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Review

Requirements for effective IgE cross-linking on mast cells and basophils

Edward F. Knol

Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands

This article reviews the characteristics of high affinity IgE receptors (FcεRI) and their role in the response to allergenic proteins. The requirements for successful cross-linking of FcεRI on basophils and mast cells and subsequent degranulation by allergenic proteins will be explained in detail. Methods for *in vitro* analysis of allergen-induced mast cell and basophil degranulation will be described and issues/problems in applying these methods will be discussed. Finally, implications for manipulation of protein allergens will be discussed.

Keywords: Allergen / Cytokines / Degranulation / Histamine

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1 Introduction

One of the characteristics of an allergenic protein is that it not only induces specific IgE production but also contains multiple IgE binding epitopes, so that IgE on mast cells and basophils can be cross-linked. As a result of IgE cross-linking, mast cells and basophils can release their well-known inflammatory mediators and cytokines, such as histamine and TNF-α. In this review, I will introduce the specific characteristics of the high affinity IgE receptors (FcεRI) on basophils and mast cells that will explain the detailed requirement for a successful FcεRI cross-linking and subsequent degranulation by the allergenic proteins. In addition to potential pitfalls in the *in vitro* analysis of allergen-induced mast cell and basophil degranulation, implication of the FcεRI cross-linking requirements for protein manipulation will be discussed.

2 IgE receptor

IgE is bound *via* its Fc part to a receptor on the basophil and mast cell plasma membrane [1]. This receptor is called the FcεRI. Studies of the interaction between IgE and FcεRI have shown that the stoichiometry of the interaction is 1:1 [2] and that the interaction is of high affinity ($K_d = 10^{-9}$–10^{-10} M) [3]. In addition, mutational analysis indicates that the third Ig constant domain (Cε3) of IgE forms the contact point between receptor and ligand [4].

The high affinity characteristics are primarily the result of slow dissociation of the IgE [5]. In a mouse model it was demonstrated that after IgE binds to FcεRI on mast cells, IgE remains on the mast cells in a fully functional state for at least 6 wk, even in the complete absence of exogenous IgE [6]. The amount of IgE on the basophils varies greatly among different donors, ranging from 6000 to 600 000 molecules *per* basophil, and appears to be related to the concentration of IgE in the serum [7]. The number of FcεRI *per* basophil varies between different donors (range 29 000–680 000) and is also related to the IgE concentration in the serum [8]. Removal of IgE by anti-IgE immunotherapy has revealed that it is IgE itself that directly regulates FcεRI expression [9]. Ligand-mediated FcεRI up-regulation by IgE is biphasic: first IgE stabilizes receptor complexes at the surface and protects them from degradation. Later, when all intracellular FcεRI is at the surface, continued expression is maintained by the synthesis of new complexes from pre-existing transcripts [10].

FcεRI consists of four polypeptide chains, αβγ2, which together form an integral membrane protein complex with seven transmembrane segments (Fig. 1). The extracellular sequence of the 50–60 kDa α-chain contains two domains, α1 and α2, that belong to the immunoglobulin superfamily.

Correspondence: Dr. Edward F. Knol, Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands
E-mail: e.f.knol@umcutrecht.nl
Fax: +31-30-2505404

Abbreviations: ITAM, immunoreceptor tyrosine-based activation motifs; **PTK,** protein tyrosine kinase

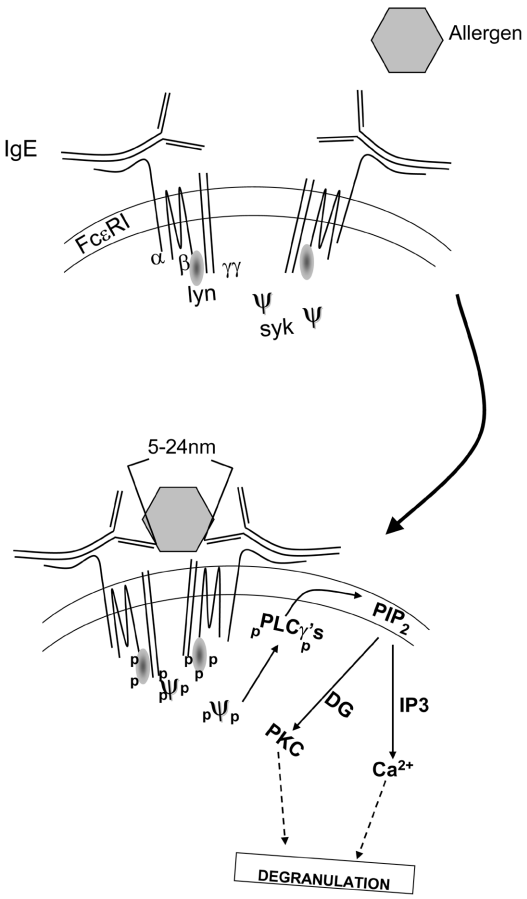

Figure 1. FcεRI cross-linking and signal transduction. FcεRI on basophils and mast cells consists of four subunits, an α subunit with one transmembrane region, a β subunit with four transmembrane regions, and two γ subunits, each with one transmembrane region and a very short extracellular region. Cross-linking of IgE bound to FcεRI by allergen leads to clustering of the receptor on the cell surface, which induces a cascade of tyrosine phosphorylation events. This results eventually in activation of PLCγ and finally degranulation.

Of these domains α2 is directly involved in IgE binding, but maximal affinity is observed only when both domains are present. Moreover, the transmembrane part of the α-chain is the most conserved region and shows strong homology with the transmembrane domain of the FcγR-III [11].

The 33 kDa β subunit consists of 243 amino acids, comprising four transmembrane domains, two extracellular loops, and a single cytoplasmic loop, and stabilizes the FcεRI complex [10]. The β subunit is lacking in the FcεRI of Langerhans cells, monocytes, and dendritic cells [12]. Together with the two 7–9 kDa γ subunits [13], the β subunit acts synergistically to promote signal transduction through the FcεRI. Each γ chain contains a transmembrane structure, including a small (five amino acids) extracellular domain, a 21 amino-acid transmembrane component, and a cytoplasmic part of 36 amino acids. In one FcεRI, two γ chains are linked *via* a disulfide linkage [14]. The γ chain is not exclusively expressed in connection with the FcεRI, because it is also a component of the FcγR in macrophages, natural killer cells, and neutrophils [15]. Moreover, there is strong homology with the ζ chain of the T-cell receptor [16]. These chains are also involved in signal transduction from the receptors to the cell interior. The β and γ-chains of the FcεRI complex contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are a conserved feature of the antigens and some Fc receptors. The ITAM consensus sequence is *D/E-XX-YXXL-X*$_{7-11}$*-YXXL-L/I*, where the tyrosine residues are phosphor-acceptor sites for the action of receptor-associated protein tyrosine kinases (PTKs) [17]. Phospho-ITAMs provide a docking site for cytoplasmic proteins that contain the Src-homology-2 (SH2) domain and hence link receptor and signal transduction cascades, as the SH2 domain has high affinity for phosphorylated tyrosine residues [18]. In the context of FcεRI, the β- and γ-chain ITAMs have slightly different structures. The β-chain ITAM is notable owing to its differences from the consensus ITAM sequence, the presence of a third tyrosine between the canonical tyrosine residues, and the short length of its spacer region. The β- and γ-chain ITAMs also have distinct functions. There are two species of FcεRI-associated PTK – the *src* family kinase Lyn and the p72Syk kinase. The former is found associated with FcεRI Iβ, whereas the latter is capable of binding both FcεRI β and FcεRI γ but has higher affinity for interaction with FcεRI γ [10] (Fig. 1).

3 FcεRI signaling events

Cross-linking of the FcεRI initiates a chain of phosphate transfers within the receptor microenvironment. The mechanism by which α-chain aggregation is sensed by the signaling subunits is still unknown. Lyn can be constitutively associated with FcεRI, but is incapable of phosphorylating the receptor subunits in the absence of aggregation. One of the first signals is the activation of β-chain bound Lyn [19]. The model of transphosphorylation by Metzger and coworkers [20] proposes that Lyn phosphorylates an adjacent FcεRI following aggregation of at least two receptors. In contrast, in a model by Baird and coworkers [21], it is stated that Lyn is constitutively active in so-called lipid rafts and that cross-linking of FcεRI promotes its proximity to active Lyn in these lipid rafts [21]. Subsequently, Lyn phosphorylates the β and γ-chain ITAMs (Fig. 1). The phosphorylated γ-chains then recruit the PTK-Syk, which upon binding to the two phosphorylated ITAMs tyrosyls also undergoes activation [10]. This mechanism illustrates the requirement that clustered FcεRIs remain in close proximity for at least the time required for these processes to take place.

The activated Syk subsequently tyrosine phosphorylates phospholipase C-γ1 and phospholipase C-γ2. These phos-

phorylalated phospholipase Cγs catalyze the hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. These second messengers promote release of Ca^{2+} from internal stores and activate PKC, respectively. Both events are essential for FcεRI-mediated degranulation [22] (Fig. 1).

4 Cross-linking the receptor

Monovalent binding of IgE to FcεRI has several consequences including: (1) increasing receptor expression, (2) increasing mast cells resistance to apoptosis, and (3) under specific circumstances, inducing cytokine release [23]. However, for degranulation, in order to activate mast cells through the IgE receptor, this receptor must be clustered by divalent antigens at a minimum. Importantly, the minimal requirements for release of mediators by natural allergens have not been reported. Artificially cross-linked IgE or linear polymers of DNP-lysine binding to DNP-specific IgE have provided insights in to the FcεRI clustering process. For example, the capacity of covalently cross-linked IgE, including dimers and higher order oligomers, to cluster FcεRI on the rat basophilic leukemia cell line RBL-2H3 cells was determined, and the role of the size of the cluster was investigated. While a rather limited secretory response was observed following stimulation by IgE-dimers (5% maximum), a significant response was induced by IgE-trimers (15%) and higher oligomers (25%). The view that only the size of the cluster determines the strength of the degranulation signal is too simplistic [24]. Other characteristics of the FcεRI clustering have to be taken into account. For instance, the lifetime of the cluster must exceed a distinct threshold. Within this model system, the threshold is approximately 100 s and is probably required to allow transmembrane signaling to complete. In addition to receptor affinity, the dissociation characteristics of the antigen–IgE complex is important [24].

Only a relatively small fraction of FcεRIs needs to be clustered in order to produce a significant secretory response. Both theoretical analysis and experiments using mast cell (line)s suggest that a half of the saturation concentration of active dimers of $\sim 7.8 \times 10^{12}$ M is required. This corresponds to 0.26% of the cell's FcεRI (10^2–10^3) of both mast cells and basophils [24, 25].

5 Properties of antigens to induce FcεRI-mediated mast cell and basophil degranulation

Mast cell/basophil degranulation has been discussed with regard to the properties of both FcεRI and IgE binding. At the other end of the spectrum, the antigens/allergens that induce degranulation will now be introduced and discussed.

It is clear that both cellular (FcεRI-IgE) and protein (allergen) characteristics determine the final outcome of the interaction. The optimal conditions for release of histamine depend on the concentration of antigen-specific IgE antibody on the membrane, the concentration of the antigen, and the affinity of the IgE for the antigen. *In vitro*, as little as 1 ng/mL of ragweed antigen E (2.5×10^{-11} M) can activate basophils from ragweed-allergic individuals. The valency of the allergen is important to mast cell sensitivity, so that relatively low affinity IgE antibodies are able to promote degranulation upon challenge with high valency allergens [26]. Moreover, after an allergen binds one IgE, the second IgE binding event can be of lower affinity.

Small bivalent haptens with a different antigenic site at each end will activate cells, suggesting that the hapten bridges adjacent IgE molecules on the cell surface. The minimal distance between cross-linked IgE molecules is estimated to be 8–10 nm (=80–102 Å) and the maximal distance to make a stable contact is estimated to be 20–24 nm [27]. The average interreceptor distance in the absence of antigen has been calculated to be only ~50 nm, whereas the minimal distance between two receptors in a cluster that results in a degranulation signal is only 5 nm [28, 29]. However, these distances were calculated using artificial antigens, and natural allergens <10 nm have been described, suggesting that the minimum distance may be lower for natural allergens. However, the actual minimum and maximum distances for natural allergens have not been determined. Importantly, for the major birch pollen allergen Bet v 1, its dimerization is essential for IgE cross-linking in mice *in vivo* [30].

6 Pitfalls in the analysis of allergenic proteins

Technical pitfalls may influence the outcome of assays of the allergenic potential of proteins, regardless of whether the assay system involves an animal model or an *in vitro* degranulation assay. In mice, IgG immune complexes can trigger mediator release from mast cells by signaling through an FcγRIII α, β, γ2 structure, as when FcεRI enables allergic anaphylactic reactions in IgE deficient mice [31–33]. Human mast cells and basophils do not express FcγRIII [34]. So whenever allergen-specific IgG antibodies are administered or induced in a murine model, a false-positive result will be obtained. In contrast, in human mast cells and basophils, allergen-specific IgG antibodies can even inhibit FcεRI-induced degranulation, because these cells bear FcγRII. The principal FcγRII species in basophils and mast cells is the ITIM-containing FcγRIIB [35]. When simultaneous FcεRI and FcγRII are primed by

anti-DNP IgE and anti-DNP IgG degranulation, IL-4 production, Ca^{2+} mobilization, and Syk phosphorylation in human basophils are inhibited [36]. Also allergen-specific IgE reactivity toward the major cat allergen Fel d1 can be inhibited by coaggregation of FcεRI and FcγRII [37]. In evaluating the allergenic properties of specific proteins in mast cells or basophils, the inhibitory role of FcγRIIB will not be a problem when serum-preloaded cells are studied, because the affinity of the FcγRIIB is too low to bind monomeric IgG antibodies. However, when allergens are preincubated with specific IgE and IgG containing serum, IgG will also bind to the allergen and will diminish subsequent activation of basophils/mast cells with the preincubated allergens. The latter approach should therefore be avoided in evaluating the allergenic potential of proteins.

There is a wide variability in the extent of histamine release from the cells of both atopic and nonatopic individuals. This phenomenon has been termed releasibility [38]. The releasing phenotype of a specific individual is relatively constant in time and about 10–20% of the individuals have basophils in their peripheral blood that release <10% of their histamine content after FcεRI cross-linking [39]. The mechanisms behind this phenomenon are not clear, because other activation signals are within normal range after FcεRI cross-linking [40]. However, Syk is selectively decreased in nonreleasing basophils [41]. To control for these nonreleasing basophils, it is important to include a positive control in the analysis of potential FcεRI cross-linking allergens, such as a polyclonal anti-IgE antibody (monoclonals are in general poor inducers) or an anti-FcεRI antibody.

In conclusion, there are several potential technical problems in analyzing the cross-linking properties of allergens on basophils and mast cells, including the fact that basophils are relatively difficult to isolate and to handle. Therefore, these analyses should only be performed in research groups with sufficient expertise, and even then, it is unclear how the information obtained can be used for standardization.

7 Manipulation of the allergenic properties of allergens

Several approaches have been developed to remove or diminish the allergenic properties of a protein. A few typical examples will be introduced. First, after establishing the IgE-binding epitopes in a protein, one can selectively engineer recombinant proteins in which the IgE binding epitopes are eliminated or reduced. For example, the peanut proteins Ara h 1, 2, and 3 were recently engineered with altered IgE-binding epitopes [42]. These modified proteins bind minimal IgE Abs from peanut-allergic patients, although they promote T-cell proliferation to a similar extent as native peanut allergens. Similar results were obtained for the major birch pollen allergen Bet v 1 and the timothy grass pollen allergen Phl p 5b [43, 44].

Another approach is to reduce the size of the protein sufficiently, so that FcεRI can no longer be cross-linked. This approach was used for the Fel d 1 protein to minimize cat allergy [45], although the T-cell activating properties of the modified Fel d 1 peptide still induce short-term clinical symptoms.

In general, the methods mentioned above aim at leaving the T-cell reactivity intact. In this way, the immune system might be directed away from an IgE inducing response and toward an IgG inducing response or even a more tolerating response. This is also the result of classical allergen immunotherapy, in which the allergen as such is not manipulated, but the allergen is administered in an immune-manipulating adjuvant to minimize the IgE-inducing characteristic of the allergen-specific response [46].

8 Summary of the specific IgE binding properties of an allergen

In brief, for a protein to possess allergenic properties, it has to contain at least two IgE binding sites and enable cross-linking of FcεRI on the cell membrane of basophils or mast cells. The number/duration of cross-links *per* basophil or mast cell should be at least 100 for at least 100 s, to induce mast and/or basophil activation, subsequent degranulation and the release of inflammatory mediators such as histamine.

9 References

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