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REVIEW ARTICLE

IgE-binding epitopes: a reappraisal

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Abstract

Here, we discuss various questions related to IgE epitopes: What are the technical possibilities and pitfalls, what is currently known, how can we put this information into hypothetical frameworks and the unavoidable question: how useful is this information for patient care or allergenicity prediction? We discuss the information obtained by (i) 3D structures of allergen–antibody complexes; (ii) analysis of allergen analogues; (iii) mimics without obvious structural similarity; (iv) mAbs competing with IgE; (v) repertoire analysis of cloned IgEs, and other developments. Based on limited data, four suggestions are presented in the literature: (i) IgE might be more cross-reactive than IgG; (ii) IgE might be more often directed to immunologically 'uninviting' surfaces; (iii) IgE epitopes may tend to cluster and (iv) IgE paratopes might have a higher intrinsic flexibility. While these are not proven facts, they still can generate hypotheses for future research. The hypothesis is put forward that the IgE repertoire of switched B-cells is less influenced by positive selection, because positive selection might not be able to rescue IgE-switched B cells. While this might be of interest for the discussion about mechanisms leading to allergen-sensitization, we need to be modest in answering the 'clinical relevance' question. Current evidence indicates the IgE-epitope repertoire is too big to make specific IgE epitopes a realistic target for diagnosis, treatment or allergenicity prediction. In-depth analysis of a few selected IgE epitope-peptides or mimitopes derived from allergen-sequences and from random peptide libraries, respectively, might well prove rewarding in relation to diagnosis and prognosis of allergy, particularly food allergy.

In 1984, Benjamin et al. (1) published a landmark paper titled 'The antigenic structure of proteins: a reappraisal'. At that time, the impact of three important technical advances (the large-scale analysis of the immune reactivity of peptides, hybridoma technology and the determination of the 3D structure of proteins, including three antigen-Fab complexes) had resulted in opposing views on the nature of protein epitopes. One view was that proteins contain a very limited number of sites that are intrinsically immunogenic (2). The opposing view, advocated by Benjamin and his 14 prominent coauthors, was that 'most of the accessible surface of any globular protein is potentially immunogenic, that one can define which sites are immunogenic only with respect to a particular responding individual, and that the total antigenic structure of a protein is the sum of all sites recognized by a large variety of responding individuals and species' (1).

There is an intriguing parallel with the current discussion on the nature of IgE-binding epitopes, and that of IgE-inducing antigens. On the one hand, it has been proposed that there are few restrictions on the properties of antigens that can induce IgE antibodies (3). On the other hand, it has been argued that only a very restricted set of antigens can induce IgE antibodies, a view forcefully promoted among others by Breiteneder et al. (4).

In the present critical review, we will attempt to make a connection between the epitope discussion as reviewed in the Benjamin paper (1) and the above-mentioned allergenicity issue by discussing the current knowledge on IgE-binding epitopes.

Epitopes, paratopes and mimotopes: concepts and misconceptions

The epitope

The term epitope was introduced by Jerne fifty years ago (5). In this visionary article entitled 'Immunological speculations',

he defined the term epitope as 'surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas' of an antigen. In the textbooks, the more concise definition soon became 'specific antigenic determinant site on a molecule'. 'Antigenic determinant' is usually considered to be synonymous with 'epitope'. Although the original definition of an epitope was coined for antigen–antibody interactions, it was later on applied also to describe the interactions between T cells and peptides recognized by the T-cell receptor (TCR). Because antigen recognition by the TCR is so different from that by antibodies, it is important to stress that we will exclusively discuss B-cell epitopes.

Benjamin et al. (1) are mostly quoted for their brave statement that the whole surface of a protein is antigenic, but this does not imply that every part of the surface is equally important from the antibody point of view: some epitopes are recognized much more often than others.

No epitope without a paratope

The paratope is the area on the surface of the paired VL-VH domains of an antibody molecule that interacts with the epitope. It incorporates most if not all of the six complementarity-determining regions (CDRs: three from the VL domain and three from the VH domain).

An epitope is defined by a paratope. The relation between epitope and paratope is degenerate. It is generally assumed that one epitope can react with different paratopes, even though this may not be strictly true (see below). Similarly, examples have been found of a monoclonal antibody interacting with nonidentical epitopes. In the latter case, there are two explanations: cross-reactivity and multi-reactivity. Cross-reactivity is relatively simple. The epitopes have structural features in common, which strongly suggest that in these cases, the epitope/paratope interactions are similar (nondegenerate). However, in other cases, no obvious common features exist. This multi-reactivity could reflect our inability to recognize common features, but in several instances, it has been shown that a single antigen-combining site on the antibody may contain more than a single paratope (6). This kind of multi-reactivity is often hard to distinguish from cross-reactivity. In this context, it is instructive to consider the analogous interaction between paratopes and anti-idiotype antibodies. A single paratopic surface can react with many different anti-idiotypic antibodies, only some of which will interfere with the interaction of the paratope with the epitope. The distinction is also important in interpreting the results of binding studies performed with structures called mimotopes (which will be discussed in more detail later).

Can one epitope react with different paratopes? Unrelated paratopes reactive with the 'same' epitope exist. These are very often different and will have different contacts with the epitope, so these epitopes are not identical. Based on such a strict definition of the epitope, a purist would predict that virtually all epitopes are defined by a single, unique paratope. However, in real life, such a distinction is not very productive. In both views (the strict as well as the relaxed definition of an epitope), it is in general not worth the effort to elucidate a particular epitope/paratope interaction to the finest details unless there is a particular interest in analysing or modifying a specific interaction (Box 1).

Box 1 Relevance of information on epitopes

The most-frequently used argument relates to the safety and efficacy of specific immunotherapy. Almost all papers on IgE epitope mapping state that epitope information is important to make allergen vaccines safer. While selective destruction of a single epitope may occasionally reduce skin test reactivity substantially, in the end, the modification needed to robustly reduce skin test reactivity involves in general a more drastic modification. More often than not it does not really require knowledge about the structure of the epitope to produce such a modified allergen. An additional issue is whether or not it is important to have a properly folded allergen rather than a small peptide or a completely misfolded protein that can still affect T-cell reactivity (as discussed earlier in this paper).

The second argument why information on epitopes is important relates to cross-reactivity. If we know the epitope of an allergen, we should, in principle, be able to predict more accurately which proteins will be cross-reactive with this allergen. Potential cross-reactivity prediction is important in relation to the risk assessment of novel foods. Until now, predictions are based on sequence similarity rather than on similarity to known epitopes. This may become the better procedure in the near future, but for now, the information on epitopes is still insufficient.

A third way in which epitope information might be useful is in helping to elucidate more fundamental questions related to the special features of the IgE immune response: epitopes as probes of the IgE repertoire, as discussed earlier in this paper. This knowledge will somehow (but presumably not in the immediate future) be translated into improving prevention and treatment of allergic diseases.

For clinical relevance, we should look at paradigmatic examples rather than at the full repertoire. In-depth analysis of a few selected peptide IgE epitopes (allergen-sequence-derived peptides as well as random-library-derived mimotope peptides) might well prove rewarding in relation to diagnosis and prognosis of allergy, particularly food allergy.

Two complications concerning the epitope/paratope definition

The definition of the epitope is focused on the actual contacts between the atoms of the antigen and those of the antibody. Two aspects are often overlooked. The first aspect is the potential influence of buried water (7, 8). Traditionally, this was assumed to be an unfavourable effect that decreased the overall entropy of the complex and thus decreased its stability. This notion was based on the stability-increasing effect of hydrophobic contacts in the interface, which were largely explained by the increase in entropy upon release of 'frozen' water molecules covering these hydrophobic patches in the noncomplexed state (9). For a recent discussion on the effect of water exclusion from hot spots in protein–protein interactions, see Morena et al. (10) and Li and Li (11). With the

advent of more very high-resolution structures (< 2 Å), it is becoming increasingly clear that buried water molecules have an important stabilizing role 'contrary to naive expectation' (12). Other physiological solutes, such as metals like nickel or zinc, can similarly be involved (13). Semantically, it is not clear whether or not these noncovalent components are to be considered part of the epitope and/or paratope. More importantly, these components are not yet part of epitope-prediction algorithms. It is technically challenging to determine their contribution (and even their number) under physiological conditions, among others because of the possible influence of crystallization conditions on the outcome (14).

The other aspect is the distinction between this traditional structural epitope/paratope interaction and their functional interaction as described by the different steps in the association–dissociation kinetics, for example the encounter-docking model (15). The crystal structure of an antigen–antibody complex is a static representation of a dynamic condition described by the affinity constant. Reaching the equilibrium state as observed in a crystal structure may take an appreciable time (up to several hours) (15). Not all amino acids that are important in the early phase are equally important in the final stage. In the context of defining the epitope and paratope, it is relevant to point out that charged residues may markedly influence the association rate without being part of the final contact area. The presumed role of these charges, which interact over a relatively long distance, is to get the antigen and the antibody into an optimal orientation during the earliest phase of the interaction.

In some biological situations, complex stability is the only important factor. However, the association rate is often more important than the dissociation rate. In toxin- and virus neutralization, there is a competitive race between the antibody and some receptor that allows the pathogen to do its dirty job. For allergen-induced mast cell triggering, the interaction between antigen and antibodies does not need to persist for

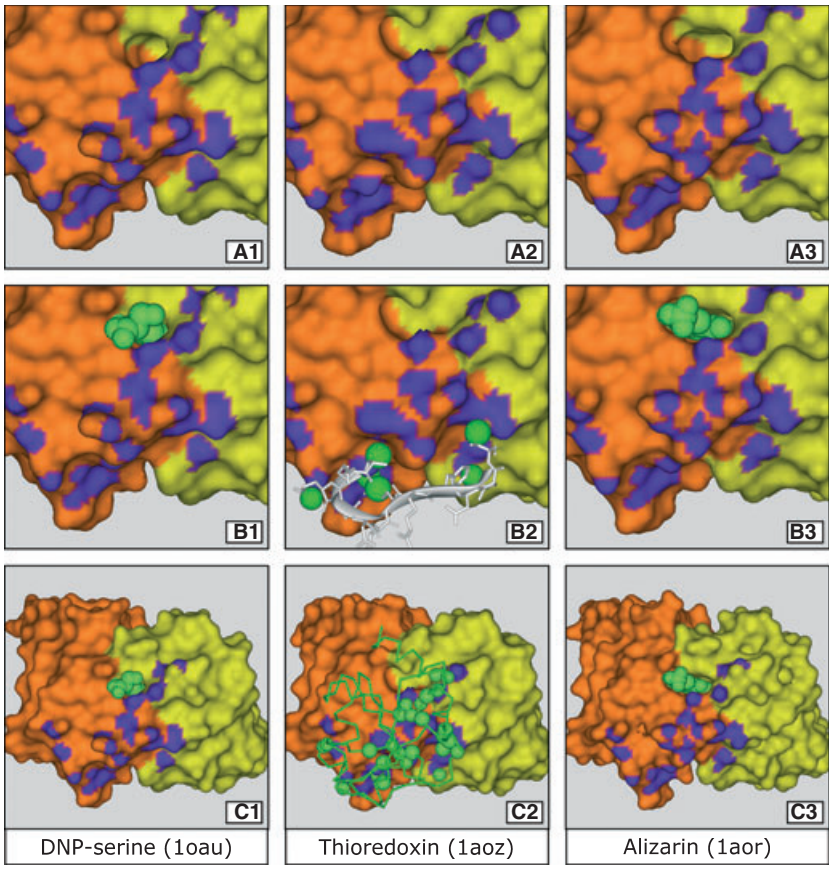


Figure 1 An example of a mimotope reacting with an anti-hapten antibody (6) is illustrated. In this example, a random library of constrained 12-mer peptides inserted into a loop of thioredoxin fused to a phage coat protein has been used. SPE-7 is a monoclonal mouse IgE antibody raised to the hapten dinitrophenol coupled to the carrier protein KLH. It binds DNP, but not close analogues of DNP, such as 2-nitrophenol and 2-nitro-4-iodophenol. However, SPE7 also binds several apparently unrelated compounds, such as alizarin red and the protein thioredoxin, mutated to contain a 12-mer mimotope peptide. In the panels A1–A3, the VH–VL fragments of these three immune complexes are shown, with the ligand stripped. The purple surface areas indicate the contact sites between the VH–VL and the peptide in its thioredoxin scaffold (as depicted in panel C2). The ligands are shown in panels B1–B3. In panels C1–C3, a zoomed-out view is presented. Panel C2 shows the full extent of the contribution of amino acids of the scaffolding thioredoxin molecule.

hours. In the latter situation, a fast association rate may be more influential. It is tempting to speculate that the poor biological activity of IgE antibodies to cross-reactive carbohydrate determinants (CCD) (16) is because of a slow rate of association attributable to the high mobility of the glycan chain. In order for blocking antibodies induced by immunotherapy to be effective, the IgG antibodies will have to bind before the allergen interacts with the IgE antibody. The technology is now available to investigate the relevance of these kinetic factors in allergic reactions (17).

Nonpeptidic epitopes

In many publications, epitopes are implicitly assumed to exclusively consist of amino acids. However, many epitopes do not contain amino acid, such as glycan epitopes and classical haptens, both of which are important as IgE-binding epitopes. Examples of IgE-binding glycan epitopes are the cross-reactive CCD (18) and alpha-gal (19, 20). The IgE reactivity of hapten epitopes derived from penicillin (21) or neuromuscular blocking agents (22, 23) has also been studied extensively. These nonpeptidic epitopes are often quite small. In complexes of antibodies with haptens, the hapten is usually bound in a pocket in the interface between the VH and VL domains (22), as also illustrated in Fig. 1. The paratope of some glycan epitopes has been found to interact tightly with often fewer than six sugars (24, 25).

The artificial dichotomy of conformational vs sequential epitopes

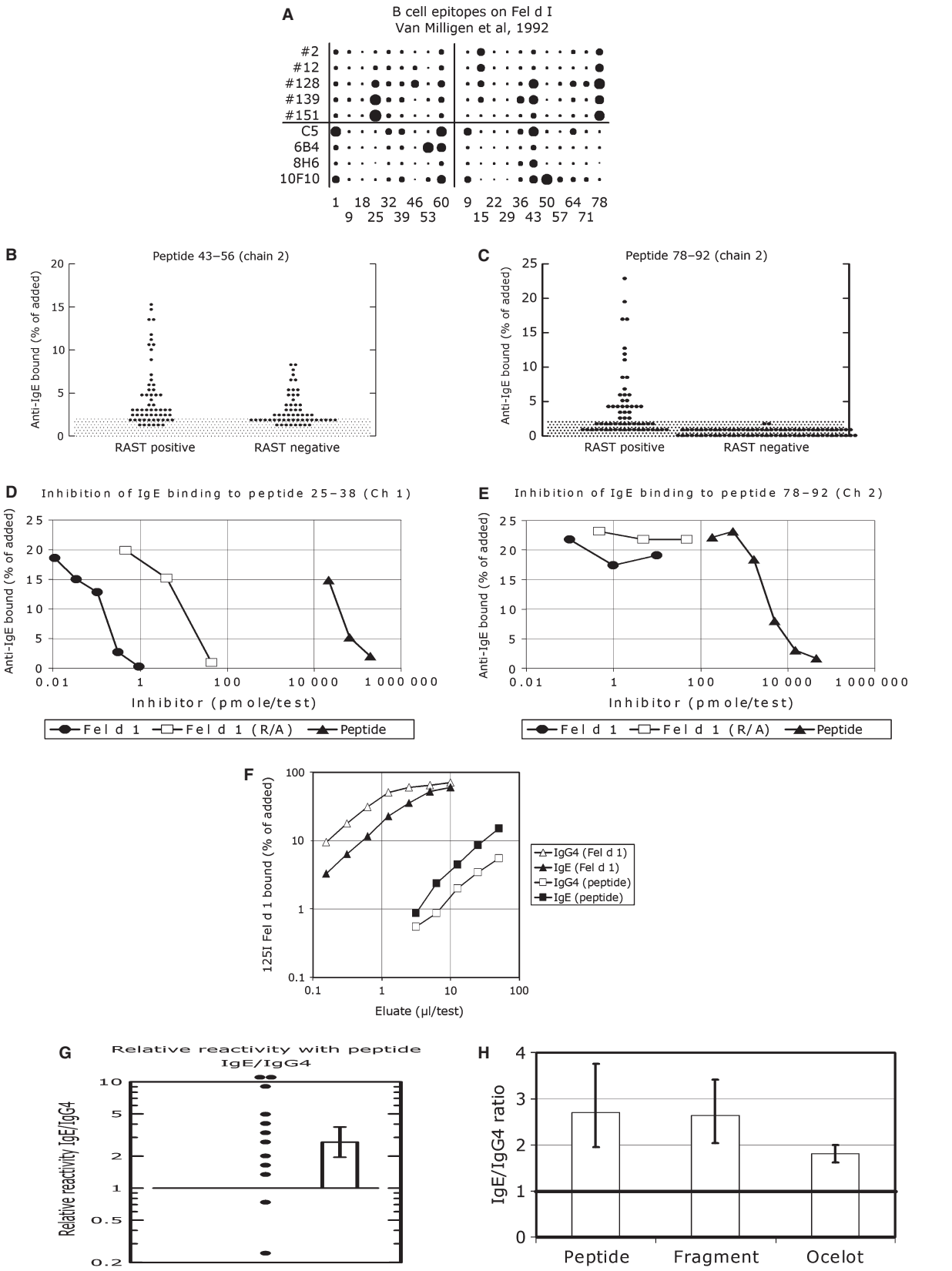
The remainder of this discussion will be restricted to fully peptidic epitopes of protein antigens. These epitopes are in general found on the surface of the native protein. In some situations, however, B cells may be exposed to fragmented and/or partially unfolded proteins. This most commonly occurs in the gastro intestinal tract, but may also happen in infected tissues in which chymase, elastase and other proteases may be active. Also upon vaccination with adjuvant, some unfolding of the antigen may occur (26). It is therefore incorrect to assume that only amino acids that are surface-exposed in the fully native protein are relevant for epitope studies.

Sometimes, the amino acids with the most important contacts are found in a sequential stretch of 6–12 amino acids. This has been referred to as a 'sequential epitope', particularly if the corresponding synthetic peptide binds to the antibody. For many antibodies, several parts of the molecule, not sequentially located in the antigen, form the epitope. The epitope is then referred to as 'conformational' to indicate that the epitope–paratope interaction needs a properly folded antigen structure to assemble the relevant amino acids into the epitope. The topographic nature of the epitope was stressed already by Benjamin et al. (1), who indicated that presumably all protein epitopes are conformational. As illustrated in Fig. 2, it is common to find a large loss in affinity (because of a large loss of entropy) when binding of the free peptide is compared with binding of the native antigen, even with strongly binding peptides (27). While some antigen-derived peptides are very useful for studying epitopes and for immunization purposes, it should be appreciated that such peptides cover only a fraction of the epitope repertoire.

Mimotopes: cross-reactivity or multi-reactivity

A mimotope is a peptide able to bind to a particular antibody, without a relation in sequence to the protein antigen used to induce the antibody (28, 29) (see Fig. 3). A mimotope is typically a structure selected by an antibody from a large library of different structures. Most commonly, a random library of peptides expressed on the surface of a bacteriophage is used (30–33), but libraries of synthetic peptides (34)

Figure 2 Illustration of an example of IgE epitope mapping by peptide scanning (27, 45, 46). In this example, overlapping 15-mer peptides derived from the cat allergen Fel d 1 have been used. In panel A, the results obtained with RAST-type assays on five sera (top) and four monoclonal antibodies (bottom) are shown (chain 1 on the left, chain 2 on the right). The quantitative immunoassay data are visualized as a dot matrix. Panels B and C show the results of testing two larger panels of sera: a cat-positive panel on the left and an atopic, but cat-negative panel on the right. With some peptides (e.g. the peptide 43–56 of chain 2, shown in panel B), a significant number of cat-negative sera reacted to the peptide, suggesting some nonspecific binding activity. With other peptides, little or no IgE reactivity was found in the cat-negative panel (e.g. the C-terminal peptide of chain 2, shown in panel C). In the experiments shown in panels D and E, IgE binding to a peptide was inhibited with the same peptide (triangles) or by Fel d 1 (filled dots). The open squares indicate the inhibition obtained with Fel d 1 denatured by reduction/alkylation. Panel D shows that peptide 25–38 from chain 1 was inhibiting, but only at a dose that was almost 10^6 times higher (on a molar basis) than was required of Fel d 1. Surprisingly, the C-terminal peptide of chain 2, which was behaving well in the experiment shown in panel C, was not behaving as expected in the experiment shown in panel E: no inhibition by Fel d 1, despite this peptide itself being an good inhibitor of IgE binding. This might indicate that in the purified natural Fel d 1, the C terminus was somehow modified. In panel F, results are shown of an affinity purification of peptide-reactive IgE and IgG4 antibodies using Sepharose-coupled peptide (in this example peptide 15–28 of chain 2, squares), compared to a similar affinity purification using Sepharose-coupled Fel d 1 (triangles). The presence of high-affinity antibodies to Fel d 1 in the two eluates was tested by a double antibody precipitation technique using iodinated Fel d 1. The results indicate that the ratio IgE/IgG4 antibody was higher in the peptide eluate than in the Fel d 1 eluate. The results expressed as (IgE/IgG4)/(peptide eluate/Fel d 1 eluate) obtained with 12 patients are shown in panel G. Overall, IgE was almost three times more reactive to the peptide than IgG4. As shown in panel H, IgE was (relative to the native Fel d 1) also more reactive with other Fel d 1 variants, either chain 1 of Fel d 1 (2nd column), or a natural variant (the Fel d 1 equivalent of the ocelot (3rd column).


A
B cell epitopes on Fel d I
Van Milligen et al, 1992
#2
#12
#128
#139
#151
C5
6B4
8H6
10F10
1 18 32 46 60 9 22 36 50 64 78
9 25 39 53 15 29 43 57 71
B
Peptide 43–56 (chain 2)
Anti-IgE bound (% of added)
RAST positive
RAST negative
C
Peptide 78–92 (chain 2)
Anti-IgE bound (% of added)
RAST positive
RAST negative
D
Inhibition of IgE binding to peptide 25–38 (Ch 1)
Anti-IgE bound (% of added)
Inhibitor (pmole/test)
Fel d 1
Fel d 1 (R/A)
Peptide
E
Inhibition of IgE binding to peptide 78–92 (Ch 2)
Anti-IgE bound (% of added)
Inhibitor (pmole/test)
Fel d 1
Fel d 1 (R/A)
Peptide
F
125I Fel d 1 bound (% of added)
Eluate (µl/test)
IgG4 (Fel d 1)
IgE (Fel d 1)
IgG4 (peptide)
IgE (peptide)
G
Relative reactivity with peptide
IgE/IgG4
Relative reactivity IgE/IgG4
H
IgE/IgG4 ratio
Peptide
Fragment
Ocelot

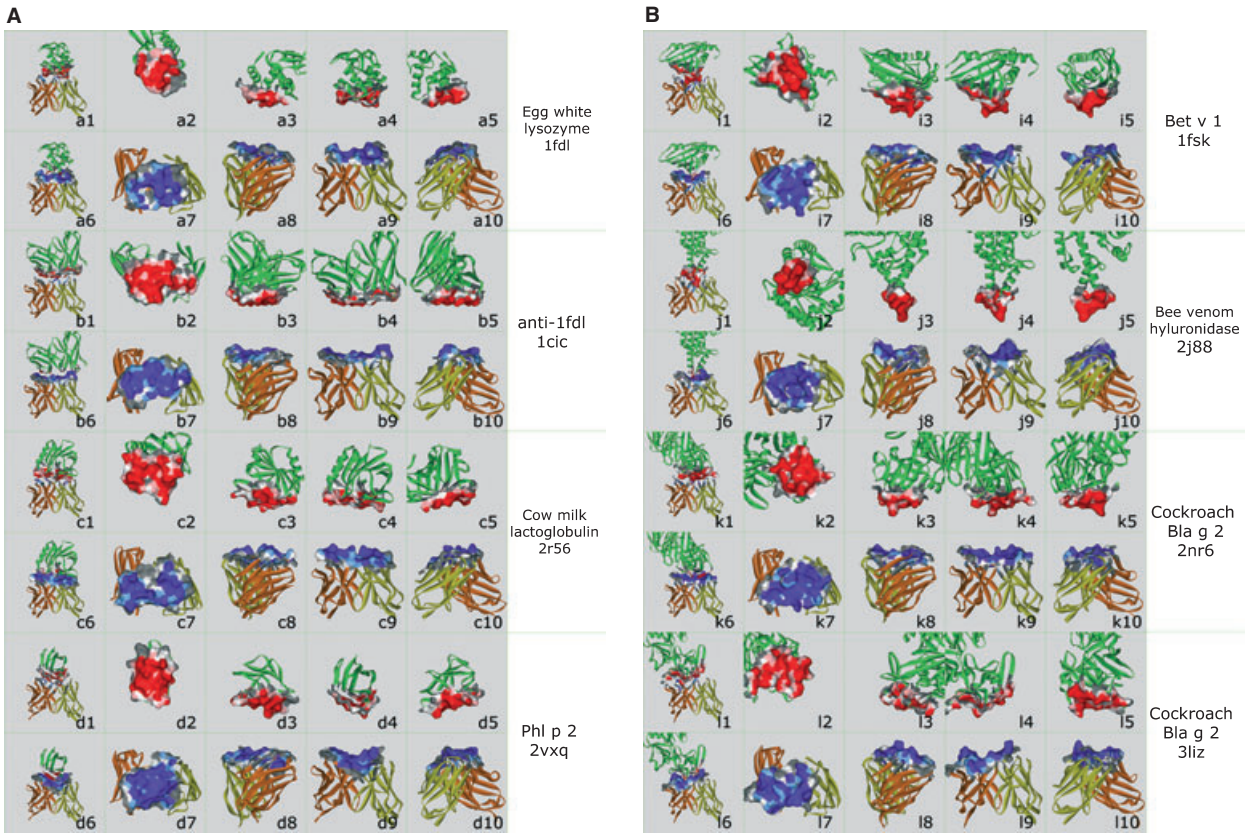


Figure 3 Epitope (red)/ paratope (purple) structures for six allergens: (a) and (b) lysozyme from egg white (89, 90); (c) beta lactoglobulin from cow's milk (58); (d) grass pollen group 2 from phleum pratense (59); (e) Bet v 1 from birch pollen (91); (f) hyaluronidase from honey bee venom (92); (g and h) Bla g 2 from cockroach(13, 41). The structures shown in (a and b) show the same anti-lysozyme antibody, in (a) in complex with lysozyme, in (b) in complex with an anti-idiotype antibody, which mimics some features of the allergen. The structures shown in (k) and (l) show the same allergen, in complex with two different antibodies. The recombinant antibody fragments shown in (c and d) are derived from human IgE antibodies, whereas the six other antibody fragments are derived from mouse antibodies. All structures are shown with the VH (orange) in the same orientation. In frames a2, b2, etc. and a7, b7, etc., the structures have been rotated forwards (2) or backwards (7) by 90° over the horizontal axis to show a frontal view of the epitope (red, 2) or paratope (purple, 7). Frame 4 and 9 show an enlarged view of the epitope and the paratope in the same orientation as in column 1 and 6. In columns 3/8 and 5/10, the structures have been rotated by 120° to the right and to the left on the vertical axis. The figures have been made using DSViewer 5.0. The epitope and paratope have been defined as the collection of atoms with a distance ≤4 Å to the antibody or allergen, respectively. The surfaces have been calculated by probing the structures with a sphere with a 3 Å radius.

or of nonpeptidic compounds, such as aptamers, have also been examined (35). Mimotopes may mimic not only epitopes of proteins, but also carbohydrates, lipids or haptens (see DNP-example in Fig. 1), including biotin.

If the binding characteristics of the selected compound indicate specificity (i.e. the compound does not react with control antibodies), the selected compound is called a mimotope, because it is often assumed that it structurally mimics the epitope. If structural similarity is indeed responsible for the interaction, the mimotope is cross-reactive with the antigen, which would experimentally be demonstrable by mutual inhibition (Box 2). However, in real life, such an inhibition experiment is not as simple as it sounds. The mimotope may potentially interact with only a small part of the paratope and thus be an inefficient inhibitor, whereas inhibition of peptide binding by the antigen (particularly if it is a large macromolecule) may be because of steric hindrance rather than to true competitive inhibition. Because both the mimotope and the epitope bind to the same paratopic surface, it is tempting to assume that a mimotope gives some information about the structure of the epitope. This may be true in some cases, but it is prudent to take into account that the alternative explanation, multi-reactivity of the paratopic surface, is also a realistic option (36, 37). A paradigmatic example is the antibody SPE7 (6), which happens to be an IgE antibody (see Fig. 1).

As illustrated in Fig. 1, there is little evidence that each contact residue in the mimotope makes contact with one or more residues in the paratope. In most cases, mimotopes cannot easily be mapped on the 3D structure of the antigen. To

Box 2 Cross-reactivity, a confusing concept

At its purest level, cross-reactivity is simple: one paratope P is found to react with two similar, but not identical epitopes (E1 and E2). This corresponds to the situation in which a monoclonal antibody is analysed for reactivity with two epitopes, each derived from a single antigen (e.g. from two recombinant allergens Bet v 1 and Mal d 1). Most of the contact residues between P and E1 are also contributing to the interaction between P and E2. A first level of uncertainty arises when the sites of interaction are not so similar anymore. Typical examples are interactions of P with mimotopes and anti-idiotypes.

Another level of cross-reactivity is the real-world situation in which a mixture of paratopes (e.g. serum of patient A) is analysed for reactivity with two sets of epitopes derived from two single antigens (e.g. Bet v 1 and Mal d 1). Some, but not all, antibodies in this single sample will be cross-reactive. The serum from another patient B may have only non-cross-reactive antibodies. Similarly, within a serum IgE antibodies may be partially cross-reactive, whereas IgG4 antibodies might lack such cross-reactive antibodies. It is tempting to conclude that the specificity of IgE antibodies is different from that of IgG4 antibodies, whereas the larger part of the IgE- and IgG4 paratopes might be similar (Bet v 1-specific, non-reactive with Mal d 1).

The situation may become confusing if the 'cross-reactivity' is used to describe the real-world situation in which a serum of a patient is found to react with a mixture of allergens from two source materials (e.g. birch pollen and apple). The confusion will further increase if clinical symptoms are included as a critical part of the definition of cross-reactivity: some of the antibodies may be cross-reactive, without corresponding symptoms of the patient (asymptomatic cross-reactivity), or the patient may have symptoms to both source materials but no cross-reactive antibodies are demonstrable (multiple sensitisation).

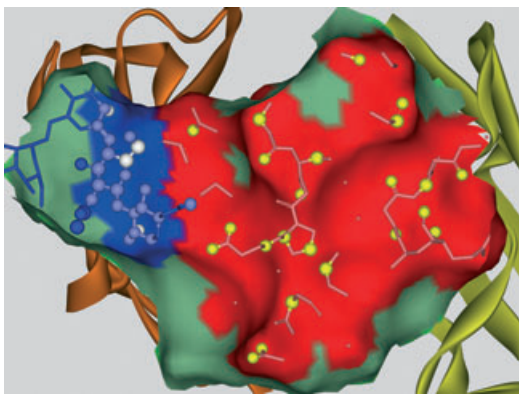


Figure 4 Corecognition of a peptide (partial) epitope (the red-coloured surface) and a glycan (partial) epitope (the blue-coloured surface). The contacting atoms of the peptidic part of the epitope are shown as yellow spheres, the four contacting atoms of the glycan epitope as white spheres. The epitope surface is seen from the inside of the allergen, which is the cockroach allergen Bla g 2 (13, 41). Behind the epitope surface, parts of the antibody are visible, the H-chain in orange, the light chain in yellow. The full structure of this allergen–antibody complex (3liz) is shown in the panels i1-i10 in Fig. 3.

Box 2 Continued

It has been suggested to introduce the term 'co-recognition' to distinguish immunochemical cross-reactivity from clinical multi-reactivity (88). Unfortunately, the term 'co-recognition' has been used already for more than 30 years in a very different context, describing the mode of recognition of the MHC-peptide complex by the T cell receptor: the T cell receptor needs to recognize both components at the same time.

For this reason we prefer to use 'cross-reactivity' to describe a situation in which one paratope can interact with either of two epitopes and in which these epitopes interfere with the binding of the other (mutual inhibition). In the absence of inhibition data, we use 'multi-reactivity'. 'Co-recognition' in the context of B cell epitope recognition might be used to indicate the situation in which the paratope interacts with two different types of epitope structures at the same time. An example, shown in Fig. 4, is the combined recognition of a peptidic epitope (the red-coloured surface, with the contacting atoms shown as yellow spheres) and a glycan epitope (the blue-coloured surface, with the four contacting atoms shown as white spheres) as found for the anti-Bla g 2 antibody 3liz (40) shown in full in Fig. 3, frames I1–I10.

qualify as a genuine mimotope, a peptide should not only be able to bind to a particular antibody, but it should also be able to elicit antibodies that recognize the epitope being mimicked, and this is not the case for many peptides selected by Pepscan^{R} or by phage surface display. It has to be realized that an epitope is not an intrinsic structural feature of a protein that could be identified without knowledge of the interactions with a paratope.

Mimotopes are conceptually similar to anti-idiotypic antibodies. Like mimotopes, the paratopes of anti-idiotypic antibodies have been viewed as mimics of epitopes. While both are probes of the paratopic surface, their contribution to the elucidation of the structure of epitopes has been, at best, modest. However, both have proven to be useful as immunochemical reagents and for synthetic vaccine candidate (38, 39) as well as for exploring the relation between IgE antibodies and antibodies of other isotypes, as will be discussed later.

Techniques to identify (partial) epitopes

At present, the most satisfactory technique (but still not perfect, see below) to define an IgE B-cell epitope is X-ray analysis of crystals of the antigen–IgE antibody complexes. This provides atomic resolution of the epitope and paratope. Similar information could be obtained by different modes of NMR spectroscopy (40). Such allergen–antibody structures will be discussed in the next section. However, these methods are not generally applicable because they require homogeneous (i.e. monoclonal) antibody.

To study epitopes of noncloned IgE antibodies, three types of analytical tools have been used:

- Structural variants, including (i) natural variants; (ii) variants obtained by site-directed mutagenesis or chemical modification; and (iii) fragments obtained by proteolysis, recombinant technology or chemical synthesis.
- Structures that mimic the allergen in its interaction with the IgE antibody, without obvious structural similarity, are mimotopic peptides and anti-idiotypic antibodies.
- Structures (mostly mouse monoclonal antibodies) that bind to (or: close to) the IgE epitope of the allergen and thus compete with the IgE antibody. One efficient approach is to (i) select a mouse monoclonal antibody that substantially interferes with IgE antibody binding; (ii) solve the X-ray crystal structure of an allergen in complex with this monoclonal antibody; (iii) perform site-directed mutagenesis targeting residues of the 'mouse' epitope; and (iv) analyse the effects of these mutations on IgE binding. This process allows the identification of specific residues involved in IgE antibody binding, as long as the mutations do not disturb the overall fold of the allergen (for an example, see Ref. 41).

Structural variants

To study epitopes of noncloned IgE antibodies, scanning with overlapping peptides (derived from the sequence of the allergen) is a popular approach. In an often-used format (commercialized as Pepscan^{R}), the peptides are synthesized on pins that can be tested in an ELISA-type protocol. A disadvantage of this, and other, *in-situ* synthesis systems is the problem of analysing the quality of the peptides. An low-technology approach is to use the 'tea bag' procedure, which enables the parallel synthesis of up to a hundred soluble peptides (42). However, automated synthesis of larger numbers of soluble peptides for use in peptide micro-arrays is also an option (43, 44). These techniques are often useful, but the results have to be interpreted with caution. Because most, if not all, IgE-binding epitopes are conformation dependent (see below), hits obtained by peptide scanning represent at best partial epitopes. Their similarity to the 'real' epitope should be studied as described for the epitope-grafted construct: by comparing (on a molar basis) the peptide with the native allergen in their potency to inhibit IgE binding to the peptide [see Fig. 2, based on (27, 45, 46)]. Some peptides can bind with high affinity to proteins, but the strategies for high-affinity binding are different from those of protein–protein interactions (47).

IgE reactivity with peptides is a form of cross-reactivity. As with conventional cross-reactivity, the number of peptides recognized by IgE in a serum is related to the level of allergen-specific IgE. The association described for peptide-reactive IgE and symptoms in food allergy (48, 49) is to some degree (perhaps completely) a reflection of this. An important reason why a peptide reacts well with a paratope is that it happens to have only a few dominant configurations, one of which happens to be close to the configuration of the most relevant amino acids in the folded protein. Peptides are a powerful tool in the analysis of the reactivity of polyclonal IgE antibodies, but it is important to carefully check the specificity of the interaction (by inhibition with the full protein) and the affinity of the interaction (by comparing on a molar basis the peptide in solution with the full protein in their capacity to inhibit IgE binding to the peptide). It is also relevant to check the completeness of the epitope repertoire found with peptides by attempting to inhibit IgE binding to the full protein by a mixture of all peptide epitopes. In our Fel d 1 experiments, the affinity of even the most potent peptide was several orders of magnitude lower than that of the protein, and the inhibition of IgE binding to the allergen by the peptide mixture was $< 30\%$. IgE reactivity to Fel d 1 is susceptible to reduction of its disulphide bridges (but reduced-alkylated Fel d 1 is not a better inhibitor of IgE binding to peptide, see Fig. 2), so it would be of interest to see data showing that peptides derived from other allergens (particularly food allergens) are able to inhibit more than 30% of the IgE binding to the properly folded allergen. However, Albrecht et al. (50) used IgE-binding peptides derived from peanut and shrimp allergens and also failed to find substantial inhibition.

The measurement of IgE-binding to natural variants or to recombinant variants subjected to site-directed mutagenesis is a useful procedure to map epitopes for polyclonal IgE antibodies. Loss of IgE binding because of point mutations is an indication that this residue is part of an epitope. Another way to identify IgE antibody-binding sites is by epitope grafting. In this approach, a chimeric protein is constructed from two proteins: the allergen (the donor) and a closely related protein without IgE binding from the serum in question (the acceptor). The aim is to introduce IgE binding to the mutated acceptor protein by replacing as few as possible amino acids from the donor protein. By comparing the chimeric protein with the native allergen in their capacity to inhibit IgE binding to the chimeric protein, the success of the grafting can be quantified. This grafting technique has been used by King et al. (51) with the aim of generating a monovalent (=noncrosslinking) variant of a vespid allergen. It was also used in the Albrecht study mentioned earlier (50). A hybrid tropomyosin carrying five IgE-binding regions of shrimp tropomyosin (Pen a 1) was grafted into the structural context of the nonallergenic mouse tropomyosin. However, one should be aware of the fact that point mutants or hybrids might have a modified overall 3D structure compared to the native allergen thus reducing or eliminating IgE binding to conformational epitopes. For a correct identification of IgE antibody-binding epitopes, these 'engineered' molecules need to be folded like the native allergen.

Structural mimics

Screening of a large number of random peptides for IgE-binding activity is also often used. These peptides are either chemically synthesized based on the linear sequence of the allergen, typically overlapping with an offset of < 8 amino acids, or from a (much larger) library of random peptides expressed on the surface of a bacteriophage, as discussed earlier in the mimotope section and illustrated in Fig. 1.

Epitope-blockade

If a monoclonal antibody to the allergen is available, it is informative to test whether this antibody is able to inhibit

IgE binding to the allergen. The most direct approach is to test the inhibition of IgE binding to the solid-phase-coupled allergen. The amount of allergen on the solid phase may have to be titrated down, and even then, it is not always possible to reach a maximum inhibition plateau (possibly because of the influence of the direct contact between the allergen and the solid phase). A more robust approach is to use the monoclonal antibody as a solid-phase-coupled allergen-catching reagent. IgE binding to this allergen–antibody complex can now be compared with IgE binding to allergen directly coated to the solid phase (52). Because the direct coupling is random, all epitopes will be available (if the allergen is in excess). This approach is analogous to the epitope mapping that is performed in optimizing monoclonal two-site ELISAs, using a coated catching antibody and an enzyme-labelled detecting antibody. For monomeric antigens, it is obvious that the second antibody should recognize an epitope that does not overlap with the epitope of the first antibody. Informative examples with mite group 2 allergen are found in (17, 53). Unexpectedly, the use of the same antibody both as catching and as detecting antibody does not work well with Fel d 1, which is a homo-dimer (of a hetero-dimer of chain 1 and chain 2) (54). For the mapping of IgE epitopes, the detecting antibody is now replaced by an IgE antibody. This configuration is sometimes referred to as 'chimeric ELISA' (55). It is also possible to perform this 'chimeric ELISA' in an inhibition mode. In this case, two monoclonal antibodies (that are selected to be directed to nonoverlapping epitopes) are used. One antibody is used as catching antibody in the chimeric ELISA, the other antibody to compete with IgE antibodies for the allergen. The read-out is with labelled anti-IgE, as in the standard chimeric ELISA.

Instead of using a monoclonal antibody, other specific ligands for the allergen may sometimes be used, e.g. binding of profilin to poly-L-proline, or an enzymatic allergen with its inhibitor. The resolution of such a competition-based epitope mapping depends on the size of the competitor. Particularly in the case of full-sized monoclonal antibodies, the chance of steric hindrance (rather than competition because of binding to the epitope) is a significant limitation of this approach. The use of Fab fragments or single-chain Fv fragments (or, even better, but not yet available: monoclonal single domain VH fragments derived from a llama or another cameloid) improves the resolution of this epitope mapping procedure.

Another approach to block IgE access to a well-defined part of an allergen is to use an allergen with an N- or C-terminal protein attached. This approach has been found to be informative in the case of the peanut allergen Ara h 2 with an N-terminal mannose-binding protein (40 kD) attached (56). Enlarging the distance between the fusion protein and the core of the allergen substantially increased IgE reactivity for a subpopulation of patients that did not react fully to the construct with the shorter linker peptide.

Are IgE epitopes special?

There is some evidence to support the notion of different epitope recognition by IgE antibodies, for example the suggestion that IgE antibodies tend to be more often cross-reactive than IgG antibodies (see Fig. 2). We will discuss three hypotheses: (i) IgE epitopes might have special structural features; (ii) IgE epitopes may have a tendency to cluster; (iii) the spectrum of IgE epitopes differs from the spectrum of IgG epitopes to the same allergen within one subject.

Structural features

An excellent review describing structural features of allergen epitopes has recently been published (57). In this paper, epitopes recognized by monoclonal mouse IgG antibodies as well as two epitopes (from lactoglobulin and from Phl p 2) recognized by cloned human IgE antibodies were discussed (see Fig. 3). There is evidence of a flat or moderately concave shape of the antigen-combining sites in the beta lactoglobulin and Phl p 2 in complex with specific Fabs from a combinatorial library constructed from lymphocytes of allergic patients (58, 59). According to both studies, IgE-binding epitopes would tend to be flat (like The Netherlands), whereas the known nonallergenic epitopes are more like Switzerland, with mountains and gorges. In more scientific terminology, IgE antibody-binding epitopes have the tendency to be planar rather than protruding/ convex (58). However, based on the data available (including the information that many allergens do not have a flat surface area, and certainly not several, as would be required if all IgE epitopes were to have this feature), there is not yet enough evidence to assume that the structure of human IgE antibody-binding epitopes is dramatically different from the structure of other B-cell epitopes.

Another interesting possibility is suggested by the structure of the mouse IgE antibody called SPE-7 (6), shown is Fig. 1. This antibody was raised against a hapten (DNP), but was found to be reactive to other, not obviously similar, haptens. Structural analysis of this antibody in complex with different haptens indicated that the paratope of this antibody has multiple configurations [antibody isomers (60)]. It is not known yet how common antibody isomerism is, nor whether it is more often found for IgE paratopes.

Spatial clustering

A polyclonal antibody to the 25-mer C-terminal peptide of Lol p 1 (a protein with two-domains, which are not closely interacting) was found to inhibit basophil histamine release by more than 90% (61). These studies were confirmed and extended by Flicker et al. (62) by the use of well-defined monoclonal antibodies. In another study by the same group (63), a recombinant antibody derived from a cloned human IgE antibody to Phl p 2 (of which the immune complex structure was described earlier) was found to inhibit sometimes more than 80% of the IgE antibodies of other grass-allergic patients. The inhibition by a full-size antibody was considerably higher than by a Fab fragment, which indicates that the antibody blocks a number of different, but spatially clustered, epitopes. Such clustering of IgE epitopes was also found in the earlier-mentioned study of Phl p 1 epitopes (62). While these results clearly support the hypothesis that at least

for some allergens IgE epitopes are not randomly distributed over the allergen surface, it is also clear that an allergen must have several nonoverlapping IgE epitopes to cross-link IgE receptors an sensitized mast cells. Experimental data supporting this for grass pollen group 1 allergens have been provided by the studies by Hiller et al., summarized in Ref. (64).

Intra-individual differences between the IgE and IgG(4) allergen epitope repertoire

It is often implicitly assumed that the spectrum of allergen-derived IgE epitopes does not fully overlap with that of epitopes recognized by other isotypes. This goes much further than the superficially similar statement that not all antigens are allergens. The latter effect is most easily explained by the observation that not all antigens induce a sufficiently polarized Th2 response to induce B cells to switch to IgE. At the epitope level, this argument no longer holds. The T cell is not so obviously involved in the selection (or lack of selection) of a particular paratope on the surface of the responding B cell. Yet, there are some indications that there are differences in the epitope repertoire for IgE and IgG4 (for the sake of discussion, we will focus on IgG4 as example of a Th2-responsive non-IgE isotype). As a model system, we compared IgE and IgG4 reactivity to variants of the cat allergen Fel d 1 (Fig. 2). We used a natural variant (the homologous protein from the ocelot), we used a synthetic peptide derived from the Fel d 1 sequence reactive with Fel d 1-specific sera (26), and we used two recombinant fragments (each of the two chains that the allergen is made of) (46). In each of these models, we found that the fraction of Fel d 1-reactive IgE that was reactive with the modified structure was 2–3 times higher compared with IgG4. Or, to put it more clearly, IgE was found to be more cross-reactive than IgG4 (43). One technical aspect is still to be resolved: to what extent avidity effects because of the monovalency of IgG4 (65, 66) might result in an underestimation of the IgG4 antibody levels in solid-phase assays with allergen variants with a lower intrinsic affinity. We are currently investigating this issue.

What could be the immunological explanation for different epitope recognition?

In earlier papers (67, 68), we argued that four features contribute to the special features of the developmental path of the IgE-committed B cell, leading us to the following working hypothesis:

From the naïve B cell two routes lead to IgE, which differ in the number of class switches. The direct switch from IgM to IgE is favoured for weak antigenic stimulation (69). It is the most relevant one for the initial sensitisation in atopic allergy (e.g. pollen and house dust mite allergens). The indirect switch (via one or more IgG intermediates) is the main pathway upon strong antigenic stimulation (69), including most animal model systems. In humans, it is responsible for the 'modified Th2 response' and for the transient post-seasonal IgE increase originating in the airway mucosa (70). For the direct switching pathway, the paratope repertoires of IgE and IgG are largely independent, whereas for the indirect switching pathway, the paratopes of IgE antibodies are a subset of the IgG paratopes.

The IgE switch is a first and large stumbling block, particularly for the direct switching pathway (71).

Following the hurdle of the IgE switch, there is another challenge for the IgE-committed B cell. In a fully active germinal centre the IgE switched B cell has a short life span, unless it rapidly initiates its terminal differentiation to IgE-secreting plasma cell and finds a survival niche, preferably in the bone marrow (alternative survival sites may be available in some mucosal tissues). In the absence of substantial proliferation, this results in a small clonal size (72), little opportunity for affinity maturation and a few IgE-switched memory B cells.

Upon subsequent allergen exposure, complexation of the allergen with the pre-existing circulating IgE antibodies facilitates the recruitment of additional naïve B cells.

If the hypothesis is correct that a weak antigen signal promotes IgE (possibly because it is insufficient to activate regulatory circuits for which the IgE response is very susceptible), would this result in a difference in IgE vs IgG epitope recognition? The human antibody response to classical atopic allergens such as Lol p 1 (73) and Der p 1 (74) is weak, in the sense that the majority of the human population develops very little if any antibody at all. If an antibody response develops, it is typically a Th2 response. The reasons for the absence of a strong response are speculative. Low exposure is likely to be one of the reasons. A weak antigen signal is known to instruct dendritic cells to signal T cells to initiate a TH2-polarized response (75, 76) and affects another T-cell population as well (77). This would have an effect on the B-cell response to the antigen as a whole, but not necessarily at the level of B-cell paratope specificity. The recruitment of a B cell in this immune response requires this weak antigen signal to activate the B-cell receptor. It is now well established (but not generally appreciated) that antigen presentation is important not only for T-cell activation, but also for B-cell activation (78). Not only is this antigen presentation to B cells important for their initial activation, it is also involved in affinity-related cell fate decisions following somatic hyper-mutation in the germinal centre (79). This weak signal in combination with the TH2 signals suffices to initiate a B-cell response, including class switching and somatic hypermutation, but does not induce the anti-apoptotic mechanisms that the B cell requires to survive in the long run to become a memory cell. Hypothetically, the IgE-switched B cells are less influenced by positive selection, because positive selection might not be able to rescue IgE-switched B cells (in contrast to IgG-switched B cells, in which anti-apoptotic factors are induced). The alternative pathway for the B cell is to initiate its terminal differentiation pathway and becomes a plasmablast. This plasmablast moves out of the germinal centre and may circulate for a short time in the circulation to find a survival niche. If this fails, the cell will die within a few days.

This weakness of the signal could be related to the antigen dose, to the milieu in which these reactions occur (including

the presence of substances such as TLR ligands entering the body together with the allergen acting as positive or negative factors) and/or to some intrinsic property of the allergen itself. A flat, featureless surface (which may be a feature of IgE epitopes, but this is at the moment still based on very limited information) is not an attractive target for the BCR and might thus act as an intrinsically weak signal.

Following the initial sensitization, subsequent allergen contact will result in IgE-facilitated antigen presentation, both to T cells and to B cells. Presentation of the allergen via IgE complexation followed by binding of the IgE-allergen-complex to CD23 on an antigen-presenting cell that is recognized by B cells is a potential (but hypothetical) way of influencing the epitope specificity of the ensuing B-cell response. Because the IgE in the IgE-allergen complex shields the epitope that was recognized during the initial sensitization, a nonshielded part of the allergen surface will be preferentially recognized. This will increase the epitope repertoire and thus the chance of cross-reactive antibodies. The IgE-switched B cell participates in the somatic hypermutation process, but exits the germinal centre without proper selection. This would explain why IgE antibodies have been found to be often auto-reactive (80).

Perspectives

The development of a database of the complete human IgE epitope repertoire is not a realistic option. It is also unlikely to be useful. A more interesting possibility is to study the IgE epitope repertoire within one individual at one point in time for a few selected allergens, each with a known 3D structure.

In principle, predicting the structure of an epitope from its paratope is like reverse engineering. Analysing IgE paratopes in plasma is still beyond our analytical capabilities, but the rapidly developing mass-spectroscopic techniques may in the future help us out. Using double affinity purification under stringent conditions, it may become possible to obtain sufficient (and sufficiently pure) epitope-specific IgE, perform a sequential fragmentation and determine the amino acid sequence of fragments based on their mass (with leucine/ isoleucine and other equal-mass ambiguities remaining).

At the moment, the more realistic option is to use cDNA technology to target the B-cell repertoire rather than the antibody. First, build the paratope repertoire by generating cDNA (both VH and VL) from single B cells/plasma cells. Allergen specificity can be achieved by allergen-specific selection of the cells. This can be achieved (with some difficulty) by FACS, but also by limiting dilution cultures of the (small numbers of) preplasma cells present in the blood, which at a single-cell level spontaneously secrete sufficient IgE to allow allergen-specific identification (L. K. Poulsen, personal communication). Alternatively, the full set of Fv fragments can be expressed on the surface of a phage to select Fvs with specificity for the allergen. The next, currently unavoidable, step is the expression of the Fab fragment as a recombinant protein and determination of the 3D structure of the Fab-allergen complex. To get access to the full repertoire of the B cells/ plasma cells involved in a particular immune response, circulating memory B cells are the obvious first choice. However, IgE-switched memory B cells are extremely rare and are unlikely to be able to provide the current IgE repertoire in full. We have already some information for the epsilon VH repertoire from RNA isolated directly from peripheral blood cells (without culturing the cells, to avoid in vitro class switching) (81–86). Expression of the VH from the patient's cells in combination with a suitable VL from a light-chain bank (rather than from the same IgE-producing cell), production of the Fab fragment and resolving the 3D structure of the Fab in complex with the corresponding allergen has been published for lactoglobulin and Phl p 2, as mentioned earlier (58, 59). So, the first steps to an individual's epitope repertoire have been taken. However, to get to the full epitope repertoire is currently a huge undertaking for even a single allergen from a single patient, because of the need for the Fab 3D structure. We will presumably have to wait until we (i) are able to predict the Fab structure from the primary sequence and (ii) have a reliable in-silico docking procedure to identify the epitope from the amino acid sequences of VH and VL. Only then, we might be able to get close to an epitope repertoire of the peripheral blood cells. However, this is still a distant dream.

This is not the whole story. An important question will be how well this repertoire covers the repertoire of the plasma cells in the tissues. This may perhaps in the future be addressed by investigating IgE paratopes at the protein level, by analysis of allergen-specific IgE in plasma and other body fluids as described earlier.

There is another approach to compare the peripheral blood repertoire with the full repertoire: the analysis of plasma cells in postmortem material using the cDNA route described earlier. In addition to informing us about the IgE epitope repertoire, it may reveal (via analysis of the switch region in the cDNA (87) the precursor of the plasma cells: a naïve IgM-positive B cell, or an IgG intermediate B cell. By combining this information with information on the repertoire of genuine IgE memory cells, it will become clear how the long-lasting IgE antibody level is maintained. We will find answers to important questions on the ontogeny of these plasma cells and thus about the way allergic sensitization develops and, hopefully, can be prevented and treated.

Conclusion

Back to Benjamin et al. (1), Our current position is: yes, all parts of a protein surface can be part of an epitope, but some parts are more often involved than others. This implies some kind of hierarchy. A similar form of hierarchy is found in allergenicity: every antigen can become an allergen (given the right conditions), but some antigens are more likely to induce IgE than others. And for IgE epitopes, we reach the same conclusion, albeit with even more the character of a working hypothesis: all epitopes can induce IgE antibodies, but some are more likely to do so than others.

To understand allergenicity, we have to adjust our way of thinking, from a dichotomy (yes/no) to a grading (low-grade to high-grade). To be able to do such grading, we need to invest in obtaining detailed information on different aspects

of the allergic response. We need detailed information on (i) the paratope repertoire of allergic patients, (ii) detailed structural information on the paratope/epitope interaction and (iii) biological activity in cellular systems such as basophil degranulation. We need experiments as reported by Christensen et al. (17) with more structural information on the epitope/paratope interaction and, in the end, find ways of fitting this information with clinical and epidemiological data.

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Conflicts of interest

The authors have no conflict of interest to declare.

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