switzerland) in a total volume of 50 μl using 2.5 U of Platinium Pfx DNA Polymerase. The first PCR was carried out at 94ºC for 5min, followed by 30 cycles of 94ºC for 40sec, 58.9°C for 30sec and 72°C for 40sec. For final extension samples were incubated for 10min at 72ºC. The second PCR of 40 cycles was done as mentioned except for an annealing temperature of 57°C. Products of the second PCR were purified and sequenced using primers of the second PCR.

Primers and TaqMan probes for real-time PCR

The primers and the TaqMan probes for real-time quantitative PCR were designed from appropriate sequences using the PrimerExpress software (Applied Biosystems). The primers and the TaqMan probes specific to the LTMα15V_H and to the housekeeping gene porphobilinogen deaminase (PBGD) are: LTMα15$V_H$for 5' CCGTATATTACTGTGCGAA AGGG 3'; LTMα15$V_H$rev 5' C ATGGACGTCTGGGGCA 3'; LTMα15V_H Minor Groove Binder (MGB) TaqMan probe 5' AAAGATGGCTACCGTCCTA 3'; PBGDfor 5' TGACC CACAGTTGGTAGGCAT 3'; PBGDrev 5' ATGCCCAAGTTCTGGGCA 3' and PBGD TAMRA TaqMan probe 5' CGTAACATTCCACGAGGGCCCCA 3'. The primers were synthesized by Microsynth (Balgach, Switzerland) and the TaqMan probes by Applied Biosystems (Rotkreuz, Switzerland). LTMα15V_H MGB TaqMan probe was labelled at the 5' end with 6-carboxy fluoroscein (FAM) and at the 3' end with MGB/ Non- Fluorescent Quencher (NFQ). PBGD TaqMan probe was labelled at the 5' end with FAM and the red fluorophore tetramethylrhodamine (TAMRA) at the 3' end was used as quencher.

Real-time PCR

Real-time PCR was performed using genomic DNA of 22 healthy donors (donor 5 – 26) and 11 urticaria patients (donors 27 – 37). All reactions were performed in triplicates (25μl each) using 12.5μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 200nM of TaqMan probe and 900nM of each primer except for LTMα15$V_H$rev primer which was used at 2 μM. To obtain equal amplification efficiency PBGD and LTMα15V_H genes were amplified using 100ng and 2μg of DNA, respectively. Real-time PCR was performed under the following conditions: 50°C for 2min; 95°C for 10min; 60 cycles at 95°C for 15sec and 60°C for 1min on an ABI prism 7700 sequence detector equipped with a 96-well thermal cycler. Negative control without template (no genomic DNA) was included on each PCR plate. Data were collected and analysed with Sequence Detector software (Applied Biosystems, Rotkreuz, Switzerland).

Quantitative analysis and statistics

Quantitative analysis of gene copy number was done using the comparative C_T (ΔC_T) method, in which C_T is the threshold cycle number (the minimum number of cycles needed before the product can be detected (15)). To ensure that quantification of LTMα15V_H was not affected by differences in the quality and quantity of the genomic DNA we used the housekeeping gene porhpobilinogen deaminase (PBGD) as internal control and for normalization. The average C_T values of the PBGD gene in the various genomic DNA samples differed less than 1.1 C_T values among each other, implying that the quality and quantity of genomic DNA per tube were comparable (data not shown). The arithmetic formula for the ΔC_T method is given by $2^{-\Delta\Delta CT}$, where ΔC_T is the C_T value of LTMα15V_H minus the C_T value of PBGD, which gives a normalized value. The normalized ΔC_T value of LTMα15V_H is then subtracted from the normalized ΔC_T value of a calibrator, which gives the $\Delta\Delta C_T$ value. The donor with the

highest C_T value was chosen as calibrator. The formula $2^{-\Delta\Delta CT}$ therefore gives a relative value when comparing the target to the calibrator. For each data point the standard deviation of the difference was calculated from the standard deviations of the LTMα15V_H and PBGD values as follows: S (std dev of difference) = $\sqrt{S1^2 + S2^2}$ (15).

Results

Determination of primers and probes specific to LTMα15

Previously we isolated identical anti-FcεRIα variable heavy (V_H) and variable light (V_L) chain genes from urticaria patients and healthy donors (4, 6, 7). To investigate via PCR whether these V genes are differently distributed between urticaria patients and healthy donors, specific primers had to be designed. To identify specific primers, the complementary determining region (CDR) sequences of V_H and V_L of LTMα15 were compared to a human immunoglobulin database. Immunoglobulin sequences containing similar $CDRV_L$ as $LTM\alpha15V_L$ were identified and analysed for potential primer annealing sites. As shown in figure 1A a total of 146, 119 and 65 potential primer annealing sites in $CDR1V_L$, $CDR2V_L$ and $CDR3V_L$, respectively, were found indicating an abundant usage of such CDRs in the V_L repertoire. We next analysed the number of potential PCR products. A PCR product would be expected if a single immunoglobulin sequence contained two LTMα15 like $CDRV_L$ regions where potential forward and reverse primer annealing sites had been previously identified. Figure 1B shows that 61 potential PCR products were obtained if primers were designed to anneal in $CDR1V_L$ and $CDR3V_L$ and 49 if using $CDR2V_L$ and $CDR3V_L$. This high number of different PCR products prevented the design of V_L primers for the detection of $LTM\alpha15V_L$. The same approach was used to identify sequences containing similar $CDRV_H$ as $LTM\alpha15V_H$. Analysis of potential forward primer annealing sites in these sequences revealed 764 and 4667 sites in the $CDR1V_H$ and $CDR2V_H$ indicating an abundant usage of such CDRs in the V_H repertoire. (figure 1A). In contrast for $CDR3V_H$ only three immunoglobulin sequences contained potential reverse primer annealing sites (figure 1A). Detailed analysis revealed that these sites were contained in the three anti-FcεRIα antibodies LTMα15, UMα16 and UGα8, which carry identical $CDR3V_H$. As shown in figure 1B a limited number of three potential PCR products were found if primers were designed to anneal either in $CDR3V_H$ and $CDR1V_H$ or in $CDR3V_H$ and $CDR2V_H$. This low number of PCR products allowed us to design V_H primers for the detection of $LTM\alpha15V_H$. As there were less potential forward primer annealing sites in the $CDR1V_H$ than in the $CDR2V_H$ (764 versus 4667) a forward primer was designed which anneals to $CDR1V_H$. Detailed analysis of the bioinformatic results also showed that four nucleotides in the CDR3 of $LTM\alpha15V_H$ are exclusively present in this $CDR3V_H$ and in no other immunoglobulin sequence. To provide specific amplification of the $LTM\alpha15V_H$ sequence we designed the reverse primer such that these four nucleotides are located at the 3' end of the primer sequence. Forward and reverse primer annealing sites for the semi-nested RT-PCR are shown in figure 2. To perform the real-time PCR the minor groove binder (MGB) probe was designed such that these four nucleotides exclusively present in the CDR3 of $LTM\alpha15V_H$ sequence are located in the middle of the MGB probe as shown in figure 3.

Semi-nested RT-PCR for the detection of $LTM\alpha15V_H$ sequence in healthy donors

The presence of the $LTM\alpha15V_H$ sequence in the human genome was monitored by RT-PCR. To increase sensitivity a semi-nested RT- PCR was done as shown in figure 2. The semi-nested RT-PCR was performed using cDNA of 26 randomly selected healthy donors. Cord blood cDNA known to be positive for the $LTM\alpha15V_H$ sequence was taken as positive control. For the first PCR a pair of primers was designed which anneal to the CDR1 of $LTM\alpha15V_H$ and the first constant region of the IgM gene, respectively. The first PCR yielded for all tested donors as well as for the cord blood sample a single product band of expected size (400bp, data not shown). The second PCR was carried out with purified first PCR products, the same

forward primer used for the first PCR and a reverse primer annealing with its 3' end to the four nucleotides unique for the CDR3 of LTMα15V_H. As shown in figure 4 all product samples of the second PCR contained a major band of the same size (254bp) as the one obtained with the control plasmid DNA containing LTMα15V_H. Sequencing revealed that in cord blood cDNA as well as in cDNA of donors 3, 10, 11, 12, 14 and 15 the same V_H was found as the one present in LTMα15. In all other donor samples, except donor 2, the V_H germline family, which is present in LTMα15V_H (V_H3-23), was amplified. However, in these donor samples different CDR3V_H sequences were amplified indicating that the four nucleotides at the 3'end of the reverse primer sequence did not provide enough specificity to amplify exclusively the LTMα15V_H sequence. We have repeated the semi-nested RT-PCR using a non-proof reading polymerase to investigate if this increases the specificity of the reverse primer (16, 17). Our results demonstrated that the use of a non-proofreading polymerase did not increase specificity of the reverse primer (data not shown). These data are in contrast to other studies showing that a mismatch of one nucleotide at the 3'terminus of a primer sequence resulted in no amplification (18). To avoid time-consuming optimisation of the semi-nested RT-PCR we chose the real-time PCR approach, which should be more specific because of the use of an additional probe.

Quantitative analysis of the LTMα15V_H sequence in healthy donors and urticaria patients

To compare the frequency of LTMα15V_H in healthy donors and urticaria patients, genomic DNA samples of 22 healthy donors (donor 5 – 26) and 11 urticaria patients (donor 27 – 37) were tested for the presence of LTMα15V_H sequence. DNA of donor 1 – 4 was no more available. Genomic DNA instead of RNA was chosen as template because of the reproducibility and sensitivity problems linked to RT-PCR. The real-time PCR was carried out using a forward and a reverse primer hybridising to the framework 3 and the CDR3V_H, respectively and a probe, which anneals to the CDR3V_H region containing the four nucleotides unique for the CDR3V_H of LTMα15V_H. To increase specificity of real-time PCR a 3' minor groove binder (MGB) probe was used instead of the commonly used TaqMan probe. The MGB molecule allows TaqMan probes to be shorter compared to conventional ones, hence increasing stability and specificity of the probe as a single base mismatch prevents hybridisation (19).

As internal control of genomic DNA quality and quantity the housekeeping gene porphobilinogen deaminase (PBGD) was chosen. Quantification analysis was performed using the comparative C_T method. This was possible because the efficiencies of amplification of the LTMα15V_H and the PBGD gene were equal ((15) data not shown).

For quantification, the LTMα15V_H rearranged gene copy number of each donor was normalized against PBGD and compared to a normalized calibrator. Donor 21 which showed the highest C_T value was chosen as calibrator. The relative quantity of the LTMα15V_H was given by $2^{-\Delta\Delta CT}$. As shown in figure 5 the LTMα15V_H sequence was detected in 9 out of 22 healthy donors and in 4 out of 11 urticaria patients demonstrating that the presence of this V_H is not restricted to urticaria patients. The results also show that the relative quantity of LTMα15V_H rearranged gene copy number varied considerably among the different donors, over a range of more than four orders of magnitude and there was no correlation with the source of the sample originating either from healthy donors or urticaria patients.

Time related analysis of LTMα15V_H sequence in healthy donors

So far our data demonstrated that the distribution and quantity of LTMα15V_H rearranged gene copy number varies from donor to donor. We next asked the question whether in a single healthy donor LTMα15V_H rearranged gene copy number is stable over a certain time period. Thus blood samples were collected from four healthy donors (6, 14, 16, and 20) at different time points. The first blood donation of each donor was defined as day 0 and used as calibrator. The normalized amount of LTMα15V_H rearranged gene copy number was calculated as described above and given by $2^{-\Delta\Delta CT}$. The healthy donors showed a varying gene copy number over time ranging up to four orders of magnitude difference for a given donor suggesting a time related fluctuation of circulating B cells carrying the LTMα15V_H rearranged gene (figure 6).

Discussion

Autoantibodies against the α-chain of the high affinity IgE receptor were claimed to be a selective marker for autoimmune urticaria (3). However we have recently shown that natural anti-FcεRIα autoantibodies are also present in the sera of healthy donors which could give false positive results in serological diagnostics for autoimmune urticaria (4). The distribution of such natural autoantibodies was further analysed at a monoclonal level in healthy donors and urticaria patients. To determine the distribution of rare antibodies, polyclonal B cell activation with limiting dilution assays and detection of specific antibodies are usually employed (20, 21). As this approach is time consuming, a simplified screening test would be desirable. Detection of *M. tuberculosis* or group B streptococcus using RT-PCR and real-time PCR, respectively have become standard procedures and are approved by the US Food and Drug Administration (FDA) (22). In recent years these approaches have also become important to predict the clinical outcome of minimal residual disease in leukaemia. Junctional regions of immunoglobulin and T cell receptor gene rearrangements can be regarded as leukaemia-specific DNA fingerprints. Using PCR analysis of these junctional regions it was possible to detect one leukemic cell in a background of approximately 10^4 to 10^6 normal cells (23-26). These studies indicated the feasibility of the detection of one single immunoglobulin sequence in a high background of other immunoglobulin sequences using PCR approaches. In this paper we report the detection of a single immunoglobulin heavy chain sequence of the natural anti-FcεRIα antibody LTMα15 using semi nested RT-PCR and real-time PCR approaches.

Specific primers for the semi-nested RT-PCR were identified by comparing the CDR regions of LTMα15 V_H and V_L to other immunoglobulin sequences. Immunoglobulin sequences containing LTMα15 like CDRs were analysed for potential primer annealing sites using a new bioinformatic program. Our results demonstrated that LTMα15 like $CDRV_L$ are abundantly used in the V_L repertoire, which prevented the specific detection of $LTM\alpha15V_L$ using semi-nested RT-PCR. Interestingly $LTM\alpha15V_L$ belongs to the Vλ6a gene family, which normally represents 1.9% - 4% of a healthy antibody repertoire (27, 28). Therefore such an abundant usage of LTMα15 like $CDRV_L$ was not expected. Detailed analysis of our bioinformatic results demonstrated that immunoglobulin sequences containing LTMα15 like $CDRV_L$ were mostly isolated from SLE and primary systemic amylodoisis patients. As it was shown that the expression of the Vλ6a gene family is ten fold increased in such patients (28-30), the observed abundant usage of LTMα15 like $CDRV_L$ in the V_L repertoire most probably represents a bias in the published immunoglobulin sequences. Using the same bioinformatic program a number of immunoglobulin sequences were identified containing CDR1 and CDR2 similar to those of $LTM\alpha15V_H$. This was expected as $LTM\alpha15V_H$ is in germline configuration of the V_H3-23 family which is abundantly used in the human antibody repertoire (31). Our bioinformatic analysis also revealed that no other immunoglobulin sequence contained a 100% identical CDR3 as that used in $LTM\alpha15V_H$. The CDR3 of $LTM\alpha15V_H$ may therefore account for its antigen specificity. This is supported by other studies showing that the CDR3 of V_H is the key determinant of specificity in antigen recognition and is sufficient to obtain highly specific IgM antibodies to protein antigens (32, 33). However, the CDR3 of $LTM\alpha15V_H$ consists of diversity (D) and joining (J) segments, which are 67 % in germline configuration. Parts of the CDR3 sequence of $LTM\alpha15V_H$ are therefore also present in $CDR3V_H$ of other immnuoglobulin sequences using the same D and J segments. Analysis of our bioinformatic results showed that only four nucleotides are exclusively present in the CDR3 of $LTM\alpha15V_H$. As it was shown that one mismatch at the 3' end of a primer sequence results in no amplification (18) we designed a reverse primer

annealing with its 3' terminus to these four nucleotides of the CDR3 of LTMα15$V_{H.}$ This reverse primer was used together with a germline forward primer annealing in the CDR1, which was expected to amplify LTMα15V_H, but not exclusively. Our semi-nested RT-PCR results demonstrated that the V_H germline family, which is present in the LTMα15V_H (V_H3-23), was detected. However in 16 out of 22 tested donors different CDR3V_H than the LTMα15V_H CDR3 were amplified indicating that the reverse primer was not specific enough to exclusively amplify the LTMα15V_H sequence. Additionally, the successful detection of LTMα15V_H sequence was not reproducible in all donors (data not shown). This may be explained by the kinetics of a PCR. Based on the assumption that in early cycles of the PCR a mispriming of the reverse primer occurs, templates containing the V_H germline family of LTMα15V_H but also other CDR3V_H than LTMα15V_H are exponentially amplified and can interfere with an efficient PCR of the LTMα15V_H sequence. To increase specificity we have performed a real-time quantitative PCR that provides a reliable method for rapid quantification of specific gene copies. This method has been successfully used to detect minimal residual disease in leukaemia (24-26). In these studies conventional TaqMan probes were used to detect CDR3 regions of rearranged immunoglobulin sequences, which were not in germline configuration. This is in contrast to the CDR3 region of LTMα15V_H, which is 67% in germline configuration. For this reason we used a novel fluorescent probe annealing to the four nucleotides of the CDR3V_H previously identified as unique for LTMα15V_H by our bioinformatic program. This probe contains a minor groove binder (MGB) at its 3'end which binds to the minor groove of DNA with higher affinity compared to ordinary TaqMan probes (19). This method reduces non specific probe hybridisation and has been successfully used for single nucleotide polymorphism (SNP) analysis (34).
Our real-time PCR analysis revealed the presence of the LTMα15V_H sequence in both healthy donors and urticaria patients, confirming our previous studies where this V_H sequence could be isolated from healthy donors and urticaria patients using phage display technology (4, 6, 7). Moreover our results showed that the rearranged gene copy number of LTMα15V_H does not correlate with the health status. The rearranged gene copy number can be even higher in healthy donors than in urticaria patients and can therefore not be used as a biomarker for urticaria. Furthermore our kinetic experiments revealed that the gene copy number ranged up to four orders of magnitude difference in one single healthy donor over a period of more than 70 days. This finding is consistent with previous studies reporting that V_H family distribution varied with time and isotype in one individual thus implying that repertoire fluctuation might occur during the lifetime of a healthy donor (35-37).

In summary we have been able to detect one single natural autoantibody sequence in healthy donors and urticaria patients. Using a new bioinformatic program we were able to determine a region in the CDR3V_H, that is unique for our natural antibody sequence. A MGB probe, which, hybridises to this region was designed and allowed us to detect one single natural autoantibody sequence by real-time PCR. To our knowledge this is the first report describing the detection of a natural antibody sequence, which is in germline configuration. Therefore our approach may be a model to monitor natural autoantibody sequences which are in germline configuration and to compare the gene copy number of such sequences in the human antibody repertoire at a monoclonal level.

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Figure legends

Figure 1
Determination of specific CDR regions of LTMα15. LTMα15 CDR regions of V_H and V_L were compared to all other known immunoglobulin sequences contained in the IMGT database. (A) The numbers of potential annealing sites are shown for forward primers either in CDR1 (white bars) or in CDR2 (grey bars) and for reverse primers in CDR3 (black bars) of V_L and V_H. (B) The results are shown in numbers of possible PCR products to be expected if a potential forward primer annealing site in the CDR1 (black bars) or in CDR2 (grey bars) is combined with a potential reverse primer site in the CDR3 of V_L and V_H.
CDR: complementary determining regions; V_H: variable heavy chain; V_L: variable light chain

Figure 2
Semi-nested RT-PCR strategy to detect LTMα15V_H sequence. cDNA is amplified by a first PCR using a 5' primer located in the CDR1V_H and a 3' primer annealing in the first constant domain of IgM (Cμ1). The second PCR is performed with purified product of the first PCR, the same CDR1V_H forward primer as for the first PCR together with specific CDR3V_H reverse primer.
CDR: complementary determining regions; FR: framework; V_H: variable heavy chain

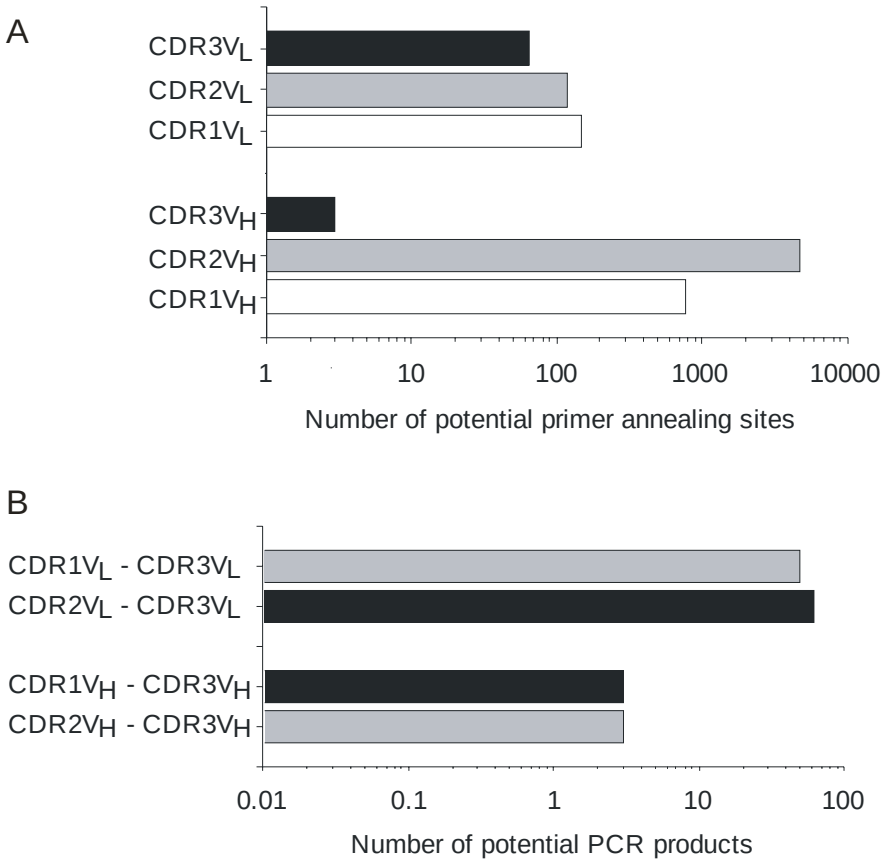
Figure 3
Real time PCR strategy for detection of LTMα15V_H CDR3 sequence. Real time PCR was performed using primers annealing to the 3' and 5' end of the CDR3 region and a TaqMan MGB probe covering 17 bases in the CDR3 region.
FAM: 6-carboxy fluoroscein; MGB : Minor Groove Binder, NFQ: Non- Fluorescent Quencher; CDR: complementary determining regions; FR: framework; V_H: variable heavy chain

Figure 4
The products of the second reaction of the semi-nested RT-PCR were analysed on a 2% agar gel. Lanes 1-26 are products from cDNA of 26 healthy individuals, lane 0 from plasmid DNA containing LTMα15V_H gene, lane 27 from cord blood cDNA. M: 100bp DNA molecular weight marker.

Figure 5
Relative quantification of LTMα15V_H rearranged gene copy number using the comparative C_T method. DNA from peripheral blood mononuclear cells of 22 healthy donors and 11 urticaria patients were used as templates for real-time PCR containing either primer and probe for LTMα15V_H or for PBGD. Relative rearranged gene copy number of LTMα15V_H normalized to PBGD and relative to the normalized calibrator (donor 21) was given by $2^{-\Delta\Delta CT}$. Black bars represent healthy donors, grey bars urticaria patients.

Figure 6
Time related relative quantification of LTMα15V_H rearranged gene copy number using the comparative C_T method. DNA form peripheral blood mononuclear cells of 4 healthy donors were isolated at different time points. The relative rearranged gene copy number of LTMα15V_H normalized to PBGD and relative to the normalized calibrator (time point 0; first blood donation) was given by $2^{-\Delta\Delta CT}$. White circles represent donor 6, black circles donor 14, white triangle donor 16 and black triangle donor 20.

Figure 1


A
CDR3VL
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1
10
100
1000
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Number of potential primer annealing sites
B
CDR1VL - CDR3VL
CDR2VL - CDR3VL
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CDR2VH - CDR3VH
0.01
0.1
1
10
100
Number of potential PCR products

Figure 2

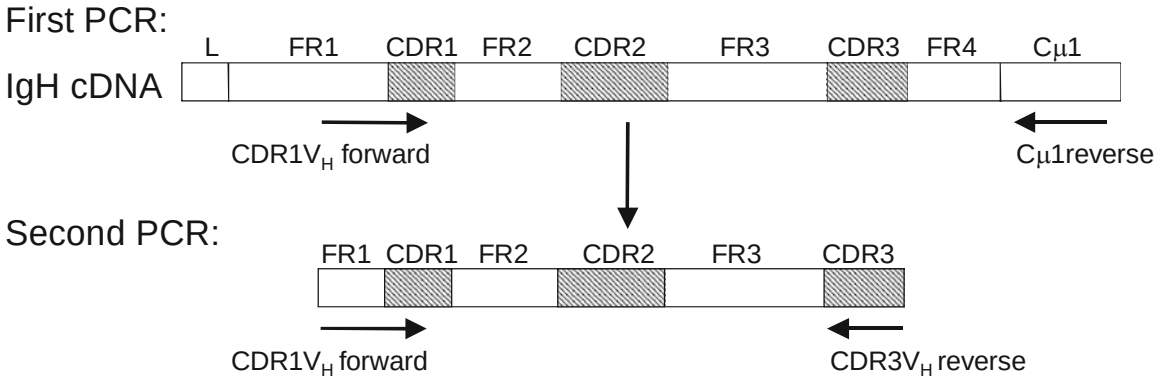

First PCR:
IgH cDNA
L
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
Cμ1
CDR1V$_H$ forward
Cμ1reverse
Second PCR:
FR1
CDR1
FR2
CDR2
FR3
CDR3
CDR1V$_H$ forward
CDR3V$_H$ reverse

Figure 3

Real time PCR:

Rearranged DNA

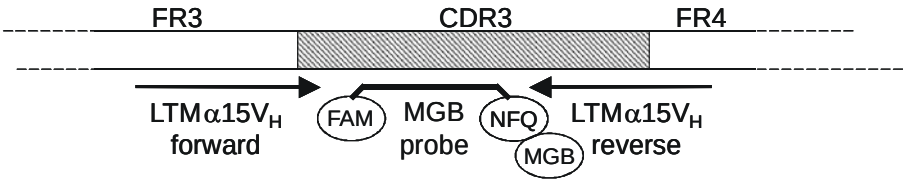


Figure 4

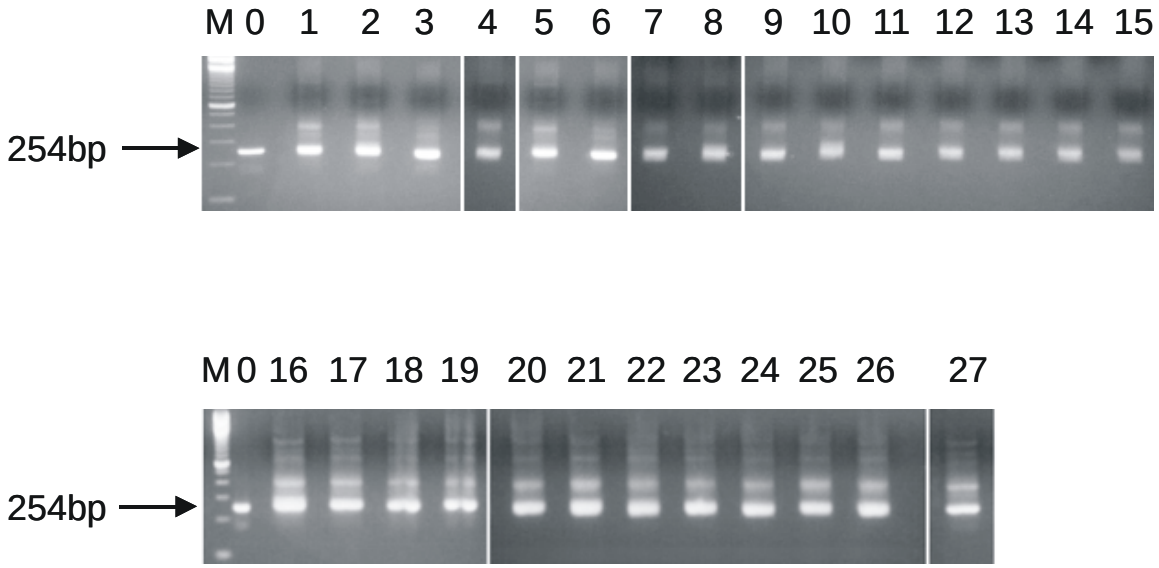

M 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
254bp
M 0 16 17 18 19 20 21 22 23 24 25 26 27
254bp

Figure 5

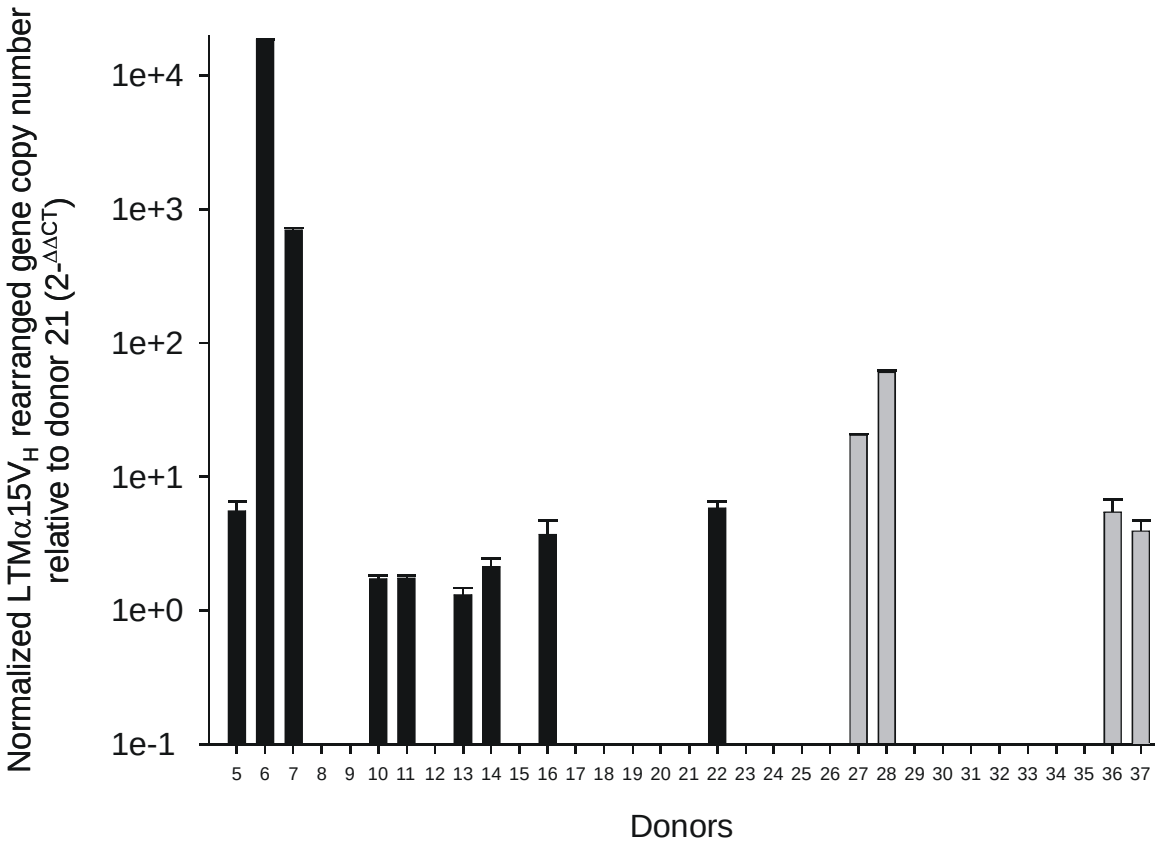
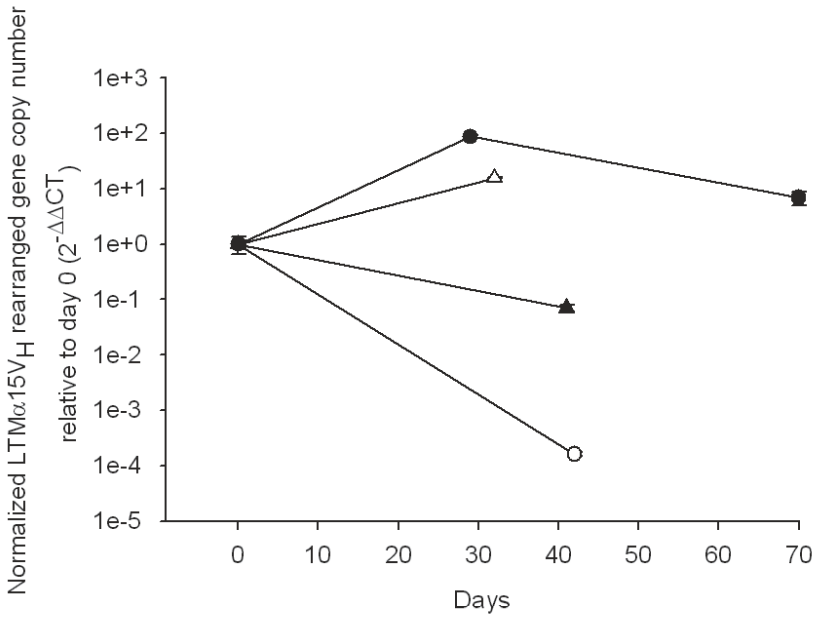

Normalized LTMα15V$_H$ rearranged gene copy number relative to donor 21 ($2^{-\Delta\Delta CT}$)
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
Donors

Figure 6


Normalized LTMα15V$_H$ rearranged gene copy number relative to day 0 ($2^{-\Delta\Delta CT}$)
1e+3
1e+2
1e+1
1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
0
10
20
30
40
50
60
70
Days

Dissertation Equivalent B

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A Highly Conserved Interspecies V_H in the Human Genome

Monique Vogel[1]*, Cornelia Tschopp[1], Tomasz Bobrzynski[1] Michaela Fux[1], Michael B. Stadler[1], Sylvia M. Miescher[1,2] and Beda M. Stadler[1]

[1]*Institute of Immunology, Sahli Haus 2, Inselspital, 3010 Bern Switzerland*

[2]*ZLB Behring, Wankdorfstrasse 10, 3022 Bern, Switzerland*

**Corresponding author*

Idiotype conservation between human and mouse antibodies has been observed in association with various infectious and autoimmune diseases. We have isolated a human anti-idiotypic antibody to a mouse monoclonal anti-IgE antibody (BSW17) suggesting a conserved interspecies idiotype associated with an anti-IgE response. To find the homologue of BSW17 in the human genome we applied the guided selection strategy. Combining V_H of BSW17 with a human V_L repertoire resulted in three light chains. The three V_L chains were then combined with a human V_H repertoire resulting in three clones specific for human IgE. Surprisingly, one clone, Hu41, had the same epitope specificity and functional *in vitro* activity as BSW17 and V_H complementarity-determining regions identical with that of BSW17. Real-time PCR analysis confirmed the presence of the Hu41 V_H sequence in the human genome. These data document the first example of the isolation of a human antibody where high sequence similarity to the original murine V_H sequence is associated with common antigen and epitope specificity.

Keywords: phage display; humanization; anti-IgE; idiotype conservation; human antibodies

Introduction

Idiotype conservation between mouse and human antibodies of the same antigen specificity has been reported in several antigen systems.[1–4] Eilat *et al.* showed that a major cross-reactive idiotype on mouse anti-DNA monoclonal antibody was also expressed on serum anti-DNA antibodies in systemic lupus erythematosus (SLE) sera.[5] Analysis of the human influenza-specific B cell repertoire has documented the presence of shared idiotypes expressed on murine and human influenza-specific antibodies.[6]

Such shared idiotypes are referred to as public, recurrent, dominant or cross-reactive idiotypes (CR1). The importance of CR1 in the pathogenesis of disease and in the idiotype anti-idiotype network interaction has been demonstrated in several systems.[7–10] In sera of patients with different forms of clinical paracoccidioidomycosis, anti-idiotypic antibodies were found that recognize both human and monoclonal murine antibodies specific to parasite glycoprotein gp43.[10] Analysis of the autoimmune response in thromobocytopenic patients has demonstrated the presence of anti-idiotypic antibodies produced against cross-reactive idiotypes shared by human and mouse anti-platelet antibodies.[9] These findings underlie the role of shared idiotypes in idiotypic modulation and may imply that specific antibody responses are associated with immunoglobulin variable-region genes that are highly conserved during evolution.[11–13]

Using a pool of non-immune human Fab phage display libraries we have previously described the isolation of two human anti-idiotypic antibodies that mimic epitopes located in the constant domain of the IgE molecule and are specific to a murine monoclonal anti-IgE antibody, termed BSW17.[14] Additionally these antibodies showed functional mimicry and were able to induce *in vivo* a BSW17-like anti-IgE response after rabbit immunization.

Abbreviations used: V, variable region; H, heavy chain; L, light chain; CDR, complementarity-determining region.

E-mail address of the corresponding author: monique.vogel@dkf6.unibe.ch

These findings suggested a conserved functional idiotype and a possible structural relationship between mouse and human associated with an anti-IgE response. Thus, we addressed of whether a human anti-IgE antibody displaying the same epitope specificity and idiotype sequence as BSW17 exists in the human repertoire.

We previously tried to isolate BSW17-like human anti-IgE from human antibody libraries using the phage technology. With this approach only two clones were isolated with an anti-isotypic specificity to IgE but not with the same epitope specificity as BSW17.[15] This might be due to the limited size of the libraries, which do not allow access to the whole anti-isotypic repertoire. Indeed previous results with recombinant antibody repertoires of non-immunized donors provided evidence for a correlation between the repertoire size and the presence of specific, high-affinity antibodies.[16,17] Thus antibody libraries have been constructed with high diversity and a size of up to 6.5×10^{10} clones from where high-affinity antibodies to many different target antigens have been selected.[17–19] However, *in vitro* selection procedures can be subject to bias especially in the case of complex antigens and it still remains difficult to isolate antibodies recognizing a particular epitope.[20,21]

During the last few years an alternative method termed "guided" selection has been developed for the generation of human antibodies. This technique combines chain shuffling and phage selection to derive the antibody with the designed specificities. The V_H or V_L of a parental murine antibody serve as a guiding structure for the selection of a human complementary V-region from a repertoire of cloned human V_L or V_H genes. Several studies have shown the feasibility of this method to generate human antibodies with different specificities.[22–32]

We describe here the humanization of the mouse monoclonal anti-IgE antibody, BSW17 by guided selection based on phage display. The V_H region of the BSW17 monoclonal antibody was first used to guide the selection of a human V_L from a V_L repertoire. In a consecutive step, three selected human V_L were used to select a human BSW17-like V_H region from a human V_H repertoire. This strategy allowed the isolation of a fully human anti-IgE antibody that retained the same binding specificity and *in vitro* functional activity as the murine monoclonal antibody BSW17. Remarkably, the selected V_H sequence shared 97% identity with the parental murine V_H. To our knowledge, this is the first time that a highly conserved human V_H sequence with the same functional specificity as the murine parental antibody has been isolated by guided selection.

Results

Light chain humanization

To obtain a human anti-IgE the strategy of guided selection, which combines chain shuffling and phage display (Figure 1), was followed using the V_H of the mouse monoclonal anti-IgE antibody, BSW17 as the guiding molecule. The V genes of BSW17 were first cloned as a Fab fragment into the phagemid vector pMVS and both V chains were sequenced (Figure 2). As the interaction of two matching human constant domains C_L and $C_{H\gamma 1}$ is preferred to stabilize the hybrid Fab, the mouse Fd was substituted by a chimeric Fd fragment composed of mouse V_H linked to the human first constant domain of the heavy chain which was then cloned into pMVS. The human light chain repertoire derived from four human combinatorial Fab libraries[14] was then cloned into pMVS containing the chimeric mouse/human Fd fragment yielding a phage library of about 2.3×10^7 different clones expressing hybrid Fab. To isolate human light chain analogs of BSW17 this

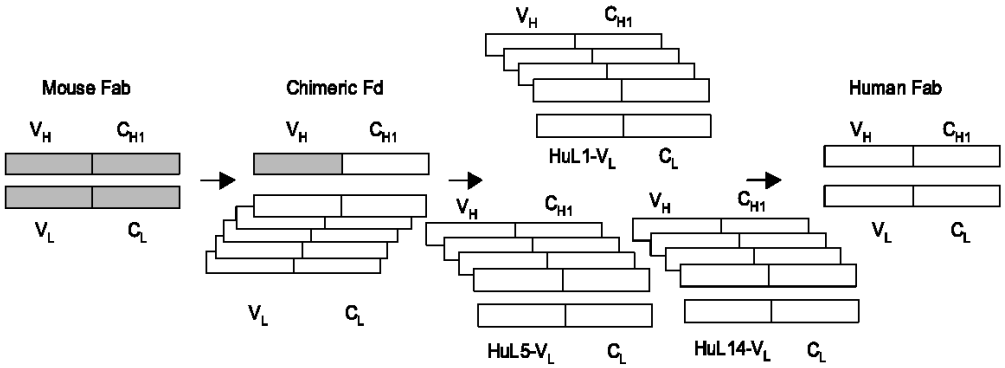

Figure 1. Schematic drawing of humanization of mouse monoclonal anti-IgE antibody, BSW17 using guided selection. Mouse sequences are indicated in gray, human sequences in white. In a first step a chimeric mouse/human Fd is used as guiding structure to select human light chains from a human light chain repertoire. In the second step three human light chain fragments were used as guiding structure for the selection of human V_H from a human V_H repertoire. The two sequential shuffling procedures were performed using phage display technology.

Light chain

Clones	FR1	CDR1 (L1)	FR2	CDR2 (L2)	FR3	CDR3 (L3)	FR4
BSW17-V_L	ELVMTQSPAIMSASPGEKVTMTC	SASSS.V..TFIH	WYRQKSGTSPKGWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISTMEAEDAATYYC	QHWSG..NPL.T	FGTGTKLEL.K
HuL1-V_L	--..----GTL-L----RA-LS-	R--Q-.-NRIYLA	--Q--L-QA-RLL--	GA-NR-A	-I-D---------DFT----RL-P--F-V---	LQYGS..S-IF-	--P---VDI.-
HuL5-V_L	---------TL-L----RA-LS-	R--Q-.-GS YLD	--Q--P-QA-RLL--	-A-NR-T	-I----------DFT----SL-P--F-V---	-QH-N.W-PW-.	--Q---V-I.-
HuL14-V_L	---V--P-SV.--A--Q---IS-	-G---NIGNNYVS	--Q-LP--A--LL--	-NN-RP-	-I-D-----K----AT-G-TGLQTG-E-D---	GT-D-SLSAGRV	--G----TVLG

Heavy chain

Clones	FR1	CDR1 (H1)	FR2	CDR2 (H2)	FR3	CDR3	FR4
BSW17-V_H	EVQLLESGGGFVKPGGSLKLSCVVSGFTFS	SYAMS	WVRQTPEKRLEWVA	SIS.SGNIIYYPDNVKG	RFTISRDNVRNILYLQMSSLRSEDTAMYYCTR	GRSTYGGFDH	WGQGTTLTVSS
Hu19-V_H	----------V-Q--R--R---AA------	T-G-H	----A-G-G-----	V-YD--SNK--A-S---	--------SE-TM----N---A----V---A-	..RGIY-M-V	------V----
Hu22-V_H	----------V-Q--R--R---AA------	N-G-H	----A-G-G-----	F--KDAYNKN-V-S---	--------SKKT-----N---A--------AK	EDRAS-AL-I	-----MV----
Hu41-V_H	------------------------------	-----	----------V---	---.----------	-------------------------V---A-	----------	--T---V----

Figure 2. Deduced amino acid sequence of selected human V_L and V_H genes. The sequences are segregated into CDR (complementarity-determining regions) and FR (framework regions) and aligned towards parental mouse BSW17 V_L and V_H fragments. Dashes indicate identical amino acids. Loop regions of V_L (L1, L2 and L3) and V_H (H1 and H2) are deduced using V Base.

Table 1. Classification and predicted canonical structures of the antigen binding loops of V-genes

Clones[a]	V-gene family[b]	Predicted canonical structures of antigen binding loops[b]			Human germline gene with closest protein sequence[c]	Amino acid sequence identity with murine BSW17 (%)
		Loop1[b]	Loop2[b]	Loop3[b]		
BSW17-V_L	V_κ7	1/10[d]	1/7	1/9	B1 +	100
HuL1-V_L	V_κ3	n.f./12	1/7	n.f./10	DPK22	65
HuL5-V_L	V_κ3	2/11	1/7	n.f./10	Vg/38K	69
HuL14-V_L	V_λ1	5/13	1/7	n.f.	DPL5	58
BSW17-V_H	V_H3	1/10	1/9	n.a.	DP58	100
Hu19-V_H	V_H3	1/10	3/10	n.a.	DP49	76
Hu22-V_H	V_H3	1/10	3/10	n.a.	DP49	75
Hu41-V_H	V_H3	1/10	1/9	n.a.	DP58	96

n.f., not found, canonical structure classes could not be assigned based on currently known classes; n.a., not applicable.

[a] Selected human V_Ls were assigned in combination with BSW17 V_H. Selected human V_H of Hu19 and Hu41 were assigned in combination with the V_L of HuL14 whilst selected human V_H of clone Hu22 with the V_L of HuL1.

[b] Canonical structures were assigned as described by Martin & Thornton.[61] Loop1, loop2 and loop3 correspond to the antigen binding loops of heavy and light chains.

[c] V-gene families and closest human germlines were deduced from V BASE (http://www.mrc-cpe.cam.ac.uk).

[d] The class numbers consist of the Chothia-equivalent structural class number followed by the number of amino acids in the antigen binding loops.

library was subjected to three rounds of panning on solid phase IgE. After the last round of panning, 15 clones were tested for IgE reactivity in the competitive immunodot assay where the binding of phage clones to IgE was inhibited by BSW17. Three clones demonstrating the same IgE epitope specificity as the monoclonal antibody (mAb) BSW17 were selected and sequence analyzed. Figure 2 shows their deduced amino acid sequences, which are aligned to the mouse BSW17 sequence. Databank screening indicated that they belonged to the same V_κIII family but to two different germlines, namely DPK22 and Vg/38K (Table 1). One clone, HuL14 carried a λ chain derived from the germline DPL5 of the V_λ1 family (Table 1). As shown in Table 1, the selected V_L have different canonical structures and amino acid lengths for L1 compared to that used by BSW17 V_L. No differences in canonical loop were observed for L2. In the case of L3, loop lengths could be determined in only two clones (HuL1 and HuL5) and were different from that used by BSW17. No known canonical structure for L3 could be found in any of the three human selected V_L sequences. As the three selected clones showed the same specificity to IgE as BSW17, the light chains of all three clones were used to guide the selection of the human heavy chain.

Heavy chain humanization

For the shuffling of the heavy chain, the selected human light chains were cloned separately into pMVS, which did not carry any V_H to avoid risk of contamination with the BSW17 V_H. Human Fd fragments were amplified by PCR amplifying from phagemid DNA of four human Fab combinatorial libraries.[14] These PCR products were pooled and cloned into the vectors containing the selected light chain sequences to generate three libraries of 2×10^7 clones each. The libraries were pooled and selected by four rounds of panning on immobilized IgE. After the fourth round, 50 phage clones were tested for binding to IgE in the competitive immunodot assay described above. One clone (Hu41) was found whose binding to IgE was completely inhibited by BSW17, suggesting that this clone recognized the same IgE epitope as BSW17. Two clones (Hu19 and 22) were also selected whose binding to IgE was only partially inhibited by BSW17. Sequence analysis of the three clones revealed two different human V_L (reselected from chimeric clones HuL1 and HuL14) and three different V_H sequences. The human V_λ1 reselected from chimeric clone HuL14 was found in two clones (Hu19 and Hu41), whereas the third clone (Hu22) carried the human V_κIII reselected from the chimeric clone HuL1. The V_H amino acid sequences of the three clones (Hu19, Hu22 and Hu41) and of the parental antibody BSW17 are aligned in Figure 2. Two clones (Hu19 and Hu22) had closely related V_H fragments that derived from the same germline DP49 of the V_H3 family. The third clone (Hu41) contained a V_H fragment that derived from the germline DP58 of the V_H3 family. Amino acid sequence alignment revealed that the V_H sequence of clone Hu41 was 97% identical with the parental murine V_H gene. As shown in Table 1, the H1 canonical lengths and structures for the three clones (Hu19, Hu22 and Hu41) were identical with that of BSW17 V_H, whereas only clone Hu41 used the same canonical class for H2 as BSW17 V_H. These results imply that a murine-like V_H sequence was selected from the human genome. However, as the selected V_H sequence differed in only three amino acid residues from the parental mouse V_H a potential reason for this selection would be a carry-over PCR contamination. To rule out this possibility, the presence of the V_H sequence from the clone Hu41 in the human genome was verified by PCR.

Identification of Hu41 V_H sequence in the human genome by PCR

The presence of the clone Hu41 V_H sequence in the human genome was monitored by conventional reverse transcriptase (RT)-PCR and real-time PCR. RT-PCR was performed using cDNA of 21 randomly chosen donors. For PCR amplification a pair of primers was designed that were specific to the complementarity-determining regions (CDR2 and CDR3) regions of the V_H fragment. A PCR band of the expected size was obtained for 17 donors (data not shown) and was sequenced. All the samples showed identical sequences corresponding to the same V_H fragment found in the selected Hu41 clone, suggesting that the selected BSW17-like V_H sequence is present in the human genome. However, this result might still be a subject of controversy due to the problems associated with conventional RT-PCR, namely specificity and carryover contamination.

To circumvent these problems and reduce the potential for false-positive results we chose the real-time PCR approach using the TaqMan technology. The TaqMan assay was performed on DNA of the same donors as used for the RT-PCR. DNA was preferred to RNA because of the reproducibility and sensitivity problems related to RT-PCR. To increase sequence specificity a 3′ minor groove binding (MGB) probe was used instead of a normal TaqMan probe. This probe prevented annealing with single nucleotide mismatches. To ensure that quantification of the V_H of Hu41 was not affected by differences in the quality and quantity of the DNA, the housekeeping gene, porphobilinogen deaminase (PBGD) was chosen as reference standard. Quantification analysis was performed using the comparative C_T method. This was possible because the efficiencies of amplification of Hu41 and PBGD genes were equal.[33] The amount of Hu41 V_H gene for each donor was given by $2^{-\Delta\Delta CT}$. As shown in Figure 3, $2^{-\Delta\Delta CT}$ values higher than 0 were obtained in all 21 samples tested, indicating that all donors contain the Hu41 V_H gene in their genome. However, these $2^{-\Delta\Delta CT}$ values vary considerably among the donors and show changes between 1.9 and 8051-fold, suggesting that the Hu41 V_H gene copy number differs greatly between the donors.

Affinity measurement and binding specificity of humanized BSW17

As clone Hu41 was the only clone showing the same IgE specificity as BSW17, it was produced as full-length IgG in eukaryotic cells for further characterization. The affinity of clone Hu41 for IgE was assessed by online monitoring of binding using the IAsys cuvette system. The affinity was 3.0×10^{-8} M, which is similar to the value of 6.0×10^{-8} M obtained for the parental murine antibody, BSW17.

In order to show that BSW17 recognizes an epitope on IgE, which is also responsible for the binding of IgE to the FcεRIα, we tested whether clone Hu41 can still bind to IgE bound to FcεRIα. Sequential binding of clone Hu41 to IgE and of the mouse monoclonal anti-IgE antibodies, BSW17 and Le27, was assessed by online monitoring using the IAsys system. As shown in Figure 4, a

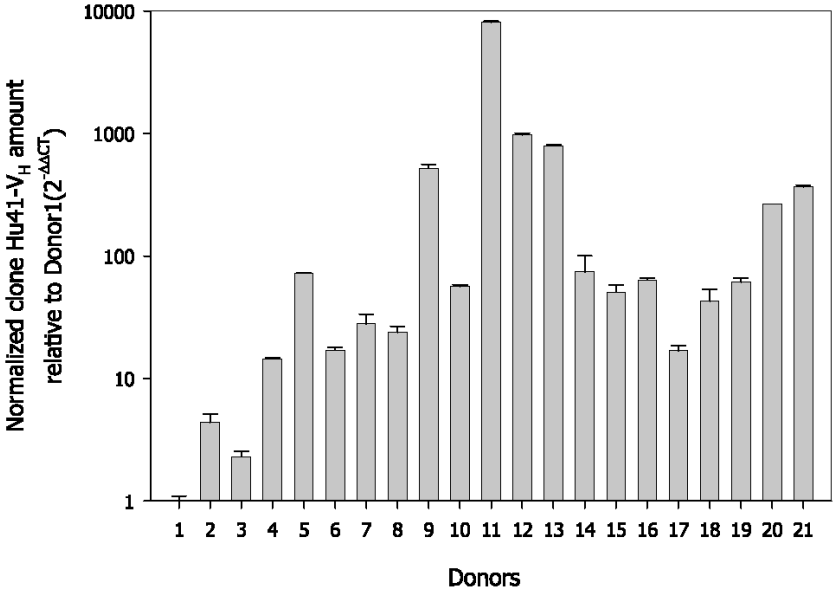

Figure 3. Relative quantification of Hu41 V_H gene using the comparative C_T method. DNA from peripheral blood mononuclear cells of 21 donors was used as template for real-time PCR reactions containing primers and probes either for Hu41V_H or for PBGD. The copy number of Hu41V_H gene normalized to PBGD and relative to the calibrator (Donor 1) was given by $2^{-\Delta\Delta CT}$.

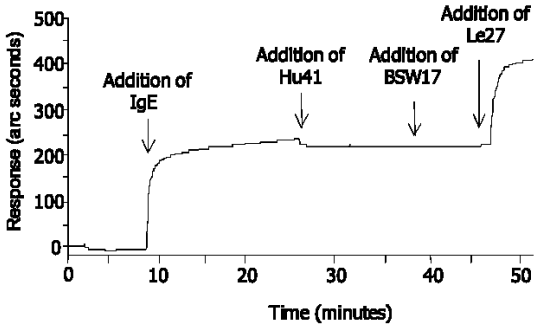

Figure 4. On-line monitoring of anti-IgE antibodies interacting with IgE bound to FcεRIα. IgE Sus11 (100 nmol) was incubated with immobilized FcεRIα for ten minutes. The volume was then replaced successively with 100 nmol of Hu41, BSW17 and Le27 and incubated for ten minutes. Finally, the cuvette was washed with 3×50 µl of PBS/Tween 0.05%. Arrows indicate the addition of antibodies. Antibody binding is displayed as arc seconds of angle correction with maximal laser reflection.

binding curve was obtained after addition of IgE, whereas no enhancement of the binding curve was observed after sequential addition of clone Hu41 and then BSW17. These results indicated that Hu41 behaved like BSW17 and that both antibodies recognized overlapping epitopes on IgE that are used for binding IgE to FcεRIα. In contrast, addition of the same amount of Le27 showed an enhancement of the binding curve, indicating that this antibody reacted with a different epitope on IgE that is still exposed in FcεRIα-bound IgE.

Non-induction of IgE-mediated mediator release from human basophils

Our previous data have shown that BSW17 is non-anaphylactogenic and does not induce mediator release from basophils.[34] Therefore, the non-anaphylactogenic properties of Hu41 were tested using freshly isolated peripheral blood lymphocytes enriched for basophils. Figure 5A shows the effect of different monoclonal anti-human IgE antibodies on histamine release after priming with IL-3. An anaphylactogenic anti-FcεRIα monoclonal antibody, 29C6, was used as a positive control for histamine release. However, stimulation with either Hu41 or BSW17 did not trigger the cells for histamine release, whereas an anaphylactogenic anti-human IgE antibody, Le27, triggered a strong histamine release response compared to the spontaneous release in the presence of IL-3 alone.

Similar results were obtained for sulfido-leukotriene (sLT) release. As shown in Figure 5B, no sLT release was observed from cells treated with Hu41 and BSW17. Again, stimulation of the cells with Le27 induced a marked enhancement of sLT production over spontaneous release.

Non-induction of IgE-mediated hexosaminidase release from human FcεRIα-transfected rat RBL-2H3 cells

We further tested the capacity of Hu41 to modulate hexosaminidase secretion from rat basophilic leukemia cells expressing human FcεRIα (RBL-2H3E5.D12.8 cells). To monitor cellular activation, we measured the extent of degranulation of the preformed mediator hexosaminidase from RBL-2H3E5.D12.8 cells that were stimulated with an optimal amount of an anaphylactogenic anti-FcεRIα mAb, 22E7. Under these conditions the release of hexosaminidase was in the range of 18% of the total cell cellular hexosaminidase (Figure 6, 22E7) with 5% spontaneous secretion (Figure 6, buffer control). We then tested the capacity of anti-human IgE antibodies to modulate degranulation of RBL-2H3E5.D12.8 cells preincubated for 90 minutes with saturating amounts of IgE Sus11. Figure 6 shows that addition of Hu41 and BSW17 did not produce an increase of release compared with the buffer control. In contrast, the anaphylactogenic anti-human IgE antibody Le27 triggered β-hexosaminidase release in the same range as 22E7.

Discussion

Antibody humanization has become a standard procedure to reduce immunogenicity of rodent monoclonal antibodies for clinical application in humans.[35] The humanization strategy termed guided selection was successfully used for obtaining human antibodies with the same functionality and antigen specificity as the parental mouse antibody.[22,25–29,36] In most of the cases the humanized antibodies showed rather low sequence similarity with the parental mouse antibody. Here, we report the cloning of a human anti-IgE antibody by guided selection that showed the same specificity but also carried a V_H sequence highly similar to the parental murine monoclonal anti-human IgE, BSW17.

In our guided selection and in contrast to other studies,[26,37] we did not retain the V_H and V_L CDR3 region of the parental murine antibody. The rationale for this strategy was to ensure full humanization of V_H and V_L sequences. Humanization of the light chain was performed by using the murine BSW17 V_H sequence as guiding structure to select human light chains from a light chain repertoire obtained from four different human Fab libraries. Three chimeric clones containing different kappa and lambda chains that had the same epitope specificity as BSW17 were isolated. Comparison of the sequences of the original murine V_L chain with the human V_L chains revealed differences in length and sequence of the CDR1 and relative low similarity in the CDR2 and CDR3. The kappa chains were not derived from the human germline closest to the mouse V_L chain. This corresponds to

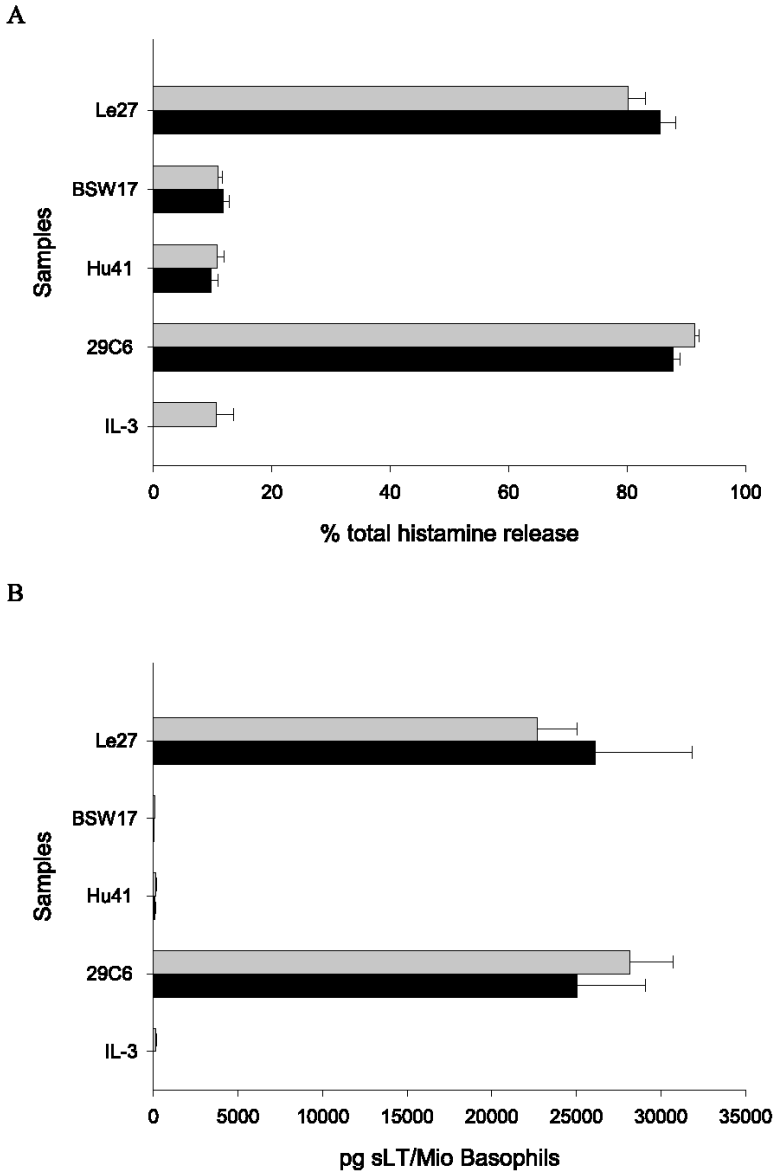

Figure 5. A, Non-induction of *in vitro* histamine release with Hu41. Freshly isolated and enriched basophils (3×10^5 cells containing 41% basophils) isolated from peripheral blood were primed with IL-3 (10 ng/ml) and then incubated with anti-human IgE antibodies (Hu41, BSW17 and Le27) at the concentrations of 10 μg/ml (black bars) and 0.1 μg/ml (gray bars). Monoclonal anti-FcεRIα antibody 29C6 was used as positive control. Results are expressed as mean percentage of total histamine cell content (TC) of triplicate cultures. B, Non-induction of *in vitro* sulfoleukotriene (sLT) release with Hu41. Basophils were isolated and treated as for the histamine release assay. Pg sLT represents the mean of triplicate assays of sLT release from 10^6 cells.

other examples of human V_L chain selection where the guided selection did not yield the most similar human counterpart sequence.[22,26,27,29,36] For the antigen binding site, more important than sequence homology are the canonical structures defined by Chothia *et al.*[38,39] Previous studies have shown the isolation of human antibodies by guided selection, which shared these structural elements with the parental mouse antibody. In our case the three light chains did not share similar L1 and L3 canonical structures with the mouse antibody BSW17. This observation suggests that the light chain can be more promiscuous and not only one chain plays a role for maintaining the antigen binding specificity.

To increase the probability of obtaining a fully human Fab in the V_H selection step the three V_L chains were used to guide the selection of a V_H

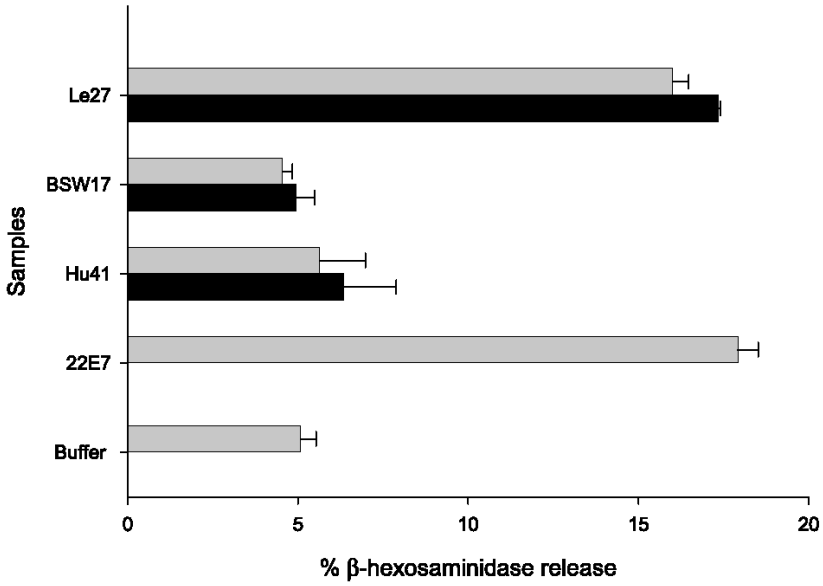

Figure 6. Non-induction of hexosaminidase release from RBL-2H3E5.D12.8 cells. Cells were pulsed with 50 μg/ml of IgE Sus11 and incubated with anti-IgE antibodies (Hu41, BSW17 and Le27) at a the concentrations of 10 μg/ml (black bars) and 1 μg/ml (gray bars). Anaphylactogenic monoclonal anti-FcεRIα antibody 22E7 was used as a positive control at 1 μg/ml. Spontaneous release was measured using a buffer (0.5% BSA in Hank's balanced salt solution) control. The results are shown as mean percentage of duplicate cultures of total hexosaminidase release. The total hexosaminidase release was calculated as the sum of spontaneous release and the content of hexosaminidase present in the lysate of unstimulated cells.

chain. Three clones were selected that paired with two different V_L chains. This differs from what was found by other groups, where only one particular light chain with the best binding characteristics was reselected.[24,26,28,36] This might be due to the fact that in our case the selected light chains did not differ in their IgE binding specificity. The heavy chains of two clones (Hu19 and 22) showed common features in the CDR1 and CDR2 and were derived from the same germline DP49 of the V_H3 family. Compared with the original murine V_H they showed minimal similarities especially in the sequences of the CDR2 and CDR3.

Surprisingly, one clone, Hu41, which demonstrated the same IgE binding specificity as BSW17, carried a V_H sequence sharing 97% identity with the original murine V_H sequence and differed in only three residues located in FR2 and FR3. For the heavy chain shuffling the selected light chains were recloned separately into a vector not carrying any V_H genes in order to prevent contamination with BSW17 V_H. Therefore the isolation of a V_H highly similar to the BSW17 V_H is highly unlikely to be due to a cross-contamination with the original or a mutated murine V_H sequence.

Interestingly Watzka *et al.*, who used a similar guided selection approach, described the isolation of a human Fab carrying V_L and V_H sequence with an overall similarity of about 90% and 80%, respectively, compared to the parental murine antibody.[23] However, this human Fab did not have the same antigen epitope specificity as the parental mouse antibody. Nevertheless this result is in line with our present data suggesting the presence of variable immunoglobulin sequences highly similar to mouse in the human genome. In fact comparison studies of human and mouse IgV_H have previously demonstrated strong similarity existing between some V_H families of these two mammalian species.[40] Our data indicate that Hu41 V_H was derived from the human germline gene DP58 of the V_HIII family. Interestingly there are reports showing that human and mice V_HIII genes have above 80% DNA sequence similarity, suggesting that they shared a common ancestor and have been conserved throughout mammalian evolution.[11,41] Additionally, pairwise comparison of human and mouse V_H allowed us and others to identify human/mouse V_H pairs demonstrating similarity above 80%, suggesting some form of environmental selection for the maintenance of specific antibody gene sequences.[13] Interestingly some of these highly similar V_H sequences have been identified in autoreactive antibody molecules of either human or mouse origin.[42] Thus, it has been reported that sera from patients with SLE contain antibodies that compete with the mouse monoclonal antibodies for binding on DNA, suggesting structural similarities between human and mouse anti-DNA antibodies.[3,4,43] In sera of patients with thrombocytopenia, anti-idiotypic antibodies were found against murine antibodies specific to a platelet glycoprotein, suggesting cross-reactive idiotypes shared by human and

mouse anti-platelet antibodies.[9] In a previous study we have isolated a human monoclonal anti-idiotypic antibody against the mouse anti-IgE antibody, BSW17, implying an interspecies idiotype conservation associated with the anti-IgE response.[14] Furthermore, shared idiotypic specificity was also observed in non-autoreactive immune responses. Analyses of human and murine influenza responses after immunization demonstrated the existence of interspecies idiotypes expressed on murine and human influenza-specific antibodies.[6]

Our data confirm and extend these findings by the isolation of a murine-like human V_H sequence in the human genome. By using RT-PCR, a transcript whose size and sequence correspond to the V_H of clone Hu41 was found in most of the tested donors. Additionally we have performed real-time quantitative PCR that provides a highly reliable method for rapid quantification of gene copies. This method eliminates the need for post sample processing and thereby prevents potential PCR carryover contamination. Until now most real-time PCR methods have been performed using sequence-specific fluorescent probes for genotyping, like TaqMan and molecular beacon probes. We used a novel fluorescent oligonucleotide that contains a 3′ minor binding group (MGB) which binds to the minor groove of DNA with higher affinity compared to ordinary DNA probes.[44] This method reduces non-specific probe hybridization and has been successfully used for single nucleotide polymorphism (SNP) analysis.[45] Real-time PCR analysis revealed the clone Hu41 V_H gene in all the donors tested, thus supporting the presence of a highly homologous murine sequence in the human genome.

Antigen specificity and non-anaphylactogenicity are critical parameters to be maintained during the humanization of BSW17, which binds to a discontinuous epitope on the IgE molecule that is involved in the binding of the IgE to α chain of the high-affinity receptor for IgE (FcεRIα).[46] By binding to this epitope BSW17 prevents the binding of IgE to FcεRIα and shows unique properties, as it is non-anaphylactogenic and thereby prevents the sensitization of the effector cells implicated in allergic diseases. Our data indicate that the humanized clone Hu41 binds to the same epitope as BSW17, as determined by competition studies and IAsys analysis. In addition, clone Hu41 demonstrated the same *in vitro* functional activity as the parental murine BSW17 antibody, namely it does not induce preformed and *de novo* synthesis mediator release from either human basophils or human FcεRIα transfected rat leukemia cells. Thus, the fact that the clone Hu41 retained the binding and non-anaphylactogenic properties of the murine BSW17 suggests that it could be used as a passive vaccine for immunotherapy against allergy. However, further *in vivo* experiments in animal models, e.g. in monkey, are required to investigate whether clone Hu41 will be a potent immunotherapeutic agent to prevent the sensitization of the effector cells implicated in allergic diseases.

In summary, we have been able to obtain a human full-length anti-IgE antibody, which demonstrates the same epitope specificity and functional activity as the original murine BSW17 antibody. Moreover this antibody carries a highly conserved interspecies V_H sequence. These results demonstrate structural similarities between mouse and human immunoglobulin V_H sequences and point out the usefulness of the guided selection approach for the isolation of human antibodies with the same functional specificity and common idiotypic sequence as murine antibodies.

Material and Methods

Vectors, bacterial strains and cell lines

The pMVS vector is a phagemid vector based on pComb3H which allows expression of Fab on the surface of filamentous phage M13.[47] The *Escherichia coli* strain XL-1 Blue and the VCSM13 helper phage were purchased from Stratacyte (La Jolla, CA). The mouse hybridoma cell line BSW17 was isolated in our laboratory from mice immunized with purified myeloma IgE.[46] The human embryonic kidney (HEK-293) cells were purchased from American Type Culture Collection (ATCC No. TIB202). The cell line RBL-2H3E5.D12.8 (RBL-2H3 transfected with human FcεRIα) was a kind gift from S. Mécheri, Institut Pasteur, Paris, France.

Antibodies and reagents

The following human IgE were used: IgE PS was kindly provided by Drs T. Ishizaka and K. Ishizaka (La Jolla, CA); IgE Savazal was kindly provided by Dr V. Savazal (Pilzen, Czech Republic). IgE JW8 is a mouse/human chimeric IgE, IgE ND is a myeloma IgE and IgE Sus11 is a monoclonal IgE.[48–50] BSW17 and Le27 are mouse anti-human IgE mAbs as described.[46] Murine anti-FcεRI mAb, 29C6, directed against the non-IgE binding epitope of the high-affinity IgE receptor α-chain[51] was a gift from Drs Hakimi and Chizzonite (Hoffman-La-Roche, Nutley, NJ). Recombinant human IL-3 was provided by Novartis (Basel, Switzerland). Recombinant FcεRIα, a fusion protein of human serum albumin as carrier, flanked by two extracellular parts of the α chain of the high-affinity IgE receptor was kindly provided by Dr H. Kocher (Novartis Pharma, Basel, Switzerland).

Humanization of the light chain

In order to use the heavy chain of the mouse monoclonal BSW17 as template for the humanization of the light chain, the V genes of BSW17 were cloned as Fab fragment into the vector pMVS[15,37,47] and sequenced at Microsynth GmbH (Balgach, Switzerland). To stabilize the hybrid Fab the mouse C_H1 region of the cloned BSW17 Fd fragment was substituted with human C_H1 and cloned into the pMVS vector as described.[28] The light chain coding sequences were obtained by restriction enzyme digestion from four human combinatorial Fab libraries[14] and cloned into the pMVS vector containing the chimeric mouse/human Fd fragment. The

ligation product was electrotransformed into *E. coli* strain XL1-Blue resulting in a light chain library and phages were produced as described.[37] Three rounds of panning against hybridoma IgE Sus11 carried out in polystyrene immunotubes (Becton, Dickinson) using 10 μg/ml of protein in 50 mM $NaHCO_3$ (pH 9.6) for coating, blocking and washing steps were performed as described.[37] Phages were competitively eluted with 100 μg/ml of BSW17. After the third round of panning single phage clones were produced and tested for binding to different human IgE in a competitive phage immunodot assay using BSW17 as competitor antibody and horseradish peroxidase (HPR)-conjugated rabbit anti-phage as developing antibody. Light chain coding genes of positive clones were analyzed by DNA sequencing. The sequences are available from the EMBL Genbank under the numbers AJ633625, AJ633626 and AJ633627 for HuL1, HuL5 and HuL14, respectively.

Humanization of the heavy chain

To prevent contamination with BSW17 Fd fragment the light chains of the three selected clones were recloned into the pMVS vector. The human Fd fragments were obtained from the above-mentioned libraries by PCR amplification using five upstream primers, which hybridize to V_H families,[28] and the downstream IgG1 primer, which hybridizes to the hinge region of the constant domain.[15] The PCR fragments encoding human Fd fragments were cloned into the pMVS vectors containing the selected human light chains, thus generating three libraries. The phage libraries were pooled and four rounds of panning on immobilized IgE were carried out as described for the light chain. After the fourth round of panning, phages were produced from single clones and were tested for binding to different IgE using competitive phage immunodot assay as described above. Light chain and Fd fragment coding sequences of positive clones were analyzed by DNA sequencing. The sequences are available from the EMBL Gen bank under the numbers AJ633622, AJ633623 and AJ633624 for Hu19, Hu22 and Hu41, respectively.

Full-length IgG from clones Hu19, 22 and 41 was produced in HEK-293 cells using an integrated vector system kindly provided Dr A. Bradbury (Trieste, Italy). Antibodies in the cell supernatant were purified on protein G-Sepharose columns (Pharmacia) and purity was controlled on an SDS/6% (w/v) acrylamide gel (data not shown).

RT-PCR amplification

Total RNA was extracted from peripheral blood mononuclear (PBM) cells of 21 unselected healthy donors and synthesized into cDNA using the Superscript reverse transcriptase system with oligo(dT) priming (Invitrogen, AG, Basel, Switzerland). Analysis of the presence of Hu41 V_H in the human genome was done with specific Hu41 V_H-CDR2 forward primer (5′-TAG TGG TAA TAT CAT CTA CTA TCC AGA CAA-3′) and Hu41 V_H-CDR3 reverse primer (5′-GTC AAA TCC CCC GTA GGT ACT-3′). Primers specific for the actin gene were used as control for testing the quality and quantity of RNA. PCR amplification was performed in 50 μl of PCR reaction mixture containing 6 μl of cDNA, tenfold PCR mix, 1 μl of each primer (20 pmol), 2.5 units of Platinium Pfx DNA polymerase (Invitrogen) and water. Amplifications were done at 94 °C for five minutes, 94 °C for 30 seconds, 51.8 °C for 50 seconds, 72 °C for 30 seconds for a total of 60 cycles followed by a final extension at 72 °C for ten minutes.

Primers and TaqMan probes

The primers and the TaqMan probes for real-time quantitative PCR were designed from appropriate sequences using the PrimerExpress software (Applied Biosystems). The primers and the TaqMan MGB probe for amplification of Hu41 V_H gene and the housekeeping gene porphobilinogen deaminase (PBGD) are Hu41V_H-for, 5′-GTG GTA ATA TCA TCT ACT ATC CAG ACA ATG-3′; Hu$V_H$rev 5′-CCG TAG GTA CTG CGG CCT C-3′; Hu41$V_H$pr 5′-TGT CAG GAA CAT CCT GTA CC-3′; PBGDfor 5′-TGA CCC ACA GTT GGT AGG CAT-3′; PBGDrev 5′-ATG CCC AAG TTC TGG GCA G-3′ and PBGDpr 5′-CGT AAC ATT CCA CGA GGG CCC CA-3′. The primers were synthesized by Microsynth (Balgach, Switzerland) and the TaqMan probes by Applied Biosystems (Rotkreuz, Switzerland). The Hu41V_H TaqMan MGB probe was labeled at the 5′ end with 6-carboxy fluorescein (FAM) and minor groove binder (MGB)/non-fluorescent quencher at the 3′ end. The housekeeping gene porphobilinogen deaminase (PBGD) TaqMan probe was labeled at the 5′ end with FAM and tetramethyl rhodamine (TAMRA) at the 3′ end.

Real-time quantitative PCR

DNA was extracted from the same donors as for RNA. Quantitative DNA PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). All reactions were done in triplicate and in 25 μl with 1.5 μg of DNA of the 21 donors, 12.5 μl of PCR mix, 900 nM each specific forward and reverse primer, 200 nM TaqMan probe. The amplification conditions for quantification were as follows: 50 °C for two minutes; 95 °C for ten minutes; 60 cycles at 95 °C for 15 seconds and 60 °C for one minute. The amplifications were performed on an ABI prism 7700 sequence detector equipped with 96-well thermal cycler. Data were collected and analyzed with Sequence Detector software (Applied Biosystems). To ensure that quantification of the V_H of Hu41 was not affected by differences in the quality and quantity of the DNA, the housekeeping gene PBGD was taken as reference standard. The average C_T values of PBGD gene in the various DNA samples differed by less than 1.1 (data not shown), implying that the efficiency of the amplifications of DNA per sample were comparable. Quantitative analysis of gene copy number was done using the comparative C_T method, in which C_T is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the comparative C_T method is described as the copy number of the Hu41 V_H gene normalized to PBGD copy number relative to a normalized calibrator and is given by the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T$ (sample)− ΔC_T (calibrator), and ΔC_T is the C_T of the Hu41 V_H subtracted from the C_T of PBGD. Donor 1 was chosen arbitrarily as calibrator sample. The standard deviation of the difference was calculated for the Hu41 V_H and PBGD values using the following formula:

S (std dev of difference) $= \sqrt{S1^2 + S2^2}$.

IAsys analysis

Affinity of full-length Hu41 IgG for IgE was determined using the IAsys cuvette system as described.[52] The subsequent binding of anti-IgE antibodies to IgE bound to FcεRIα was assayed by on-line monitoring using the IAsys cuvette system. The cuvette was immobilized with 10 μg of FcεRIα as described[52] and incubated with 100 nM IgE diluted in PBS/0.05% Tween 20. Each anti-IgE antibody was diluted in PBS/0.05% Tween 20 and added in 50 μl samples (100 nM) to the cuvette.

Mediator release from human basophils

Basophil-enriched peripheral blood leucocytes from a randomly selected healthy donor were prepared as described[53,54] by dextran sedimentation and Percoll gradient (Pharmacia). Cells were stimulated with 10 ng of IL-3 for 15 minutes, followed by the addition of different antibodies at the concentrations of 10 μg/ml and 0.1 μg/ml for 30 minutes at 37 °C. Triggering of the basophils was stopped by incubating the cells on ice for 20 minutes. Histamine release was measured in the cell supernatants using an automated fluorimetric method[55] and calculated as percentage of total histamine. Soluble sulfidoleukotriene (sLT) was quantified in an RIA assay.[56,57]

Hexosaminidase release from RBL-2H3-huα cells

The release of hexosaminidase from the cell line RBL-2H3E5.D12.8 was performed in a similar manner as described.[58–60] Briefly, the cells were distributed to 24-well tissue culture plates (5×10^4 cells in 0.5 ml of antibiotic-free complete Dulbecco's modified Eagle's medium (DMEM)/well). The medium was supplemented with 0.1 mM hydroxyurea to induce the expression of FcεRIα and the cells were cultured for two days. The medium was removed and cells were treated with 50 μg/ml of IgE Sus11 in prewarmed complete DMEM (100 μl/well) for 90 minutes at 37 °C. The cells were washed with Tyrode buffer (Sigma) and triggered with antibodies (at 1 μg/ml and 10 μg/ml, in 100 μl of Tyrode buffer) for 45 minutes at 37 °C. Secreted and intracellular hexosaminidase levels were measured as described.[58] The percentage of secreted hexosaminidase was calculated as the ratio of secreted to total available hexosaminidase × 100.

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F. CURRICULUM VITAE

Name	Michaela Fux
Date and place of birth	26 January 1977 Brig, Switzerland
Citizenship	Switzerland
Private address	Sonnenweg 30 3073 Gümligen b. Bern Switzerland Tel. ++41 31 951 79 33
Work address	Institute of Immunology University of Bern Inselspital, Sahli-Haus 2 CH-3010 Bern Switzerland Tel. ++41 31 632 13 07 Fax. ++41 31 381 57 35 E-mail: mfux@student.unibe.ch
Marital status	not married

EDUCATION

1992-1997	Gymnasium, Brig, Switzerland.
1997-2000	University of Berne, Switzerland, Major: Medical Microbiology
2000-2001	Licentiate in biology: " Generation of Recombinant Anti-Diphtheria Neutralising Antibodies." Institute of Immunology, University of Berne, Switzerland Supervisor: Prof. Beda M. Stadler
2001-2005	Doctorate in Immunology (PhD): "A New Molecular Approach for Monitoring Autoantibodies against FcεRIα in Healthy Donors and Urticaria Patients" Institute of Immunology, University of Berne, Switzerland Supervisor: Prof. Beda M. Stadler