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Abstract

IgE is a key mediator in allergic disease. Yet, many details of the composition of the human IgE repertoire are, in strong contrast to other antibody isotypes, poorly defined. The low levels of human IgE in circulation and the rarity of IgE-producing B cells are important reasons for this lack of knowledge. In this review we summarize current knowledge of these repertoires both in terms of their complexity and activity, knowledge that despite the difficulties encountered when studying the molecular details of human IgE has been acquired in recent years. We also take a look at likely future developments, for instance through improvements in sequencing technology and methodology that allows isolation of additional allergen-specific human antibodies mimicking IgE, as this certainly will support our understanding of human IgE in the context of human disease in years to come.

1. Introduction

In patients suffering from type I allergy, which represents a hypersensitivity reaction affecting >25% of the worldwide population [1], IgE antibodies specific for foreign proteins or glycoproteins are produced in the process of sensitization. At subsequent allergen contact, receptor (FcεRI)-bound IgE can be cross-linked on mast cells and basophils, a process that leads to the onset of allergic symptoms (reviewed by Larche et al. [2].

Despite its important role in disease, IgE has only been known as a molecule since the late 1960'ies. Due to its low concentration (i.e., ng/ml even in highly allergic individuals) in the most readily available clinical sample, blood, it has been difficult to discover and study. Indeed the IgE discovery process [3-5] and the development of first generation assays of specific IgE [6] was in part made possible through the availability of large quantities of a myeloma-derived IgE.

Allergic reactions are initiated through the specific interaction between allergens and IgE. Studies describing these interactions had for decades to rely largely on complex mixtures of allergens and IgE antibodies. Although such complex reagents are still in common use, research and clinical diagnosis related to allergy have substantially moved forward through the development of well-defined allergens. As methodologies to purify natural allergens were developed they provided access to more defined preparations of such proteins that bind to IgE. By the late 1980'ies a critical breakthrough occurred as the first genes encoding allergens were cloned [7-10] paving the way for the production of recombinant allergens in homogeneous form. Subsequently, numerous allergens have been cloned and characterized in quite substantial detail as summarized for instance by the Allergome, AllFam and IUIS allergen databases [11-13]. These efforts to clone genes encoding individual allergens have allowed the development of component-resolved diagnosis [14, 15], of tools able to achieve multiplexed analysis of IgE responses [16], and of defined vaccines (reviewed by Valenta et al. [17]). Therefore, the use of recombinant allergens will certainly improve the clinical management of allergic patients.

Our knowledge of IgE repertoires is however lagging behind our understanding of allergens. Monoclonal antibody technology and in particular modern tools in molecular engineering hold promise to reduce this gap by enhancing our knowledge of the IgE component. We live in an exciting era where such molecular tools have the potential to reveal several aspects of allergy research and modernise clinical practice. We will herein discuss the current status and future prospects of analysis of human IgE repertoires and how information on IgE and its reactivities can be used to support our understanding and management of allergic conditions.

2. IgE-encoding genes – the IgE transcriptome

Human antibodies, including IgE, are made up of heavy (H) and light (L) chains, of which the variable (V) domains of the H chains are believed to contribute much to their antigen-binding properties [18-20]. As monoclonal IgE are reagents that are hard to generate while gene sequences that encode the H chain (but not the L chain) V domains of IgE can be isolated more easily from transcripts derived from IgE-producing human B cells, much of the analyses of the detailed molecular nature of IgE have had to rely on studies of such gene sequences. These genes are found in a locus on human chromosome 14, a locus that is extensively described in the ImMunoGeneTics (IMGT) database (http://www.imgt.org) [21]. Briefly, three sets of genes, variable (V), diversity (D) and joining (J) genes are present in this locus. They had largely been mapped in an initial form already by the end of the 1990'ies [22-25]. A basic knowledge of many of the major genes that shape the antibody repertoire has thus been available for quite some time. Yet, as outlined in sections 2.2-2.3, there is still substantial controversy with respect to the genetic composition and processes involved in the generation of human IgE responses.

The functional V genes encode most of the H chain V domain. This set of genes, 39-55 of which are available in a single individual's genome [26], is divided into 7 gene subgroups based on sequence similarity. Among these subgroups, subgroup 3 is the largest subgroup (≥19 different genes in any given individual [27]). It is also the most frequently used H chain V gene subgroup as it is used by about one half of all rearrangements [27]. Interindividual differences in the available repertoire (in terms of V, D and J gene makeup) exist [26] and may affect antibody responses but the biological

significance of such differences in general [28] or in relation to allergic disease is not well established.

Key diversity of the H chain of antibodies derives from the rearrangement that occurs in pre-B lymphocytes that brings one V, (usually) one D and one J gene together to form a functional H chain V domain-encoding gene. The precise site of joining of these genes differs between different rearrangements and the incorporation of non-template-encoded nucleotides between the fused genes further adds to the diversity. This particular process results in the creation of sequences that encode a loop, the third complementarity determining region (CDR) of the H chain (CDRH3), that due to the diversity of these processes varies greatly both in terms of length and sequence between antibodies produced by different sets of B cells. This particular loop is very important for the specificity of many antibodies [19, 29]. Further diversity is introduced into the antibody repertoire through combination of such initially created H chain diversity with diverse L chain V domain sequences. Apart from these initial rearrangement processes, antibody-encoding genes may be diversified by somatic hypermutation following antigen encounter in a process believed to select for antibodies with improved recognition of the antigen.

Several analyses of IgE-encoding transcriptomes have been performed over the years, many of which are summarized in Supplementary Table 1. It is the H chain's general key importance for antigen binding [18, 19] that lends credibility to this approach. These studies mostly rely on relatively low throughput sequencing of randomly picked transcripts encoding the H chain V domain of IgE found in biological specimens following amplification of IgE-encoding sequences by PCR. Technological aspects (as further outlined in section 2.2) or the inclusion of very few subjects and/or very few IgE-encoding sequences in several of these studies may explain some of the different conclusions made in these studies of IgE repertoires. Nevertheless, there are some main conclusions and aspects that deserve particular attention as discussed in the following sections.

2.1. IgE repertoires are oligoclonal.

Several studies report that a quite limited set of IgE H chain V domain-encoding sequences are present in a sample. Similar sequences, evolving from the same rearrangement as defined by an origin in one specific V gene, an identical length and highly homologous sequence of the CDRH3, and shared mutations are frequently found in individual samples (see for instance Supplementary Figure 1). As these sequences frequently carry different mutations, they likely developed through a hypermutation process from a single progenitor B cell. In conclusion, a single sample (tissue or peripheral blood sample) at a given point in time contains IgE-producing B cells with an origin in only a few heavy chain rearrangements, but often multiple differently mutated variants of several of these clones.

Many studies of IgE-encoding transcriptomes have been carried out using samples derived from peripheral blood. This may not be the most likely long-term survival niche of IgE producing cells. Indeed, antigen-specific cells of the B cell lineage tend to circulate only during a very limited window after antigen challenge [30, 31] suggesting that the snapshot taken at a given point in time may be restricted to clones involved in responses to very recently encountered allergens. Given this fact and the likelihood that IgE responses anyway are mostly directed towards a limited set of allergens it is not unexpected that the diversity found in circulating IgE-producing B cells is limited. This finding does not really set the IgE response apart from other isotype-switched responses. Indeed, polyclonal IgG repertoires specific for a single target are also oligoclonal and diversified by mutation [32, 33]. The extent by which the clones encoding IgE isolated from peripheral blood are truly representative of the subjects' total repertoire is a matter of controversy. Future studies of other tissues, such as bone marrow and mucosal tissue involved in local IgE production (see section 2.4), and of IgE-producing cell types harbouring fewer transcripts than e.g. plasma cells and plasmablasts that are favoured in common transcriptome-based analysis will shed further light on this aspect of diversity of polyclonal IgE.

2.2. Are certain H chain variable domain genes or gene subgroups overrepresented in the IgE repertoire?

The binding domains of antibodies are as outlined above created by rearrangements of V, D and J genes. The nature of these processes and subsequent phases that determine the survival and death of B cells result in repertoires of antibodies in which certain genes and gene subgroups are more common than others. For instance, human antibody repertoires in general are dominated by antibodies encoded by the largest gene subgroup, IGHV3, while antibodies with an origin in IGHV2, IGHV6 and IGHV7 are rarely found. However, several studies that reported on the IgE repertoires have an origin in rearrangements that preferentially uses certain IGHV subgroups. Snow et al. [34-35] and Coker et al. [36] suggested that IgE repertoires in nasal biopsies are encoded by B cells that more frequently than expected employ rearrangements involving IGHV5 germline genes. Similarly, Edwards et al. [37] and Janezic et al. [38] noted in small studies an overrepresentation of IGHV6 and IGHV1 subgroups among transcripts encoding IgE, respectively. Many other studies (summarized in Supplementary Table 1) in contrast note a rather random usage of IGHV gene subgroups and a diverse origin of individual genes that encode the IgE repertoire.

It has been suggested that IGHV gene origin-restricted repertoires of IgE are the result of responses induced by superantigen activities that preferentially target and expand B cells carrying antibodies derived from particular genes or subgroups of genes [34, 36] rather than conventional antigen-specific antibody responses. Such notions have found support from findings demonstrating a lack of evidence for selection by antigen in IgE (further discussed in section 2.3). Others have however argued that technical artefacts in extensive PCR amplification procedures involving complex primer mixtures may cause the observed skewness of IgE-encoding repertoires [39]. We envisage that improvements in procedures, for instance the development and use of high throughput sequencing technology that can address 1. larger sets of IgE-encoding sequences at great depth, with 2. less dependence on bias-prone amplification processes, from 3. larger cohorts of subjects with different IgE-mediated conditions eventually will resolve the matter if restricted IgE repertoires exist and if superantigens are involved in the shaping of (at least some) IgE repertoires.

2.3. IgE H chain V domains are often mutated.

Several studies as outlined above report that IgE-encoding transcripts found in allergic subjects show evidence of a somatic hypermutation process (Supplementary Table 1). There are numerous point mutations that differentiate these sequences from the closest germline IGHV gene. In contrast, IgE-encoding transcripts derived from non-allergic subjects show less evidence of hypermutation [40]. However, parasitized but non-allergic subjects produce IgE from somatically hypermutated genes [39]. Similarly, allergen-specific IgE selected from phage-displayed libraries are extensively mutated (mutation frequency of genes encoding the H-chain of such IgE (Table 1) was determined to be 5.4 ± 3.3 %; n= 35) indicating that they have undergone an extensive hypermutation process. Two different pathways have been proposed for the development of IgE, one involving a direct switch from IgM to IgE production and the other involving a sequential switch from IgM via IgG (or conceivably IgA$_1$) to IgE. The direct switch that is suggested to be most relevant for atopic disease, has been proposed to be accompanied by the presence of fewer mutations in resulting antibodies and not so much by an oligoclonal response (for review, see Davies et al. [41]). The high level of mutation of several IgE repertoires [42] and selected allergen-specific antibody fragments described briefly above might speak against a dominance of the direct switching pathway at least in the conditions that have been studied most extensively. However, the limited set of data certainly suggests that further investigations on this matter are needed.

The interpretation of the role of mutations in human IgE is a matter of controversy. Some studies indicate that the mutation pattern of IgE-encoding genes show evidence of an antigen selection process [43, 44]. Other studies suggest that there is little evidence of antigenic selection in IgE repertoires of allergic [40, 45] and non-allergic but parasitized subjects [39]. Some studies even indicate, as outlined above, that mutations accumulate at patches of the V domain in a manner that indicates an involvement of superantigens in the shaping of the IgE repertoire [34, 36]. Studies of antigen selection in antibody responses are however complicated by extensive methodological problems [46]. It has indeed been proven difficult to analyze if immunoglobulin sequences (including IgE) have undergone positive selection or not, by using sequence data analyzed by extensively controlled mathematical methods alone [42]. We believe that it will not be possible to resolve this matter until methodology has been developed to

retrieve multiple human monoclonal IgE in a form identical to those that occur in vivo in similarity to studies of affinity maturation of human IgG [33]. Even if such antibodies are available, the problem to unequivocally demonstrate affinity maturation in a context that involves antigenic stimulation with complex mixtures of allergens is very extensive. Unless methodological advances to analyse the imprint of selection in antibody-encoding sequences occur, we expect it to be a truly challenging endeavour to prove beyond doubt whether or not selection by antigen shapes the repertoire of IgE as it develops in humans through natural exposure to allergens.

2.4. IgE may be produced locally.

Antibody responses are generally believed to be generated and selected in secondary lymphoid organs. However, several lines of evidence suggest that IgE-producing B cells can be generated and differentiated at other sites. Mucosal tissue has been implicated as a site where IgE class-switch, receptor revision and somatic hypermutation may occur [47-51] and IgE-producing plasma cells have been identified at the site of allergic inflammation in nasal mucosa [52]. Conditions where the patients show no evidence of peripheral IgE nevertheless may show local responses indicative of IgE-mediated disease and IgE-encoding transcripts have been isolated at such sites [53, 54]. These factors complicate analysis of the role of IgE in disease as locally produced IgE, which is not measured by standard tests, may load IgE receptors on effector cells present in the affected organ [55]. Importantly, several of the studies referred to in Supplementary Table 1 have indeed attempted to assess IgE-encoding transcriptomes found in tissue likely to be directly involved in allergic reactions. Further analyses of such transcript populations as well as transcripts found at other sites such as bone marrow are needed to increase understanding of differences and similarities of IgE-encoding B cell (or plasma cell) populations at different sites.

3. Monoclonal human IgE

The development of monoclonal antibody technology certainly was a gigantic step that revolutionized many aspects of biomedical research. Hybridoma technology has indeed been used to develop monoclonal IgE-producing cell lines mostly from malignant human B cells without known allergen specificity. Technological developments since the turn of the millennium, including the introduction of 1. novel fusion partners for use in effective cell-cell fusion to raise human hybridomas [56], and improved procedures to 2. immortalize human B cells through Epstein Barr Virus infection [57], 3. sorting and subsequently cloning of antibody-encoding genes from single antigen-specific B cells [32, 58], and 4. retrovirus-mediated *Bcl-6* and *Bcl-xL* gene transfer into, and culture of B lymphocytes in the presence of CD40L and IL-21 [59] have certainly improved our ability to study human antibody repertoires as they occur in vivo. Nevertheless, so far they have found little demonstrated application in studies of allergen-specific IgE although comments made in the literature [60] have suggested that efforts are underway to implement such technologies for this purpose. The rarity of IgE-producing cells of the B cell lineage (even in atopic subjects <1% of peripheral blood CD138+ plasma cells produce IgE [61, 62]) is a major hurdle in these efforts. On the other hand the likely oligoclonal IgE response to a limited set of well-defined allergens is a fact that favours efforts of scientists to analyse the IgE repertoire. Interestingly, Epstein Barr virus infection of B cells [63], implementation of retrovirus-mediated gene transfer into, and defined culture conditions of B lymphocytes [64] as well as cell sorting approaches [64-66] have been used to isolate genes encoding allergen-specific antibodies of isotypes other than IgE (as further discussed in section 3.3). These findings demonstrate the potential of such cell manipulation technologies for research on human allergen-specific IgG antibody responses and raise hope to use these techniques also for IgE.

3.1. Combinatorial antibody libraries and phage display technology – basic considerations

As it has been proven difficult so far to use conventional B cell immortalization technology to isolate IgE-producing cell lines or genes, our current knowledge of allergen-specific human IgE relies on the isolation of genes encoding allergen-specific

antibody fragments from combinatorial libraries [67] using filamentous phage display technology [68]. These technologies (its process is summarized in Supplementary Table 2) have been developed and implemented during the past two decades for the isolation of antigen-specific antibodies for a range of targets with great efficiency. Although often used to isolate entirely novel antibodies with no relation to an in vivo antibody response the technology has certainly also proven its performance (first demonstrated in 1991 [69]) in terms of assessment of antibody responses as they develop in a range of clinical conditions such as autoimmunity, xenoreactivity and responses against a vast range of infectious agents. Although the technology suffers from some technological difficulties (some of which are outlined in Supplementary Table 3) its proven robustness, high performance and adaptability to high throughput makes it an attractive choice for the study of human allergen-specific IgE responses. Importantly, recent technological developments, for instance in high-throughput sequencing [70], offer new opportunities to identify specific binders by using sequencing and library technologies in combination [71]. Such combined processes appear to reduce problematic losses of specific binders that are present in libraries but that are nevertheless not efficiently selected by a phage-display procedure alone [72, 73].

Phage display technology was first successfully implemented for the studies of human IgE in 1996 when Steinberger et al. [74] demonstrated isolation of allergen-specific antibody fragments from a library created from genes encoding the IgE Fd regions in combination with genes encoding L chains. All future efforts have essentially followed this plan although with certain modifications, such as the implementation of libraries encoding antibody fragments in the single chain fragment variable (scFv) format and in one case by use of synthetic instead of B-cell derived L chain diversity [75]. Antibody libraries have been created from a diverse range of lymphocyte sources and used to identify allergen-specific binders specific for e.g. grass and tree pollen, mite, milk and latex allergens from IgE repertoires (Table 1). Combinatorial libraries developed from the human IgE-encoding transcriptome suffer similar to other such libraries (Supplementary Table 3) from shortcomings like the in vitro recombination of H and L chain sequences. Nevertheless, identified binders are in our view much more closely related to IgE as found in vivo than allergen-specific antibodies generated by the other approaches (see section 3.3). The present report thus focuses mainly on specific binders

retrieved from the IgE-transcriptome itself, as we see them as better representatives of in vivo-derived human IgE.

3.2 Combinatorial antibody libraries and phage display technology – allergen-specific IgE

More than 15 years have passed since the first application of phage display technology to isolate allergen-specific IgE-derived binders from combinatorial libraries based on the diversity of H chains of IgE-producing B cell. However, only few allergen-specific IgE binders have been selected to date (Table 1). Nevertheless, an inspection of selected binders so far identifies a few points of particular interest. Firstly, the collection of allergen-specific IgE is highly diverse and these proteins originate from rearrangements involving a range of V genes and V gene subgroups. All immunoglobulin H chain V gene subgroups except two very minor subgroups (IGHV6 and IGHV7) are used even in this relatively small set of specific antibodies. Several clones from IGHV6 have been isolated but they are highly multireactive and able to bind to a range of unrelated target molecules [37]. Antibodies with such spurious, poly-reactive binding properties are not included in this description. The allergen-specific IgE repertoire is dominated by the IGHV3 subgroup, the dominant subgroup of human immunoglobulins in general [27]. Several different genes of this subgroup (such as IGHV3-07, 3-09, 3-11, 3-21, 3-23, 3-30, 3-30-3 3-43, 3-53) have been identified as being represented in this collection of IgE. The repertoire is thus highly diverse in terms of its genetic origin. The CDRH3 sequences, a key specificity-determining feature of antibodies [19, 29], of selected allergen-specific IgE have an average length (15±4.5 residues; n=39) very close to that of other human antibodies [92-94]. Average allergen-specific IgE responses thus appear to be quite diverse and in terms of overall genetic origin similar to antibody responses of other isotypes.

Antibody-antigen interactions may vary dramatically in terms of affinity, for instance as illustrated by the interaction of IgG human antibodies with tetanus toxoid following active immunization [33]. The procedures whereby allergen-specific IgE antibodies are selected from combinatorial libraries using phage display technology may certainly affect the range of observed affinities (Supplementary Table 3) and it should not be

anticipated that affinities of clones by this technology truly mimic those of IgE found in vivo. Importantly though it has been possible to isolate IgE with a large range of affinities, even above 10^9 M^{-1} (see for instance Christensen et al. [87], Gadermaier et al. [20], Jylhä et al. [90], and Levin et al. [78]). The availability of reagents with such different behaviours is important both for technical development, for instance in assay standardization, but also for the elucidation of biological outcomes of IgE-allergen interactions, as further discussed in section 6.

The details of human IgE recognition of most allergens are not well established, but with access to human recombinant IgE it has been possible to assess some of these interactions. Indeed, individual allergens just as other antigens may be targeted by quite different populations of antibodies. For instance, the IgE repertoire targeting Phl p 5 (Table 1) includes a diverse variety of antibodies binding to several different epitopes present on the allergen's N- and C-terminal domains (Levin et al., manuscript in preparation). Similarly, the major grass pollen allergen Phl p 1 is recognized by antibodies of diverse genetic origin but with shorter than average CDRH3 sequences [78] and the epitopes appear to be clustered in proximity to each other on its C-terminal domain (Table 1) [77, 78]. In contrast, the IgE response to the group 2 grass pollen allergen appears to be dominated by a very restricted set of antibodies [79] targeting a limited number of epitopes (Table 1) [80]. These findings illustrate the diversity of modes whereby antibodies recognize different allergens, even as these originate from the same allergen source.

Due to the importance of IgE in a major set of diseases and our relative ignorance of how IgE contributes to the allergic reaction there is an unmet need to establish reagents that can be used to analyze its mode of action. Initial studies have indeed uncovered the potential of such reagents and how they can be used for instance to define in detail the parameters that govern the role of IgE in allergic reactions (see section 6). We envisage that with access to much larger cohorts of antibodies that define the human IgEome we will be in a position to define how these antibodies and their interaction with a plethora of allergens cause allergic disease. We also envisage that human IgE may serve additional roles in research and in the development of appropriate diagnostic single- and multiplex assay procedures and of new tools such as novel hypoallergens, as described by Levin et al. [78]. Human monoclonal IgE with an origin in allergic patients'

IgE repertoires represent a renewable, well-defined resource of reagents that are likely to perform well for such purposes.

3.3 Monoclonal IgE from animals or allergen-specific antibodies derived from other human isotypes

Apart from human IgE, there are other sources of antibodies that target antigens also targeted by human IgE. Such antibodies range from straightforward polyclonal antisera to sophisticated recombinant antibodies.

The ground-breaking work of Köhler and Milstein and their colleagues in the 1970s [95] who developed the first successful technology for the production of monoclonal antibodies by fusing antibody-secreting plasma cells with immortalized neoplastic plasma cell lines provided the first possibility to create monoclonal antibodies of known specificity. Such antibodies offer distinct advantages over polyclonal antisera, such as being a single defined entity and an essentially unlimited resource. The first monoclonal IgE antibodies of mouse origin were subsequently generated by hybridoma technology [96-98]. These developments paved the way for more detailed studies of the effects caused by IgE-allergen interactions.

The introduction of recombinant antibody technologies in the 1980s and the increased understanding of antibody structure and function suddenly provided new tools to produce monoclonal antibodies for the use in studies of processes involved in allergy. Improved gene cloning procedures, and in particular the implementation of PCR for amplification of diverse genes encoding antibody V domains [99] now facilitated this process. For instance, antibody library and display technology have been used to isolate murine allergen-specific IgE-derived Fab clones from libraries created from IgE-producing mice [100]. Genes isolated from such libraries or from hybridomas may be cloned into plasmid vectors for production of intact glycosylated immunoglobulins in a variety of mammalian cell lines. Cutting and pasting of the recombinant genes enabled the replacement of V region genes to any constant gene of desire and the first recombinant murine IgE was produced by Gritzmacher and Liu in 1987 [101]. Recombinant approaches further enabled a transition from exclusively mouse-derived monoclonal antibodies to chimeric molecules consisting of V regions from mouse with

constant regions of human isotypes [102, 103]. Already in 1985, Neuberger et al. described a human monoclonal IgE of known specificity (4-hydroxy-3-nitro-phenacetyl) with preserved human effector functions. Its H chain was a combination of a mouse V region and a human epsilon constant region, but the L chain still remained fully of mouse origin [104]. In an analogous way a mouse/human chimeric IgE specific for the major house dust mite allergen Der p 2 was constructed by Schuurman et al. [105]. Subsequent chimerization efforts produced IgE carrying both H as well as the L chains with human constant domains [106, 107]. Altogether, several different routes can be used to exploit murine antibody specificities in the context of human IgE functionality for a variety of purposes. Several of these murine antibodies have been used together with one set of clonally related human IgE in ground-breaking studies investigating factors affecting effector cell degranulation through IgE-allergen interaction [88] as outlined in section 6.

However, murine IgE V regions may recognize epitopes that differ from those bound by human IgE. This has been seen in a number of immune responses and lately also in antibodies specific for the grass pollen allergen Phl p 1 [78]. Particular care must thus be taken when mouse antibodies are used as models for human IgE-allergen interactions.

As another alternative to human IgE, human allergen-specific antibodies of origins other than IgE have been developed. For instance, monoclonal IgE antibodies specific for the major birch pollen allergen Bet v 1 were selected from human antibody libraries created from human B cells that had been induced to undergo isotype switching to IgE in vitro (i.e. from B cells that produced other isotypes in vivo) prior to the isolation of mRNA for library construction [108]. Furthermore, Visco et al. [63] stimulated human B cells obtained from desensitized subjects to isolate Bet v 1-specific human IgG-producing clones. Very recently, van de Ween et al., [64], James et al. [65] and Patil et al. [66] succeeded in isolating B cells producing antibodies of isotypes other than IgE and with specificity for a bee venom allergen, a grass-pollen allergen and a peanut allergen, respectively, by the use of technologies involving cell sorting approaches. If these approaches prove able to isolate large numbers of allergen-specific binders at least from the more abundant IgG repertoire it will certainly add tremendously to our understanding of human allergen-specific immune responses, for instance in relation to the development of protective antibodies following specific immunotherapy of allergy.

Finally, synthetic human antibody libraries (methodologies to develop such repertoires were recently reviewed by Geyer et al. [109]) with synthetic sequence elements incorporated in vitro as sources of antibody diversity have been used to isolate allergen-specific antibodies. De Lalla et al. [110] used such technology to isolate grass pollen-specific synthetic antibody fragments. More recently, Braren et al. [111] exploited the synthetic library Griffin-1 to isolate scFv specific for the allergens Api m 1 from honey bee venom, Bos d 5 from bovine milk and Mal d 1 from apple and these binders were further converted into intact human IgE.

Altogether, the origin of many of these antibodies available today suggests that they may not precisely mimic the properties of patients' IgE and IgE repertoires. Despite the shortcomings of binders selected by methodologies like phage display from combinatorial IgE libraries, such antibodies offer the best opportunity to view human IgE-allergen interactions as they occur in allergic disease.

4. IgE-allergen interactions – a closer look into the interface

Two parts build up the contact area between IgE and its specific allergen. There is the antibody's paratope on the one hand facing the B cell epitope on the surface of allergen on the other hand. Whereas the paratope results from the quaternary structure of paired H and L chains with their CDR loops in the V regions making the allergen contacts, the corresponding epitope is composed of surface areas on the allergen formed by stretches of amino acids in sequential or conformational order in which the latter depend on the three-dimensional structure of the allergen [112].

Focussing on the epitope, several strategies have been developed to identify IgE binding residues but most of these techniques just allow their rough localization. IgE-binding regions may be determined directly by screening allergen-derived synthetic peptides or recombinant fragments with monoclonal IgE antibodies [77, 80]. The injection of these derivatives into rodents leads to the production of specific antibodies which can be alternatively used to locate the epitope by applying them for the competition with the IgE of interest for allergen binding [113]. Further, peptides that represent structural mimics of antigenic determinants but have no sequence relation with the parental

allergen may be used to identify epitopes [114, 115]. These mimotopes can be isolated by screening random peptide libraries displayed on the surface of bacteriophages [116, 117] or synthetic peptide libraries [118] with the IgE of interest and appropriate algorithms (e.g. EpiSearch [119]) help to match the mimotopes on the surface of the three-dimensional allergen structure. Additional in silico methods have been developed targeting the prediction of epitopes like the software SPADE which combines structural and cross-reactivity data and therefore localizes epitopes [120]. All these mentioned methods have in common that they only encircle the epitope. The tricky business to precisely pinpoint the interacting residues on the epitope and the paratope can only be fulfilled by the determination of the three-dimensional structure of the antibody-allergen complex by X-ray crystallography.

Only two human IgE-allergen complexes (in both cases the IgE had been isolated from combinatorial libraries) have been resolved so far [81, 89]. In the first case an IgE-derived Fab fragment was complexed with a major food allergen, β-lactoglobulin from bovine milk [89] while the second complex was formed between an IgE Fab and the major timothy grass pollen allergen, Phl p 2 [81]. These Fabs recognized flat [89] or slightly concave [81] epitopes that were comprised of discontinuous stretches of residues mainly located within the four-stranded β strands of the allergens. In contrast, in the small number of solved mouse IgG-allergen complexes, IgG tend to favour the binding of protruding regions of the allergen [121-123]. However, the temptation to set up a new paradigm supposing IgE epitopes to be the planar/concave counterparts (located on β-strands) to looping IgG epitopes is defeated by two arguments. First, the scan of available allergen structures reveals that there is no common conformation in allergenic molecules. There are allergens with almost exclusive β-sheet structure next to allergens with mixed β-sheet/α-helical structure or exclusively α-helical structures (reviewed in [124]). The second argument that prohibits the immediate acceptance of such a paradigm is the latest mouse IgG-allergen complex [125] that shows that the CDR3 region of the antibody is grabbing into a concave epitope. Determination of several additional complexes of allergens and human IgE and IgG are indeed needed to resolve these controversies.

As already mentioned, crystal structures enable the visualization of contact-making residues in the B cell epitope but additionally they allow for the first time a close view

into the IgE paratope. In the β-lactoglobulin-complex, residues from all six CDR regions participate in allergen binding and in the Phl p 2-complex residues from five of the six CDR regions take part, and in both cases the same amount of amino acids in the variable regions of heavy and light chains are involved in making direct contacts with the allergen. Structural knowledge of IgE-allergen interactions may form the basis for a downstream detailed assessment of the energetic contribution made by each contact residue to accomplish complex formation. However, such detailed analysis, which for instance would allow for determination of potential differences of IgE-, IgM- and IgG-antigen interactions, has yet to be performed in the case of immunoglobulin-allergen interactions.

These simple observations may cause speculations that the CDR regions from both chains contribute equally to specific epitope binding and binding affinity. However, a closer look into allergen/antibody complexes questions this equal status. Firstly, Padavattan et al. [81] report many more apolar interactions of the allergen with the H chain than with the L chain. Secondly, in both IgE-allergen complexes the H chain buries a much larger surface on the allergen than the L chain (544 Å^2 versus 347 Å^2 [81] and 550 Å^2 versus 385 Å^2 [89]). Does this indicate that the H chain has a leading part in allergen binding, as outlined in section 2 above?

Several assessments that have been performed over the years to contribute to this subject revealed controversial outcomes. During the course of isolation of monoclonal human IgE antibody fragments, it was found that some but certainly not all allergen-specific H chains were able to combine with different L chains with sequence variations of different extents. Their maintained allergen-binding potential suggests a dominant role for the H chain in terms of specificity in these cases [74, 76, 83]. In contrast, due to the results of other studies the conclusion might be drawn that L chains have an essential role in the definition of specificity: some light chains combined with different H chains and the resulting antibody fragments bound to the same target [90]. In this context it was further reported for Phl p 2-specific [79] as well as other [76] allergen-specific monoclonal human IgE antibody fragments from combinatorial libraries that a given set of H chain sequences is mostly selected together with similar L chains. Addressing the aspect of L chain importance for allergen affinity, it was reported that shuffling of L chains between different human Der p 2-specific IgE derived from murine

monoclonal IgG antibodies resulted in antibody fragments with altered affinity for Der p 2 [88].

A recently published study [20] provides new results that may help to shed some light on these controversies. It investigates the involvement of the L chain in creation of an allergen-specific paratope in ten IgE-derived Fab fragments which were specific for the major timothy grass pollen allergens Phl p 1, Phl p 2 or Phl p 5 [20]. Light chain shuffling was performed by pairing H chains from one specificity with L chains from the other specificities. The resulting antibody fragments were analyzed for binding specificities, affinities and epitope recognition. It was shown that the H chains in allergen-specific IgEs are mainly responsible for the determination of specificity, whereas pairing of H chains with different L chains has an effect on the affinity of the fragments. Interestingly, the pairing with different L chains may also influence the fine-specificity in terms of epitope recognition as some of the shuffled fragments recognize an epitope that is different from the parental epitope. So, whereas this study reports the recognition of different epitopes due to light chain shuffling, in similarity to an in vitro evolution study by Boström et al. [126], it was previously described that the recombination of the H chain of a virus-neutralizing human IgG_1 with other L chains resulted in a drift of epitope recognition within a single epitope [127]. All these results indicate that when allergen-specific antibody fragments have been selected from combinatorial libraries both chains often contribute to allergen binding but they do so in different ways.

5. Evolution of the IgE repertoire over time

To address the question whether the IgE repertoire in a given individual progresses over time (i.e., the initiation of antibody responses to new allergens or the recruitment of new antibody clones targeting new epitopes on previously recognized allergens), analysis of polyclonal IgE antibody titers in general as well as molecular approaches screening of V gene repertoire of monoclonal IgE antibodies (similar to e.g., the characterization of the tetanus-specific IgGs of Poulsen et al. [33, 128]) are needed.

It was only with the introduction of component resolved diagnosis (CRD) a little more than a decade ago that a detailed dissection of the individual patient´s IgE reactivity profile became possible [129]. Since then a lot of studies have been performed using

mainly the ISAC microarray technology [16] to evaluate IgE specificity of allergic patients to a broad panel of allergens. However, many of these recent studies report on the dissection of IgE reactivity in large patient cohorts by microarray technique focussing on sera derived from just one point in time of observation [130-132]. In these cross-sectional evaluations the general distribution of IgE reactivities to allergenic molecules in terms of different age, environment and gender was investigated [130, 131, 133, 134]. In fact, it could be proven that food-related components (i.e., milk and egg) were the most frequently recognized allergens early in life (0-4 years) whereas birch- and grass-specific IgE invariably appeared later (not before 3 years of age). IgE specific for house dust mite components was the most represented specificity in all investigated age classes (0-65 years) reaching a maximum between 6 and 17 years of age [130, 131]. These findings confirmed earlier but much less comprehensive data collected when patients´ IgE profiles were still determined towards grass and birch pollen using singleplex assay systems [135-137]. It thus appears that the "IgE repertoire" evolves over time as assessed at the polyclonal level. Nevertheless, to fully appreciate the dynamics of the "IgE repertoire", sera from single individuals collected over several years have to be analyzed by the ISAC technique. Two recently published studies cover exactly this important topic [138, 139]. Lupinek et al. assessed whether and how frequently adult allergic patients and non-allergic subjects experience IgE sensitizations to new allergen molecules. By testing sera from patients obtained in 1997 and 2007 using the ISAC technology they found no sensitization to new allergen families. They concluded that de novo sensitization seems to be a rare event in adults [138]. The second study dealt with the evolution of IgE sensitization in children affected by seasonal allergic rhinitis caused by grass pollen. The investigated blood samples derived from a period of 1-13 years. It revealed that Phl p 1 was an initiator of sensitization in more than 75% of the individuals meaning that many children started as mono-sensitized patients. Only later, IgE levels were detectable for Phl p 5 and/or Phl p 4 (oligo-sensitized) and finally for Phl p 2 and/or Phl p 6 and/or Phl p 11 (poly-sensitized) [139]. These results clearly indicated that the IgE response became progressively stronger and more complex during childhood and early adolescence. As these examinations were conducted only with sera from german children deriving from one birth cohort, more comprehensive investigations on changes of specific serum IgE levels including huge cohorts of children and adolescents are needed. A recently started

European Union project, MeDALL (Mechanisms of the Development of ALLergy) [140] is currently performing such comprehensive investigations on IgE sensitizations. The outcome of these assessments will certainly increase our understanding of the mechanisms of sensitization, the initiation of allergy, the development of an "IgE repertoire" and might propose the need for early diagnosis of allergy.

The information we will get by following levels of specific polyclonal IgE antibodies by techniques such as allergen microarrays will not define the details of the evolution of IgE repertoires. Questions like: "Does the diversity and character (e.g., epitope recognition profile, affinity) of IgE antibodies evolve over time?" will remain unsolved. However, we already know (as further outlined in section 6) that these parameters are aspects that are important for the biological outcome of the IgE-allergen interaction. Precise measures that are able to capture the behaviour/specificity and quality/affinity of individual human IgE over time will be required to address matters such as: "Can the IgE repertoire be further diversified following its initial establishment?" To deal with such issues researchers currently largely have to rely on the analysis of sequences of IgE antibodies of mono-sensitized individuals in terms of their choice of sequences, i.e., immunoglobulin H chain gene usage, CDR composition, and the nature of mutations within the CDR.

A first effort to gain insight into the development of allergen-specific IgE repertoire was carried out in Bet v 1 mono-sensitized patients over several years of repeated allergen contact revealed that highly similar IgE IGHV transcripts i.e., IGHV 3-23, IGHV 4-61, IGHV 1-2, IGVH 4-31 occurred in all investigated allergic subjects. Additionally, these IgE IGHV genes could be detected in all patients over time [141]. These findings indicate that Bet v 1 might directly select and stimulate allergen-specific IgE production (i.e., secondary IgE responses) via activation of a limited and pre-defined set of B lineage cells [141] suggesting that IgE repertoires change relatively little over time. This contrasts to findings describing the evolution of tetanus toxoid-specific IgG repertoires, where many clones detected following a vaccination event were unique to that vaccination event [128]. Such differences might be caused by the more aggressive immunization method used in active immunization but these differences certainly deserve attention. Therefore, additional studies with much more detailed investigations are needed to confirm or disprove these first observations.

Taken together, the lack of detailed knowledge of the evolution of the IgE repertoire over time hampers to understand the plasticity of the set of antibodies that cause allergy. Future efforts involving high throughput sequencing of IgE-encoding transcripts and isolation of allergen-specific monoclonal IgE antibodies by combinatorial library technology or even better by cell sorting technologies/immortalization at different points of time will likely support our understanding if evolution of the IgE repertoire over time really does happen. Exciting questions like if and how repeated contact with allergens change/shape the IgE repertoire will probably find their answers through these approaches.

6. The IgE repertoire and its impact on effector cell function and antigen presentation

The complexity of the IgE repertoire and its impact on effector cells and antigen presentation has been difficult to study for many years. It had early on been suggested even that IgE epitope density and perhaps geometry of epitopes might be responsible for the allergenicity of certain allergens [77, 82, 142]. With access to defined monoclonal allergen-specific antibodies it has however recently been clearly demonstrated how several defined parameters alter the magnitude of both effector cell and T cell activation. These parameters include epitope density, allergen-specific IgE clonality, affinity of individual IgE and the concentration and relative ratio of allergen-specific IgE [87, 88, 143-146] (Gieras et al, submitted for publication).

Through the recent availability of larger sets of defined monoclonal allergen-specific antibodies it became feasible to confirm such ideas and to study these processes in greater detail. Already in 2007, Gieras et al. [146] showed that the number of IgE epitopes on a given allergen determines its allergenic activity. Using a defined peptide monomer (i.e. one epitope), dimer (i.e. two epitopes) and multimer (i.e. several epitopes) of the major grass pollen allergen, Phl p 1, the authors were able to demonstrate that the increasing number of epitopes led to an enhanced release of inflammatory mediators. These observations were confirmed in studies of the major house dust mite allergen, Der p 2 [88]. By using a panel of monoclonal Der p 2-specific IgE antibodies that bind to non-overlapping epitopes they could demonstrate that increase of the IgE clonality as well as higher affinity of individual IgE antibodies

augmented basophil sensitivity. Moreover, raising the concentration of allergen-specific IgE caused an increased effector cell degranulation [146]. By using different concentrations of IgE mixtures consisting of 20% equimolar quantities of three monoclonal Der p 2-specific IgE antibodies with 80% of non-Der p 2 specific IgE to imitate a typical allergic IgE repertoire, it turned out that higher total IgE concentrations increased basophil sensitivity and maximal degranulation levels [88]. By changing the ratio of Der p 2-specific IgE /non-Der p 2-specific IgE it could be clearly shown that 100% of equimolar Der p 2-specific IgE induced maximal degranulation and sensitivity. Altering the concentrations of individual Der p 2-specific IgE antibodies to levels other than equimolar the maximal degranulation was reduced remarkably [88]. Altogether, these studies demonstrate that the makeup of the IgE population, i.e. the IgE repertoire, is an important factor that determines the outcome of the IgE-allergen interaction.

Besides affecting basophils and mast cells, IgE plays an important role by affecting T cell activation. Indeed, IgE has been shown to promote facilitated antigen presentation (FAP) [147]. Based on these studies and the disposability of defined antibodies, it was demonstrated that the IgE repertoire complexity and the affinity of IgE antibodies have a strong influence also on the FAP-mediated T cell activation [144]. By measuring the formation of allergen/IgE/CD23 complex on B cells as surrogate parameter for FAP-mediated T cell proliferation, the combination of three high affinity non-overlapping IgE antibodies were proven to elicit maximal complex formation. In contrast, the combination of one high and one low affinity IgE resulted in an intermediate response. Only when a third low affinity IgE binding to a non-overlapping epitope was added to this mixture maximal complex formation could be achieved. Through these important experiments it turned out that for FAP-mediated T cell activation IgE clonality is more important for providing the avidity required for efficient complex formation than for basophil activation [144].

The above-described data clearly demonstrate that distinct properties of the IgE repertoire determine variable effector cell and T cell activation in response to allergen challenge. We foresee that further investigations of these important aspects of allergic disease will require new defined antibody reagents. As human monoclonal antibodies are likely to best represent the relevant antibody populations in patients, we firmly

believe that there is a substantial need for further developments in the field of generation of such antibodies against allergens.

7. The state of the art – now and in the future

The development of our understanding of the IgE antibody repertoire has relied on rather low throughput methodology both in terms of transcriptome analysis and the generation of specific binders derived from the human IgEome. Yet such technology has been able to provide a first picture of IgE-encoding transcriptomes as well as a first generation of human monoclonal antibodies from that population of transcripts. We anticipate that developments in high throughput sequencing technology, approaches that already have made substantial impact on our understanding of other human antibody repertoires in health and disease [27, 148], will contribute substantially to our understanding of IgE in disease. We also envisage that by exploiting high throughput methodology [149, 150] in selection of binders from libraries will make substantial contribution to increase the number of antigen-specific antibodies derived from the IgE transcriptome thereby providing access to a large source of antibodies that mimic the repertoire of IgE found in vivo. Furthermore, the realization of efficient techniques to identify human IgE-producing single cells and to rescue IgE-encoding genes directly from such cells will certainly further improve our confidence in human monoclonal IgE in the future as such technology holds promise to eliminate biases that may be introduced by combinatorial antibody procedures. Independent of the precise procedures used we foresee that access to larger sets of human monoclonal IgE will greatly facilitate 1. analysis of basic mechanisms of human IgE in health and disease, 2. analysis of structures of allergens targeted by IgE, 3. development of improved treatments of allergic disease for instance through generation of next-generation hypoallergens, and 4. implementation of human IgE in highly successful standardization of diagnostic procedures. In all these areas first steps have already been taken to improve procedures and to enhance our understanding of IgE but access to larger sets of human IgE will certainly be necessary to facilitate future exciting advances.

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Table 1. Human allergen-specific antibodies carrying immunoglobulin H chain V domain sequences derived from IgE-encoding transcriptomes of allergic human subjects.

Allergen	Number of clone types	Lymphocyte sources *	IGHV gene subgroup	CDRH3 length	References
Phl p 1	5	AR: PBL CRS: Sinus mucosa	1, 3	6-14	76-78
Phl p 2	3	AR: PBL AR: Nasal mucosa	4	10	76, 79-81; Levin et al., unpublished
Phl p 4	2	AR: PBL	3, 4	12-24	76
Phl p 5	8	AR: PBL	1, 3, 5	15-23	74, 76, 82-86; Levin et al. manuscript in preparation
Phl p 6	2	AR: PBL	3	9-13	76
Phl p 11	2	AR: PBL	3	16-18	76
Der f extract	2	Allergy: PBL	3	16-17	87
Der p 1	2	AR: PBL	3	10-21	Levin et al., unpublished
Der p 2	1	Allergy: PBL	3	18	87, 88
Alt a 1	1	CRS: Sinus mucosa	3	20	Levin et al., unpublished
Pen n extract	1	NAFES: Sinus mucosa	1	11	Levin et al., unpublished
Bet v 1	4	AR: Nasal mucosa Allergy: PBL	3, 5	12-19	75; Levin et al., manuscript in preparation
Bos d 5	4	Milk allergy: PBL	1, 3	10-17	89, 90
Hev b 6	2	Latex allergy: PBL	2	18-21	91

* Condition of patients from which lymphocytes were derived (AR: allergic rhinitis; CRS: chronic rhinosinusitis; NAFES: non-allergic fungal eosinophilic rhinosinusitis): Sample material from which RNA was derived (PBL: peripheral blood lymphocytes)