Dependence of Optimal Histamine Release on Cell Surface IgE Density on Human Basophils: Nature of the Stimulus

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Abstract

Background: The characteristics of the aggregation reaction that follows allergen binding to cell surface IgE on basophils and mast cells depend on a variety of factors that include the density of IgE and the affinity of the allergen for IgE. For simple bivalent stimuli, one prediction is that the location of the optimum for aggregation is not dependent on IgE density, only the affinity for IgE. However, this behavior does not occur for stimulation with an anti-IgE antibody (Ab) during the treatment of patients with omalizumab. **Methods:** This study re-examined the stability of the optimum for histamine release, relative to cell surface IgE density, using the simple bivalent penicillin hapten (BPO₂) or a bivalent monoclonal anti-IgE Ab. **Results:** The results validated one prediction for one bivalent hapten, BPO₂. Across a range of BPO-specific IgE density of 270–23,500/cell, optimal histamine release remained constant (10 nM BPO₂). In contrast, across a range of approximately 6,000–110,000/cell, optimal histamine release shifted 8- to 30-fold for anti-IgE Ab. The distinguishing characteristic between the 2 bivalent stimuli was the differ-

ence in their crosslink re-equilibration. Recent modeling of histamine release suggested that the SYK-to-receptor ratio could determine the position of histamine release optimum. The study showed that there were significant shifts in the SYK-to-receptor ratio (from 1:6 to 5:1) but the basophil's ability to sense this ratio was restricted to transient crosslinks, as occurred with anti-IgE Ab. **Conclusions:** The results suggest that ligand crosslinking dynamics couple with SYK and receptor expression levels to determine qualitative characteristics of the dose response curve for secretion.

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Introduction

IgE-mediated secretion from mast cells and basophils is a reaction dependent on the ability of allergens to induce the aggregation of cell surface-bound IgE. The reaction can be quite complex because branched aggregates of the allergen (with its myriad of different epitopes) and IgE antibody (Ab) (with its myriad specificities and affinities

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for allergen epitopes) occur, and these aggregates can be quite large in size [1]. Allergic disease research often uses pan-crosslinking stimuli like anti-IgE or anti-FcεRI Abs to induce the activation of basophils or mast cells. In the early years of study, polyclonal anti-IgE Abs were used to explore characteristics of basophil or mast cell secretion but today, monoclonal Abs are often used. One monoclonal Ab in common use behaved in ways that are not currently understood, and this study sought to determine if there were properties of this reagent that revealed more subtle aspects of the IgE-mediated basophil response.

It is difficult to analytically model allergen-induced crosslinking reactions due to the branched nature of the aggregates formed on the cell surface. But it is possible to analytically model simple bivalent antigens [2, 3]. For example, the simplest antigen derived from a known allergen would be bivalent penicillin (BPO₂), 2 penicilloyl groups linked by a 6–10 carbon aliphatic linkage. This type of molecular species can only form linear aggregates, of variable size, of IgE. With a simple model in hand, it is possible to make some predictions about the characteristics of secretion induced by this simple ligand. One such prediction is that the optimum for secretion occurs at 1/2K_a, where K_a is the intrinsic one-site affinity constant between hapten and IgE Ab [2]. This prediction has been shown to be correct [4]. A second expectation for the aggregate size distribution is that the position of the optimum for maximum crosslinks is not dependent on the density of antigen-specific IgE on the cell surface [3]. A very limited examination of this prediction suggested it was probably true as well [3].

In this context, recent studies on the responsiveness of peripheral blood basophils to stimulation with the crosslinking anti-IgE Ab, 6061P, during treatment with omalizumab, were not expected [5]. The 6061P monoclonal Ab is a monomeric mouse IgM (i.e., it is not a pentamer of the single Ab unit). As such, it should act as a bivalent crosslinker and therefore follow the rules described for other bivalent haptens. During treatment with omalizumab, the relative absence of free IgE reduces the density of receptor and bound IgE on basophils and mast cells [6–8]. The position of the peak response to stimulation with 6061P Ab shifts to 200-fold higher concentrations [5]. As noted, the position of the optimum should not be sensitive to the cell surface density of IgE. Therefore, the observed behavior is not understood and warranted closer study, because it might be indicative of signal transduction reaction behavior that separates the characteristics of aggregate formation from the secretory reaction. Although the prediction of a nonshifting optimum was pre-

viously examined experimentally, only 1 experiment was done to test the prediction and the conditions were not well defined [3]. In fact, more recently, it was shown that a more complex aggregation reaction, stimulation with multivalent BPO-HSA, was found to be sensitive to the density of BPO-specific IgE with a rightward shift at lower densities [9]. This is a far more complex reaction and the predictions less clear for the dependency on IgE density, but it suggested that the original prediction needed a closer inspection. These studies began with this question and then explored one potential explanation for the optimum shift with 6061P Ab. The results suggested that there were other characteristics of the basophil response and aggregation that needed exploration, and so the second half of the report examines 2 candidate explanations for the experimental observations.

Methods

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF, Na₄P₂O₇, Na₃VO₄, 2-ME, NP-40, and FMLP (Sigma, St. Louis, MO, USA); crystallized HSA (Miles Laboratories, Elkhart, IN, USA); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD, USA); Percoll, (Pharmacia, Piscataway, NJ, USA); anti-SYK mAb, 4D10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); a penicillin (BPO)-specific IgE was partially purified from the sera of penicillin allergic patients as previously described [7]; BPO₂ (benzylpenicilloyl-di-octamine) was synthesized and purified as described previously [10, 11]; monoclonal anti-human IgE Ab (6061P) (Hybridoma Labs, Baltimore, MD, USA); anti-HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated sheep anti-mouse Ig Ab, protein G sepharose beads (Amersham Life Science, Arlington Heights, IL, USA); monoclonal anti-FcεRI Abs 22E7 and 15A5 (a gift of former Hoffman-La Roche; measured total FcεRI and unoccupied FcεRI, respectively). Countercurrent elutriation and labeling with Abs for flow cytometry was conducted in PIPES-albumin-glucose (PAG) containing 0.25 % BSA in place of 0.003% HSA. ESB is a Novex electrophoresis sample buffer containing 5% 2-mercaptoethanol. SDS stripping buffer for Western blots was 65 mM Tris (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS.

Buffers

PAG buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Lactic acid buffer for removing endogenous cell-bound IgE consisted of 0.01 M lactic acid, 0.14 M NaCl, and 0.005 M KCl (pH 3.9) [12].

Basophil Isolation, Sensitization, and Histamine Release Reaction

Blood was partially enriched for basophils using single-step Percoll gradients. After washing to obtain a mononuclear cell

preparation, cells were resuspended in saline one centrifugation before treatment for 15 s to 2 min with lactic acid stripping buffer. After washing the cells once, they were aliquoted and sensitized at 4 °C for 1 h with anti-BPO-IgE at several concentrations or with a monoclonal IgE (SE44, anti-gp120-specific IgE). After 1–3 washes (depending on the sensitization reagent), the cells were resuspended in PAG (to prepare for fixation and flow cytometry) or PAGCM buffer (to assess histamine release). Total histamine content was obtained by lysis of the cell preparation with 1.6% perchloric acid. These experiments included 2 different buffers for stimulation. The standard measurement is in PAGCM buffer, but a second condition included 44% deuterium oxide (D₂O) to replace the water in the PAGCM. D₂O is known to enhance secretion of any type of stimulation [13, 14].

Histamine Measurements

Histamine was measured by automated fluorimetry [15]. The percentage of total histamine release was calculated after subtraction of spontaneous histamine release.

Basophil Cell Surface IgE Measurement

An aliquot of cells was used to determine the total basophil count by Alcian blue staining, and the remaining cells were treated with acetate buffer (pH 3.7) to dissociate endogenous IgE. The recovered supernatant was neutralized to pH 7 and IgE concentration was measured by a total IgE assay (ImmunoCAP). A calculation of the number of IgE molecules present and the cell number allowed the cell surface IgE density to be determined. Calibration of the Ab (22E7) used to detect FcεRI by flow cytometry involved a comparison of flow cytometric results with an explicit measurement of IgE eluted from a counted number of basophils, as described previously [16]. The flow cytometric results were then equated with this measured density of IgE/basophil ([17] offers a more complete description of the calibration procedure). To calibrate the signal that detects BPO-specific IgE, 1 aliquot of cells was sensitized with a high concentration of BPO-specific IgE and 1 at a lower concentration. The cells were labeled with either 15A5 (or appropriate isotype control Ab) or by the procedure for detecting BPO-specific IgE [18] (see below). By flow cytometry (and running with pre-established PMT settings and calibrating against a standard bead), the net difference in the BPO-specific signal between the 2 loading conditions, and the net difference in the 15A5 signal was determined (as the BPO-signal increases, the 15A5 signal decreases). Since 15A5 has been calibrated against an absolute receptor measurement, the delta for BPO-specific signal and the delta for the 15A5 signal sets the equation: BPO = 15A5*(calibrated value for 15A5).

To assess the BPO-specific IgE density, basophils were sensitized with penicillin-specific IgE; its presence was detected with a sandwich assay. Prior to fixing the cells, the suspension was chilled to 4 °C, 10 µg/mL of BPO₃₂-HSA was added for 10 min, and the cells were washed twice in ice-cold buffer and then fixed with 2% paraformaldehyde for 20 min prior to blocking and storage overnight. Before flow cytometry, the fixed cells were incubated with rabbit anti-BPO IgG ± BPO-EACA at 1 mM. The presence of BPO-EACA (monovalent penicillin, penicillin coupled to ε-aminocaproic acid) generates a negative control for the measurements. A second background control was included; the labeling procedure above was also performed on cells not sensitized with BPO-specific IgE.

Assessing Binding Re-Equilibration

Leukocytes were stimulated with either BPO₂ or 6061P anti-IgE Ab. At intervals early in the reaction, the reaction mixtures were diluted 20-fold with buffer containing either (1) nothing (so the bivalent ligand concentration decreased 20-fold), (2) stimulus at the same initiating concentration (therefore, no dilution of the stimulus), or (3) EDTA (4 mM final to immediately stop the release). In the case of BPO₂ stimulation, an additional dilution series included 1 mM BPO-EACA (monovalent hapten). The reaction was allowed to proceed until the 30-min time point, at which point the suspension was centrifuged to recover the supernatant for the analysis of histamine.

Analysis of SYK Protein Expression by Intracellular Flow Cytometry

All analysis was performed on a BD FACSCalibur flow cytometer. The measurement of SYK protein expression by flow cytometry using the anti-SYK Ab 4D10 has been previously described and validated with respect to standard Western blotting [19, 20]. This method has been used on both impure as well as purified cell fractions with equivalent results. Briefly, fixed mixed leukocytes were labeled with anti-IL-3R (BD Biosciences, Franklin Lakes, NJ, USA) and anti-BDCA2 Abs (phycoerythrin and FITC, respectively) and permeabilized (Fix and Perm Kit, Caltag, Carlsbad, CA, USA) in the presence of 4D10 or IgG2a Ab. Cells were then incubated with an anti-mIgG2a-Alexa647 Ab (Molecular Probes, Eugene, OR, USA) to complete the labeling. SYK protein expression is reported as normalized net MFI, the difference between 4D10 and isotype-labeled cells was corrected for instrument variability using CaliBRITE APC calibration beads (BD Biosciences). As described previously, the flow cytometric method of determining SYK was calibrated against a quantitative Western blot that used a recombinant SYK protein as a standard.

Omalizumab Study

Data were drawn from 2 distinct omalizumab studies. Since this report is not concerned with the primary outcome metrics of either the nonclinical or clinical part of studies, the study design details will not be presented; these details can be found in the published study [5]. Informed consent was obtained via a protocol approved by the Johns Hopkins Hospital Institutional Review Board and the National Institute of Allergy and Infectious Diseases' Data Safety Monitoring Boards. The cells from the 1-step Percoll gradient were analyzed by flow cytometry for cell surface levels of FcεRIα and IgE, internal levels of SYK, or used to assess histamine release in response to stimulation with anti-IgE Ab.

Results

Sensitivity of Dose Response Curves to Cell Surface IgE Density

BPO₂ Dose Response Curve Optimum and Density

Basophils, enriched on either 1- or 2-step Percoll gradients, were treated briefly with lactic acid to dissociate a portion of the surface IgE and generate unoccupied receptors for sensitization with BPO-specific IgE. The den-

Fig. 1. Concentration-dependence of histamine release from basophils sensitized with 2 densities of benzylpenicilloyl (BPO)-specific IgE and stimulated with BPO₂. **a** Average of all experiments ($n = 10$). High-density (●) versus low-density (□) sensitization. **b** Subset of the experiments in **a** chosen to maximize the difference in BPO-specific IgE density ($n = 2$). **c** Using all the experimental results ($\pm D_2O$), the relationship of BPO-specific IgE density and the optimum of the dose response curve was plotted. The line fit (heavy) excludes the 2 lowest data points while the grey line includes all points.

sity of BPO-specific IgE was assessed with a new flow cytometric methodology. However, this method had not been previously calibrated for absolute levels, so this was performed (see Methods). The sensitization curve for BPO₂ is fairly steep, making it difficult, a priori, to choose 2 disparate densities of BPO-specific IgE that capture a

complete enough dose response curve to BPO₂ so that an optimum can be determined. A variety of relative concentrations of BPO-specific IgE for sensitization were tried. To maximize the difference in density ultimately required that cells sensitized with a high density of BPO-specific IgE be challenged in normal buffer, while those

sensitized with a much lower density were challenged in buffer containing 44% D₂O. This experimental change is a well-described way to enhance secretion [13, 21, 22], but it is possible that the conditions could distort the results. Therefore, a variety of experiments were done to explore various relative densities of BPO-specific IgE where the buffer conditions were symmetric or asymmetric with respect to the presence of D₂O, i.e., both conditions in standard buffer, both conditions in D₂O-containing buffer, or 1 condition with standard buffer and 1 with D₂O-containing buffer. In no case was there an observed difference in the position of the optimum for secretion. Indeed, these studies generally showed that the position of the optimum for secretion always occurred at 10 nM BPO₂, ranging from a density of 270 to 23,500 BPO-specific IgE/basophil across 10 experiments. Figure 1c plots the center point optimum as a function of the cell surface BPO-specific IgE density (the ordinate range is chosen to be similar to the range of shift observed with anti-IgE Ab 6061P below). A fit of the data suggests a slight inverse relationship, but this trend is driven by the 2 data points at the low end; the heavy fit line excludes these 2 data points. However, taking the results at face value, for a 100-fold range of density, at best, the optimum (8–10 nM for most results) shifts 2- to 3-fold or not at all.

For the analysis of paired experiments (2 sensitization conditions for the same subject), Figure 1 shows a couple of ways to average the results since the differential loading of the cells varied across experiments. Figure 1a averages all the experiments regardless of the buffer used (i.e., $\pm$ D₂O). In this plot, there is an average 4-fold difference in BPO-specific IgE density. It is worth noting that the EC₅₀ for the BPO₂ response, estimated here as approximately 2,500–3,000 molecules/cell, is similar to our previous measurements for BPO₂ (and BPO-HSA, where the EC₅₀ for BPO-HSA was 500–1,000 molecules) [23], suggesting that the calibration of the flow cytometric method was yielding results similar to our older method of measurement BPO-specific IgE. Figure 1b isolates 2 experiments that produced the greatest paired difference in BPO-specific IgE density, on average, 8-fold in these 2 cases. The results were similar, with no significant difference in the optimums for release.

6061P Dose Response Curve and Density

The most direct way of testing whether the density of IgE alters the optimum for release when using 6061P anti-IgE Ab is to dissociate IgE from basophils, and then resensitize the cells with different densities of IgE. In this

design, most factors that can alter the IgE-mediated dose response curve stay constant. Basophils were treated for 2 min at room temperature with lactic acid, and the cells were divided into those sensitized with 10 μ g/mL IgE (SE44, a monoclonal human IgE) in buffer containing 1 mM EDTA and 10 μ g/mL heparin (to aid in sensitization) at 4°C, or cells incubated in the same buffer but without IgE. After washing, a portion of the cells from each condition was set aside for flow cytometric measurement of cell surface IgE and the remainder were challenged with a range of concentrations of 6061P monoclonal anti-IgE Ab. In a pilot experiment, a third condition (the dose response curve without prior dissociation) was included, and it demonstrated that the resensitization by IgE behaved as with endogenous IgE, with a similar optimum at similar high densities of cell surface IgE.

Figure 2a shows that there is a marked shift in the optimum for stimulation with 6061P anti-IgE Ab with a 13.8-fold difference in the density of cell surface IgE: $9,400 \pm 1,700$ versus $130,000 \pm 34,000$ /basophil.

A second method to determine the contribution of IgE density to the position of the optimum is to compare 2 extremes of the natural population density distribution. For this study, the basophils of 14 subjects were examined for the dose response optimum to 6061P anti-IgE Ab, and for their surface IgE density as measured by flow cytometry. For the data analysis, 7 donors were chosen at the 2 extremes of the density distribution (low: $n = 4$; high: $n = 3$) in this group, and the anti-IgE dose response curves for the 2 groups were compared. Figure 2b shows the comparison. The difference in surface IgE expression was 6.5-fold ($7,200 \pm 2,700$ vs. $46,000 \pm 2,600$ IgE/basophil) and the optimum shifts were approximately 8-fold.

As part of another study [24], 13 patients were evaluated for changes in their response to 6061P prior to and 16 weeks after treatment with omalizumab. These data (not presented in here, but similar to a previously published result with fewer samples [5]) are shown in Figure 2c, cast in 2 ways, 1 absolute and 1 normalized for the change in maximum release. It shows a 200-fold shift in the optimum response to 6061P during treatment following an approximately 3-fold decrease in Fc ϵ RI (see below for further analysis).

Basophil Characteristics That May Distinguish Aggregation Dynamics

Mathematical models of aggregation characteristics for simple bivalent stimuli indicate that the crosslinking curve is simple in structure, rising and falling according

Fig. 2. Concentration-dependence of histamine release from basophils stimulated with 6061P monoclonal anti-IgE Ab in 2 experimental designs. **a** Endogenous IgE on basophils was dissociated with lactic acid treatment and the cells were resensitized to obtain 2 densities of cell surface IgE and the cells were then stimulated with monoclonal anti-IgE Ab ($n = 4$). **b** From a group of 14 basophil donors with a natural range of cell surface IgE densities, 2 groups of the extremes of density were formed at high ($n = 3$) versus low ($n = 4$) density. The anti-IgE Ab concentration-dependence curves for histamine release for the 2 groups are shown as the absolute density of cell surface IgE density. **c** ($n = 13$) 6061P concentration-dependence curves obtained from patients being treated with omalizumab, (●) before and during (□) treatment [24].

to classical solution-phase aggregate formation long appreciated for Ab-antigen reactions. However, the signal transduction pathways sense non-equilibrium aspects of the aggregation reaction, such as the rate at which aggregates form. A recent model, presented in further detail in

the Discussion section, suggests that the ratio of SYK expression to FcεRI density is a characteristic of the reaction that contributes to the shape of the dose response curve. This question of the ratio of SYK:IgE-occupied FcεRI (SYK:FcεRI) was considered next.

Fig. 3. Kinetic dilutions were used to assess the equilibration rate of an aggregating stimulus. **a** Leukocytes were sensitized with BPO-specific IgE and stimulated with BPO₂ (80 pM). At the time points shown, the reaction mixture was diluted (described in Methods), and the reaction was allowed to proceed until 30 min before harvesting the supernatant ($n = 5$). EDTA kinetic (■), BPO-EACA (0.1 mM) kinetic (□), dilution kinetic (○), and maintained concentration kinetic (●). **b** Similar in design to the experiments in **a** but using 6061P anti-IgE Ab (0.8 µg/mL) to initiate the reac-

tion ($n = 3$). EDTA kinetic (■), dilution kinetic (○), and maintained concentration kinetic (●). Data are expressed as a fraction of the release measured at the 30-min time point without dilution. Also shown as the bar at the right of the plot is the release obtained with the concentration of diluted (20-fold) stimulus for the entire 30 min (relative to the initiating concentration release at 30 min). The initiating concentration, on average, induced $36 \pm 5\%$ release for BPO₂ and $70 \pm 12\%$ release for 6061P. The arrows indicate the time interval until the dilution kinetic matches the EDTA kinetic.

Evaluation of SYK:FcεRI

All 4 of the omalizumab studies performed in this laboratory [5, 9, 25] have included the measurement of SYK and receptor expression. The first study included the measurement of cell surface IgE, and from its results, it was possible to use the receptor density determined in the other studies to estimate the surface IgE density. In addition, at the time of the first study [25], the flow cytometric measurement of SYK was cross-calibrated with a quantitative Western blot method, so that we could estimate absolute SYK expression (molecules/basophil) from either the flow cytometric measurement of SYK or the average histamine release. With this information, an estimate of SYK:FcεRI could be made for any experimental series.

Figure 3 shows the average SYK:FcεRI for 4 published omalizumab studies and the estimates of this ratio from the stripping and natural IgE differences studies (Fig. 2) in this report. Figure 3a shows the relative difference in

this ratio for the 3 types of studies, with the greatest change occurring in the omalizumab studies. The rank-order is commensurate with the amount of dose response curve shift observed in Figure 2 and the results in MacGlashan and Saini [5]. An estimate of the ratios is shown in Figure 3b–d. It is notable that the ratio of SYK:IgE/FcεRI flips in each of these experiments, from SYK expression being less than IgE/FcεRI density to the reverse.

Crosslink Stability of BPO₂ and 6061P Anti-IgE Ab

The studies above resolved why 2 bivalent stimuli would sense SYK:FcεRI differently. This ratio would be different for different densities of BPO-specific IgE, and yet the optimum for release is fixed across densities. A distinguishing characteristic for the 2 stimuli may be found in the stability of the crosslinks formed. This is a characteristic that is dependent on the affinity of the stimulus for its IgE. Crosslink stability can be assessed by testing how a rapid change in ligand concentration alters an

Fig. 4. Ratio of SYK:FcεRI expression in basophils from the 6061P experiments in this report. **a** Changes in the ratio over the 3 experiments. O, omalizumab study; S, IgE dissociation and resensitization series; N, natural variation in IgE density series. **b–d** The estimated absolute SYK:FcεRI ratio for each of the 3 experiments. resens., resensitized.

ongoing histamine release reaction. This approach accounts for several variables, including the cell's ability to sense re-equilibration of crosslinks. The rate of equilibration could be directly demonstrated for the aggregates formed, by initiating a crosslinking reaction on basophils and then rapidly diluting the reaction [26]. If the concentration of the BPO₂ was chosen to be in the ascending limb of the dose response curve and the crosslinks initially formed re-equilibrated rapidly, then the dilution would re-establish crosslinks commensurate with the lower concentration of BPO₂. The result is a rapid cessation of release that could be detected by comparing the kinetics of histamine release to the rate of histamine release stopped by the addition of EDTA, which itself immediately stops the reaction. For the anti-BPO-specific IgE Ab used for the density studies in Figure 1, we performed this measurement. Figure 4a shows the results of this experiment. One advantage of working with the BPO-based system is that a monovalent BPO (BPO-EACA) could also be used to stop the reaction. The kinetics when using BPO₁ and EDTA to stop the reaction are similar. However, for this set of reagents, bound BPO₂ did not rapidly re-equilibrate; dilution had no effect on the continuation of the reaction. In contrast, when performing a similar experiment with 6061P anti-IgE Ab, this bivalent ligand does equilibrate (Fig. 4b), not fast enough to yield a kinetic curve precisely like that generated with EDTA but requiring between 1 and 2 min to slow the reaction.

Discussion

Sensitivity of Dose Response Curves to Cell Surface IgE Density

The results shown in Figure 1 demonstrate that over a broad range of densities (250–24,000 IgE/basophil) that the position of optimum response for a simple bivalent ligand is nearly stable (at least relative to 6061P). This result anchors the idea that the variation observed for 6061P is unexpected.

The experiments with 6061P anti-IgE Ab demonstrate that a shift in the optimum can be observed by directly reducing the density of IgE with lactic acid treatment or in the natural behavior of dose response curves among subjects whose basophils express different densities of IgE but were otherwise similar in their maximal IgE-mediated response (which results from roughly equivalent SYK expression). The more recent omalizumab results (and the results in Fig. 2c) recapitulate a smaller sampling of omalizumab-treated patients from a prior study [25], providing a better sense of the average behavior during treatment with this biological agent.

All 3 types of experiments led to the conclusion that cell surface IgE density controls the concentration for an optimal response to 6061P. Individually, 2 of the tests for this conclusion could be interpreted differently. For example, after dissociating IgE to perform the experiments in Figure 2a, the cells were incubated with and without exogenous IgE. Therefore, 1 group had a mixture of en-

ogenous-bound IgE and exogenous IgE while the other group had only endogenous-bound IgE. The affinity of 6061P for the 2 situations may be different. However, as noted, in the pilot studies not shown here, the dose response curve after resensitization was similar to the dose response without dissociation of endogenous IgE.

In the comparison of subjects with different IgE densities (Fig. 2b), there is no guarantee that the surface IgE Abs were not different between the 2 groups. However, unless there is a property of IgE generation that leads to IgE Abs that differ with respect to binding 6061P anti-IgE Ab when IgE titers are low versus high, the mixing of different subjects would be expected to lead to a similar average IgE behavior in both groups.

For the case of omalizumab treatment, the comparison is within the same subject at 2 free IgE titers. There is no evidence that omalizumab modifies the generation of IgE in such a way that could lead to different IgE Abs that would bind to 6061P with different affinities.

Therefore, these experiments demonstrated the dependence of the optimum for 6061P on the density of cell surface IgE. Indeed, in many subsequent experiments, we have found that cell surface IgE density is broadly predictable from a simple histamine release study by noting the position of the optimum.

Why the Dependency on IgE Density?

As noted above, these results create a problem for the theoretical underpinning of crosslinking reactions. However, also noted above, the predictions for where the optimum for crosslinking occurs and its predicted lack of dependency on IgE density are only relevant to the actual aggregation reaction, which is not necessarily concordant with the function of the cell. Secretion, in this case, is dependent on the entirety of the signal transduction reaction that senses the presence of crosslinks.

Mathematical modeling of the early IgE-mediated reaction has suggested a possible explanation [27] for IgE density dependence with simple aggregating stimuli. Figure 5 presents a simplified view of the results from a recent model (an abstraction of a similar figure in the published report [27]) where the ratio of SYK and aggregated receptor could be varied as needed. This model and its results were based on considerable experimental data, but this particular prediction from the model has not been examined experimentally. It can be seen in Figure 5 that low SYK-to-receptor ratios alter the dose response curve by creating a curve with 2 peaks. Overall, the curve retains symmetry, but if one is only capable of testing 1 side of the full curve, illustrated by graying out the right half, a

Fig. 5. Predicted activation of a downstream signaling molecule in a model of the early IgE-mediated reaction. The results were abstracted from data presented in [21] to highlight the differences in activation at 3 SYK:FcεRI-IgE ratios. The right half of the dose response curve is shaded grey to emphasize the portion of the curve that is practically accessible for 6061P anti-IgE Ab. Note that, despite the “saddle” shape at the low ratio, the curve remains symmetric, and that aggregate size is maximal at the center of the curve. It is only the downstream steps that experience the “saddle” characteristic.

change in ratio produces a shift in the optimal functional response (not the crosslinking curve, see the report for this information). The various characteristics of this curve are largely recapitulated in the 6061P dose response curve before and after treatment with omalizumab (it would be practically difficult to examine the full breadth of the 6061P dose response curve since concentrations >10 mg/mL would be needed on the high concentration side of the curve). Note that the middle portion of the curve at the low SYK:FcεRI ratio does not return to baseline, but rather flattens at a level well above baseline. This is the characteristic of the 6061P dose response curve normally observed [5] and it disappears when the ratio increases, as it does after treatment. The dip in the middle varies according to the ratio as well, and natural individual 6061P curves show variant forms of this behavior.

In the model, the maximum response does not change, and this was found with the stripping experiments. In Figure 2, the maximum release remained constant but the optimum shifted rightward. During treatment with omalizumab, the optimum shifted rightward and the maximum increased. Another unexpected characteristic of the basophil during omalizumab treatment is an increase in

the expression of SYK [25]. In conjunction with the decrease in cell surface receptor and its bound IgE, SYK:IgE-FcεRI is exaggerated and the cell acquires more of a critical signaling element, in this case SYK. As we have previously demonstrated, SYK is a rate-limiting component of the IgE-mediated reaction in basophils, typically tuned to lie on the cusp of allowing an IgE-mediated response [28]. Thus, the response to anti-IgE Ab results in shifts in both its optimum and maximum response, unlike the results following stripping where absolute SYK levels do not change (as noted in the Methods section, SYK expression pre- and poststripping was not found to change).

Therefore, 6061P reveals an interesting aspect of signal transduction, probably because it operates on such an excess of receptors. The typical basophil has 100,000–150,000 receptors [14] while only expressing 25,000 molecules of SYK [28], but normal allergens do not interact with this density of receptors since the average ratio of allergen-specific IgE:total IgE is 1% [29]. In other words, allergens do not create the conditions that a pan-crosslinker like anti-IgE creates and do not typically probe the relationship between SYK and receptor density. The behavior is probably also unique to monoclonal anti-IgE Abs, since only they are bivalent linkers. A long-used goat polyclonal anti-IgE Ab does not show this characteristic despite being a pan-crosslinker [6]; however, we and others have found that these curves are quite complex in structure, showing partial engagement of CD32b [30] and creating observable super-aggregates that change the signaling dynamic [31]. The curve-shift effect may also be restricted to monoclonal Abs that do not interact with CD32b. We have found that mouse IgG₁ Abs interact with CD32b and this would complicate the character of a dose response curve, but 6061P is a monomeric mouse IgM and it does not bind to CD32b [30].

The shift in the optimum was consistent with the relative difference in IgE density and SYK expression in the groups being compared. (1) The greatest shift occurred during omalizumab treatment (a 200-fold shift), followed by (2) what it was possible to create with lactic acid treatment (an 11-fold shift), and finally, (3) the natural difference among subjects with different IgE densities (an 8-fold shift), at least for the survey done for this study.

Why the Difference in Behavior between BPO₂ and 6061P?

The question that follows is why there is a density dependence with 6061P, a bivalent stimulus, and the behavior for another simple bivalent ligand, BPO₂? The results shown in Figure 4 differentiate these 2 stimuli: 6061P re-

equilibrates rapidly after dilution while BPO₂ (with the BPO-specific IgE in this study) does not. We found a similarly rapid re-equilibration of 6061P when stimulating human lung mast cells [32]. The BPO-EACA results show that the BPO ligand is dissociating rapidly but the crosslink remains “broken” only if there is a vast excess of a monovalent version present; rapid rebinding disallows crosslink “breaking” and retains a signaling-effective crosslink. This kind of rapid rebinding apparently does not occur effectively for 6061P. This would be consistent with forward rate constants being slower for large proteins. It may also result from only dimeric aggregates being formed with this stimulus. If the forward binding is slow and the dissociation fast, smaller aggregates form. This difference in the dynamics of crosslink formation is interesting for its implications because stability of the crosslink is important in signal generation [33, 34]. Kinetic proofreading of the initial signal determines the nature of downstream signaling and reveals a sensitivity to conditions not as apparent with stable signals. The IgE-mediated aggregation reaction appears to sense crosslink transience [33, 34]. This leads to the tentative speculation that an unstable crosslink exposes the relevance of SYK-to-receptor ratios, unlike BPO₂, leading to shifting optimums for histamine release.

Conclusion

This study demonstrates the unique characteristics of a low affinity bivalent monomeric IgE Ab. Although it was unexpected for a bivalent ligand to show sensitivity to receptor density, a recent modeling of the reaction suggests a possible influence (SYK:IgE ratio) in the reaction. It provides support for some of the modeling of this complex reaction proposed by other study groups [21]. The results also provide an additional indication that the expression of SYK in human basophils is a rate-limiting step in its activation cascade, and that expression has functional consequences beyond determining the magnitude of the basophil response to IgE-mediated stimulation. On a practical level, we demonstrated that interpretation of changes in dose response curves for commonly used reagents may require considerations not normally part of the analysis, i.e., in this instance, that changes in receptor density (bound to IgE in this case) change the optimal response.

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Disclosure Statement

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