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Subthreshold Desensitization of Human Basophils Recapitulates the Loss of syk and FcεRI expression Characterized by Other Methods of Desensitization

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Abstract

Background—Clinical desensitization of patients to drugs involves progressive exposure to escalating doses of drug over a period of 24 hours. In prior studies, this method was recapitulated *in vitro* to also demonstrate loss of mast cell or basophil responsiveness. However, most signaling studies of human basophils have identified changes in signaling by using other methods of inducing cellular desensitization.

Objective—This study examined two well-described endpoints of basophil desensitization, loss of syk or FcεRI expression, under conditions of subthreshold desensitization.

Methods—The loss of FcεRI and syk was examined in human basophils.

Results—It was shown that both loss of syk and FcεRI/IgE occurred during an escalating series of stimulation (anti-IgE Ab) and that expression loss occurred despite the presence of little histamine release. If basophils were first cultured for 3 days in 10 ng/ml IL-3, the concentration-dependence of histamine release shifted to 100 fold lower concentrations of stimulus. However, loss of syk did not show any change in its EC50 while loss of FcεRI also shifted 100 fold. From the perspective of early signal element activation, the marked shift in the EC50 for histamine release was not accompanied by similar shifts in the EC50s for several signaling elements. The EC50s for phospho-Src, phospho-SHIP1, phospho-Syk, or phospho-Cbl did not change while the EC50s for phospho-Erk and the cytosolic calcium response did shift 100 fold.

Conclusions—These studies show that under normal conditions, subthreshold desensitization leads to loss of two critical signaling molecules (FcεRI and syk) but under at least one condition, treatment with IL-3, it is possible to markedly blunt the loss of syk, but not FcεRI, while executing a proper subthreshold titration. These data also suggest that IL-3 modifies only the sensitivity of signaling elements that are downstream of syk activation.

Keyword list

Human; Basophil; Allergy; Fc Receptors; Syk

Introduction

Clinical desensitization is used in the treatment of some allergic conditions. Most generally, it is only applied to allergies of drugs such as penicillin and is used to allow patients with hypersensitivity to the drug to be treated therapeutically [1, 2]. The protocol includes titration of the drug into the patient, progressively escalating the amount of drug by starting

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at doses that do not induce overt symptoms of a hypersensitivity reaction. With careful timing and dosing, the patient does not experience a reaction but is ultimately able to be dosed with therapeutic levels of the drug. The mechanism underlying clinical desensitization is not yet understood.

To explain the operative mechanism underlying clinical desensitization, an obvious place to turn is the mast cell response biochemistry. All forms of receptor stimulation result in the engagement of signal termination events, otherwise external signaling would result in runaway activation of cells. There are a wide variety of mechanisms that mediate termination. A common mechanism is down-regulation of the receptor from the cell surface but the list of mechanisms is long. During IgE-mediated activation of basophils (or mast cells), the mechanisms depend on the species being studied. Early studies of RBL cells (rat basophilic leukemia cells) strongly suggested that FcεRI down-regulation was a major component of the termination process [3, 4]. In contrast, studies in human basophils suggested that down-regulation of FcεRI was not even operative [5]. Subsequent studies of human basophils have shown that down-regulation of receptor expression does occur but the timing cannot account for termination of signaling; down-regulation of FcεRI in human basophils requires many hours to days [6].

There have been two operational methods to study desensitization mechanisms in basophils and mast cells. Both operate on a variation of the principle of suboptimal stimulation. The protocol that has generated most of the mechanistic details of cellular desensitization involves stimulation in the absence of extracellular calcium [7, 8]. But the method that recapitulates the technique of clinical desensitization relies on progressively escalating concentrations of allergen exposure [9–11]. It is found that if the timing of escalation is properly chosen, very little secretion is ever observed although one does eventually reach concentrations that would have induced secretion if not for the escalating method. The question for this study was whether the mechanisms discovered using the first operational protocol, calcium-free stimulation, were recapitulated with the progressive protocol. This information would be required to properly design a mechanistic study of clinical desensitization.

There have been 3 mechanisms discovered that may contribute to termination of the secretory signals in human basophils. These include activation of SHIP [12, 13], processing of syk by ubiquitination [14, 15] and degradation and loss of cell surface receptor [6]. Parenthetically, there is a fourth mechanism whose characteristics can be described and which appears to involve resorting of receptor in the membrane, but without details for how it occurs [16]. A study of subthreshold activation requires a way to examine signals too weak to induce secretion and at this point in time, all signaling steps thus far studied titrate like histamine release and are therefore undetectable at subthreshold levels of stimulation. However, two of the identified desensitization mechanisms appear to result from an integration of weak signaling. For example, it is known that syk down-regulation occurs at concentrations of allergen that do not induce secretion [14, 17]. While the process requires long times (relative to secretion), the integrative characteristic may allow detection during the subthreshold method of inducing desensitization. Likewise, loss of receptor may be detectable over a period of time. However, studies in mice indicate that receptor down-regulation that occurs during stimulation that is optimal for secretion, it does not occur at all during a subthreshold desensitization protocol [18]. Therefore, as a first step in testing whether the known mechanisms of desensitization apply to the subthreshold method, and therefore may be applicable as an explanation of clinical desensitization, we examined whether subthreshold desensitization results in loss of syk and receptor expression on human basophils.

Methods

Materials&Buffers

Materials, Buffers and Antibodies—The following were purchased: PIPES, bovine serum albumin (BSA), EGTA, EDTA, D-glucose, NaF, Na₃VO₄, 2-ME (2-mercaptoethanol), NP-40,; PP1 (Calbiochem, San Diego, CA); crystallized human serum albumin (HSA) (Miles Laboratories, Elkhart, IN); fetal calf serum (FCS) and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); Percoll (Pharmacia, Piscataway, NJ); Tris(hydroxymethyl)-aminomethane, Tween-20 (Bio-Rad, Hercules, CA); 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) (Sigma-Aldrich, St. Louis); Anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Lake Placid, NY); Rabbit anti-phospho-ERK Ab, anti-phosphoSHIP, anti-phospho Src (Tyr 416) (New England Biolabs, Beverly, MA, now Cell Signaling); anti-syk mAb, 4D10 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-human IgE Ab (6061P) (Hybridoma Labs, Baltimore, MD); antiHRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated Sheep anti-mouse Ig Ab, protein G sepharose beads (Amersham Life Science, Arlington Heights, IL). PIPES-albumin-glucose (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. Countercurrent elutriation and labeling with antibodies for flow cytometry was conducted in PAG containing 0.25 % BSA in place of 0.003 % HSA. ESB is 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.

A penicillin (BPO)-specific IgE was partially purified from the sera of penicillin allergic patients as previously described [5]. A gp120-specific monoclonal IgE was the gift of Tanox, Inc, (Houston, TX). This antibody was biotinylated and could be detected by flow cytometry by streptavidin-alexa 647, which was calibrated with absolute IgE/basophil by methods described elsewhere [6].

Basophil purification

Residual cells of normal donors undergoing leukapheresis were enriched in basophils using Percoll density gradients and countercurrent-flow elutriation, as previously described [19]. Basophils were further purified by negative selection as described above. Basophil purity exceeded 99%.

Subthreshold desensitization

Prior to culture, basophils were sensitized with either BPO-specific IgE or biotinylated gp120-specific IgE at 5 µg/ml, on ice for one hour, washed and resuspended for culture. Basophils were incubated in RPMI-1640 media (0.03% HSA, 8 µg/ml gentamycin, with media supplemented to generate a final Ca⁺⁺ concentration of 1 mM). Each experiment included a full range of single concentrations for the 24 hour incubation as well as various versions of escalating concentrations of anti-IgE Ab. The primary series for day-0 basophils was 0.006, 0.02, 0.06, 0.15 µg/ml separated by 6 hours. The final volume of the cultures in tilted microtubes was 200 µl and at each step, 1µl of an appropriate concentration of anti-IgE was added in order to minimize the volume change during sequential additions. To determine if the subthreshold series blunted histamine release, one condition included a final incubation with 0.5 µg/ml anti-IgE for 45 minutes. In some experiments, partial subthreshold series were included to examine some partial endpoints. Histamine release was measured from supernatants of the cultured cells while the cell pellets were either lysed for measurement of syk expression by Western blotting or the cells were fixed (2% paraformaldehyde) for subsequent analysis by flow cytometry for the presence of either

gp120-specific IgE or BPO-specific IgE. Spontaneous histamine release (non-stimulated) in the 24 hour cultures averaged $9\pm4\%$, a value typical for purified basophils under these conditions.

Detection of cell surface IgE

Two methods were used to detect loss of cell surface IgE/Fc ϵ RI when using anti-IgE antibody to stimulate the cells. The first method has been previously described [6]. Basophils were sensitized with a biotinylated IgE (basophils generally express a low density of unoccupied Fc ϵ RI) and after fixing the cells, its presence was detected with streptavidin-alexa647. A second method was developed. Basophils were sensitized with penicillin-specific IgE and its presence detected with a sandwich assay. Prior to fixing the cells, the suspension was chilled to 4°C, 10 μ g/ml of BPO(32)-HSA added for 10 minutes, the cells washed twice in ice-cold buffer and fixed with 2% paraformaldehyde for 20 minutes prior to blocking and storage overnight. Before flow cytometry, the fixed cells were incubated with rabbit anti-BPO IgG $\pm$ BPO-EACA at 1mM. The presence of BPO-EACA (monovalent penicillin, penicillin coupled to ϵ -aminocaproic acid) generates a negative control for the measurements. Using calibrated flow cytometry it was determined that this assay can detect fewer than 50 molecules of penicillin-specific IgE/basophil (online supplement figure 1E, panel A, shows that this assay is sensitive to very low densities of BPO-specific IgE). In a desensitization experiment, activation with the pan-crosslinking reagent, anti-IgE antibody (6061P) results in an average of $52\pm13\%$ loss of detectable BPO-specific IgE after 24 hours, a result commensurate with prior studies (figure 2E of the online supplement).

Multiplex Western blots

Pelleted cells were lysed in 20 μ l of hot ESB, the tube placed in boiling water for 5 minutes and the samples stored at -80°C until electrophoresis. Samples were run in a 15 well 8% tris-glycine gel with molecular weight markers in the first and last lanes. The nitrocellulose was cut horizontally at the 64 kD marker level. The >64 kD and <64 kD portions were blocked with 4% BSA or 2% milk, respectively. The nitrocellulose was blotted on two or three successive days with a choice of antibodies that did not interfere with each other. No stripping step occurred between blotting. The blotting order was phospho-Src followed by phospho-Erk for the portion <64 kD and phospho-Cbl followed by phospho-SHIP followed by p85 α . The order was chosen to take advantage of differences in the strength of the signals detected and the selectivity of the antibodies, with the weaker antibodies analyzed first. We have previously established that p85 α is a good lane-loading control antibody [6, 13, 20] because its expression is unmodified during a wide variety of experimental designs and because it is easily detected with few cells and is easy to detect as the last step in this multiplex Western blot design (e.g., as an average ($\pm$ S.D.) of these experiments, the band intensity of p85 at optimal stimulation was 1.06 ± 0.17 fold of the non-stimulated band intensity). Multiple film exposures were made in order to optimize linearity, the films were digitized and the band intensities determined by NIH Image.

For the analysis of syk expression, cell lysates were run on 8% gels and syk detected with 4D10 antibody by Western blotting.

[Ca²⁺]_i and mediator measurements

Basophils were labeled with 4 μ M fura-2/AM and cytosolic calcium determined by digital microscopy as previously described [21].

Results

Pilot studies

Two series of pilot studies were needed to perform these studies. These experiments used a pan-crosslinker, anti-IgE, to induce a response. Pilot studies roughly determined the dose response curve for the anti-IgE in use (6061P). Below 0.01 $\mu\text{g/ml}$ of 6061, histamine release in an overnight culture was generally zero although small levels of release can occasionally be observed from some preparations.

A further series of pilot experiments refined the method of detecting cell surface IgE/receptor. There have been several methods used in previous studies [5] and most recently, flow cytometric methods appear the most sensitive. But the 3 flow cytometric methods previously developed show some discrepancies [6]. Notably, detecting loss of receptor with anti-receptor antibody is confounded by aggregation. The best methods rely on loading a small amount of specific IgE antibody and using loss of this specific IgE as a marker of overall IgE & receptor loss (only when a pan-stimulus like anti-IgE is used). The methods section describes a new method (sensitizing the cells with penicillin-specific IgE) that relies on this approach and this method provides support for a previously described but related method (sensitizing the cells with a biotinylated IgE) [6]. An important test of the reliability of the method is whether the src-family kinase inhibitor, PP1, can reverse the loss of receptor to the same extent as it inhibits desensitization. Previous studies demonstrated that 10 μM PP1 would reverse desensitization by approximately 80% [22]. Likewise, 10 μM PP1 reverses loss of IgE/Fc ϵ RI, using either biotinylated IgE or BPO-specific IgE to detect loss, by $73\pm 11\%$ and $68\pm 11\%$, respectively (online supplement figure 2E shows these loss studies). Parenthetically, this assay should also have utility in the measurement of BPO-specific IgE densities on basophils of penicillin allergic patients.

Subthreshold stimulation

Detecting loss of either syk or IgE/Fc ϵ RI requires extended times but this requirement is compatible with the long process of subthreshold desensitization. Using the pilot experiments as a guide, basophils were cultured with progressively escalating subthreshold concentrations of anti-IgE spaced over 24 hours. Four concentrations, differing by one-half log units, were used prior to stimulation with an optimal concentration for 45 minutes to ascertain the extent of desensitization. This is an abbreviated subthreshold approach (half-log increments vs. 2 fold increments) that captures the important outcome of not observing significant histamine release at any point, including after optimal stimulation. Figures 1 and 2 show results for histamine release, syk and IgE/Fc ϵ RI expression, respectively. For each experiment, the natural response to the 5 concentrations of anti-IgE antibody is included in the protocol and it can be seen that the starting concentration, 0.006 $\mu\text{g/ml}$, is a good starting point for the subthreshold protocol, showing no release. It can also be seen that at this concentration, there is some loss of syk expression, $\approx 10\%$, over the ensuing 24 hours. As demonstrated previously [14], the EC50 for loss of syk expression ($\approx 0.017 \mu\text{g/ml}$) is about 7 fold lower than the EC50 for histamine release (0.11 $\mu\text{g/ml}$). The histogram to the right of the figure shows the total (cumulative) histamine release in the samples that were treated with the progressively escalating concentrations of anti-IgE antibody, in these experiments, an average of 2% release (6% of the response without the serial dosing). While histamine release was clearly blunted, loss of syk was unaffected, showing a loss similar to that observed at optimal levels of stimulation alone.

Figure 2 shows the results for loss of IgE/Fc ϵ RI. The differential between the EC50 for loss of receptor vs. histamine release is not as apparent, approximately 2 fold, although the EC50 for loss of receptor is still less than for histamine release. There continues to be loss of

receptor in the subthreshold series although it is, on average, not quite the level observed for optimal stimulation. In a separate series, with an abbreviated dosing schedule to examine the two lowest concentrations for loss and using BPO-specific IgE to label the cells, figure 2C shows that the sequence of the first two doses generates more loss than either single dose alone.

Effect of IL-3

While exploring the optimal conditions required to perform these culture experiments, it was noticed that treatment with IL-3 for 3 days alters the behavior of the system in an unexpected way. It has been known for some time that 24 hour culture with 10 ng/ml IL-3 significantly enhances IgE-mediated histamine release [23–25] but we noted that after 3 days, the concentrations required for release shift leftward by 100 fold. This is shown in the online supplement figure 3E. This remarkable shift in sensitivity led to a re-examination of the subthreshold style experiment but in this case with the starting subthreshold concentration necessarily being 100 fold lower, 0.00006 $\mu\text{g/ml}$ of anti-IgE Ab. Figures 3A and 3B summarize the experience with this protocol. Quite unexpectedly, the loss of syk expression did not shift with the increased sensitivity for histamine release. Under these conditions, the EC50 for loss of syk was essentially the same as for the studies discussed above, 0.012 $\mu\text{g/ml}$ while the EC50 for histamine release was 0.00018 $\mu\text{g/ml}$, in other words, a reversal of sensitivity with syk no longer displaying the unique feature of occurring in the absence of histamine release. It can be seen that syk loss still occurs with the subthreshold method (figure 3C) but if the dosing were halted as one approached the near maximum for histamine release, the loss of syk would have been only modest. In contrast, loss of receptor expression shifted precisely with the shift in histamine release; the EC50 for receptor loss was 0.00005 $\mu\text{g/ml}$ vs. 0.00018 for histamine release, retaining the 2–3 fold difference observed in the cells not first treated for 3 days with IL-3. In addition, a subthreshold protocol induced loss of receptor expression (figure 3C).

The requirements for syk loss appear unaltered by the culture with IL-3 while the requirements for receptor loss mimic the requirements for histamine release. This led to experiments to determine which signaling steps shift with histamine release and which do not. Six signaling elements were examined, phosphorylation of lyn, syk, c-cbl, SHIP and Erk, and the elevation in cytosolic calcium. Figure 4 shows that in the basophils treated for 3 days with IL-3, the phosphorylation of lyn, syk, c-cbl and SHIP do not track with the relative shift in histamine release (figure 4E in the online supplement shows representative Western blots from these experiments). Figure 5E in the online supplement shows a synopsis of prior published studies for several signaling steps in freshly isolated cells, demonstrating the similarity in concentration-dependence for histamine release and the signaling steps (see discussion). For the purposes of this study, this was done again for the current anti-IgE Ab and once again, the concentration-dependence was concordant with histamine release in the non-cultured basophils (data not shown). In contrast, the phosphorylation of Erk and the cytosolic calcium response moved leftward in a manner commensurate with the shift in histamine release (figure 6E in the online supplement shows an example cytosolic calcium response – note the delay in cytosolic calcium at the 0.006 $\mu\text{g/ml}$ concentration, consistent with prior observations on the timelag characteristic [26]). Note that while c-cbl is already shifted leftward of the other signaling species, it does not shift its relative position following culture with IL-3.

In addition, in the 3-day cultured cells, despite the apparent absence of syk phosphorylation at very low concentrations of anti-IgE Ab ($<0.005 \mu\text{g/ml}$) that continue to induce histamine release, treatment of the cells with a syk inhibitor, NVP-QAB205 (at a concentration considered reasonably selective for syk, 0.3 μM [13, 15]), inhibited histamine release at both high and low concentrations of anti-IgE Ab (95 $\pm$ 4% inhibition, n=3).

These results suggest that IL-3 induces changes in signaling downstream of the earliest steps in signaling, a result consistent with previous studies of shorter treatments with IL-3 [27], although with much greater effect. Indirectly, this conclusion suggests that the crosslinking that occurs at concentrations of anti-IgE Ab in the range of 0.0001 to 0.01 $\mu\text{g/ml}$ generate an undetectable signal that is revealed by the changes that occur to the signaling apparatus downstream of syk activation. The results also suggest that perhaps the nature of the crosslink is different. Notably, the anti-IgE antibody used is a monomeric monoclonal which at low concentrations may only generate small aggregates. Several studies in both basophils and RBL cells have suggested that dimeric-only aggregates generate both quantitatively and qualitatively different signals [28, 29]. Previous studies have noted that a bivalent hapten vs. a multi-valent haptenated protein generate either pre-dominantly small vs. large aggregates respectively [5]. To determine if small aggregates (primarily dimeric) generate signals are not readily translated to loss of syk expression, basophils were loaded with a series of penicillin-specific IgE densities and stimulated with either BPO2 or BPO(21)-HSA. Although histamine release differed for the two types of stimulation (the EC50 for BPO2 being 4 fold shifted from the EC50 for BPO-HSA or 10-fold for equivalent histamine release), the loss of syk was similar for either stimulus at a given density of IgE. Figure 5 demonstrates that for similar loading of penicillin-specific IgE, BPO2 and BPO-HSA induced only slightly different amounts of syk loss. Therefore, the type of aggregate does not appear to be the determinant of whether syk is lost during activation.

The ability of the basophil to induce marked secretion when the phosphorylation of syk is not detectable (figure 4) is now seen as compatible with earlier observations that antigens like BPO-HSA induce very transient syk phosphorylation (unlike anti-IgE Abs) [12] but nevertheless induce expected levels of IL-4 as a rate much slower than histamine release in even day 0 cells. In figure 7E (panel A) of the online supplement is shown an experiment (representative of 3 similar experiments) that demonstrates the transient nature of syk phosphorylation with antigenic stimulation while simultaneously measuring the secretion of IL-4. After syk phosphorylation has returned to near resting levels, IL-4 secretion is still occurring. We have previously published that the addition of monovalent penicillin (BPO-EACA) at 30 minutes immediately stops the IL-4 secretion [30] and figure 7E (panel B) also shows that it stops IL-4 mRNA generation also, supporting the idea that signaling must be maintained, although syk phosphorylation is not detectable.

Discussion

These studies demonstrate that subthreshold desensitization of human basophils results in two outcomes, loss of syk or cell surface Fc ϵ RI, that are also characteristic of desensitization performed in the absence of extracellular calcium or during full activation at optimal concentrations of stimulus. These two outcomes may be considered integrative outcomes because they occur progressively over an extended period of time and in the case of day-0 basophils, occur with little histamine release. Figure 5E summarizes our previous experience with various signaling steps and their relationship to histamine release. With the exception of c-cbl phosphorylation, all these signaling steps essentially coincide with the concentration-dependence of histamine release. It is probably reasonable to assume that during subthreshold stimulation, where histamine release is not occurring, that there remains some presence of signaling that can not be readily observed but can mediate the desensitization that is the ultimate result of escalating subthreshold stimulation.

This latter point is highlighted in the experiments using basophils cultured for 3 days with IL-3. The results of these experiments raised a number of interesting questions about the signaling apparatus. First, signaling at, or before, the activation of syk is not observable at low concentrations of anti-IgE Ab that induce nearly maximal release. Both lyn and syk

phosphorylation are easily apparent at 0.5 $\mu\text{g/ml}$ anti-IgE Ab, a concentration that is optimal in day-0 cells, but are not apparent at concentrations below 0.01 $\mu\text{g/ml}$, the new maximum in day-3 cells. In previous studies, phosphorylation of SHIP has appeared dependent on lyn activity rather than syk activity [13] so that the absence of observable phosphorylation at low concentrations of anti-IgE Ab is not surprising. Phosphorylation of c-cbl has already been observed to be shifted leftward of histamine release by several fold [14] and this characteristic is consistent with the previously noted leftward shift (relative to histamine release) of syk loss. But like syk loss, c-cbl phosphorylation does not appear to shift further leftward with IL-3 treatment. However, both Erk phosphorylation and the cytosolic calcium response are shifted with histamine release. Taken together, these observations suggest that IL-3 induces a change in signaling that is downstream of the earliest signaling events, remarkably amplifying signaling at some point downstream of syk itself (with the caveat that phosphorylation is a reasonable surrogate for syk's activity). These characteristics are consistent with the phenotypic changes that are induced by much shorter incubations with IL-3 [27], which suggests that both early and late changes induced by IL-3 follow a similar pattern of not modifying the early steps that occur during Fc ϵ RI aggregation.

Second, these results indicate that the process that causes loss of syk expression is not influenced by IL-3. A priori, there is no expectation for this behavior but given that syk is a step that follows aggregation of the receptor, which is presumably necessary to observe its loss from the cell surface, it is unexpected that cell surface Fc ϵ RI loss tracks with the shift in histamine release while syk does not. In addition, we have previously shown that receptor loss is not sensitive to the activities of syk [31] (but is sensitive to the activities of src-family kinases – as shown in the pilot studies and previous studies [22]). This result suggests that receptor internalization is a process that is sensitive to a change induced by IL-3 although it may be a change that is not the same as the change(s) that markedly amplify early signals like syk. Whatever the mechanism, these studies point to the possibility that loss of syk and receptor expression can be disconnected from each other under the proper conditions.

Third, the observation that the EC₅₀ for stimulated syk loss does not follow changes in the EC₅₀ for histamine release also implies that subthreshold desensitization could either be arranged to minimize the loss of syk or that subthreshold desensitization doesn't work as well. In the experiments done, it was found to be harder to choose concentrations of anti-IgE Ab that still induced a desensitized state without greater histamine release (see figure 3, panel C). But this could be a technical issue, related to the need for a more subtle escalation of subthreshold concentrations of anti-IgE Ab.

The nature of the aggregates induced by very low concentrations of a monoclonal anti-IgE Ab aren't known but since monoclonal antibodies should be capable of only inducing linear aggregates of IgE/Fc ϵ RI -- a condition for other bivalent haptens like BPO2 -- theoretical considerations would predict that a higher percentage of the aggregates would be dimeric at low concentrations [29, 32]. This possibility led to the hypothesis that perhaps small linear Fc ϵ RI aggregates (3) are inefficient for induction of syk loss but when IL-3-induced amplification steps are engaged, these same small aggregates are sufficient for enhanced downstream signaling. However, when we compared the ability of BPO2 and BPO(12)-HSA to induce loss of syk in basophils sensitized with BPO-specific IgE, there was only a small difference in the loss of syk for similar loading of the cell even though histamine release differed considerably. Therefore, it seems unlikely that aggregate size is a strong determinant of syk loss.

It is also apparent from both the day-3 results and results like those shown in the online supplement, figure 7E, that syk activity (assuming phosphorylation is an indicator of activity) does not need to be measurable for there to be a signal that induces mediator

secretion. Despite that apparent absence of phosphorylation, the reaction remains sensitive to a syk inhibitor, or dis-aggregation by monovalent hapten [30].

In summary, these studies demonstrate that with respect to two well studied outcomes of basophil desensitization, loss of syk or FcεRI expression, subthreshold methods are efficient at engaging these mechanisms of down-regulation. However, these studies also demonstrated that it may be possible dissociate the loss of syk from mediator release and loss of FcεRI under the right conditions, notably incubation in cytokines that prime the basophil response; conditions that are common in cell cultures of mast cells derived from progenitors. These studies also demonstrated that long exposures to IL-3 markedly alters signaling steps downstream of the earliest steps in signaling without any measurable effect on the early signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Subthreshold dosing effects on syk expression (n=3). Panel A: Concentration-dependence of histamine release ($\square$) and loss of syk expression ($\bullet$) in 24-hour cultures of purified human basophils. The EC50 for histamine release is approximately 0.11 $\mu\text{g/ml}$ and EC50 for loss of syk expression is approximately 0.02 $\mu\text{g/ml}$. Histamine release was sampled from the supernatants after 24 hours and the cells lysed for syk expression and analyzed by Western blotting. Panel B: Loss of syk expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.006, 0.02, 0.06, 0.15 $\mu\text{g/ml}$) over a 24 hours period. Histamine release was sampled from the supernatants after 24 hours and the cells lysed for syk expression.

Figure 2.

Subthreshold dosing effects on FcεRI expression. Panel A: Concentration-dependence of histamine release ($\square$) and loss of FcεRI expression ($\bullet$) in 24-hour cultures of purified human basophils ($n=3$). The EC₅₀ for histamine release is approximately 0.11 $\mu\text{g/ml}$ and EC₅₀ for loss of syk expression is approximately 0.06 $\mu\text{g/ml}$. Histamine release was sampled from the supernatants after 24 hours and the cells analyzed by flow cytometry for the presence of the probe IgE, biotinylated gp120-specific IgE (see methods). Panel B: Loss of syk expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.006, 0.02, 0.06, 0.15 $\mu\text{g/ml}$) over a 24 hours period. Histamine release was sampled from the supernatants after 24 hours and the cells analyzed by flow cytometry. Panel C: the design was similar to that used in panels A & B but the IgE probe was penicillin-specific IgE. An abbreviated stimulation series was used to determine the sensitivity of FcεRI loss in the early phase of the series where single concentrations had little effect ($n=3$).

Figure 3.

Subthreshold dosing effects on syk or FcεRI expression on basophils first cultured for 3 days with 10 ng/ml IL-3. After 3 days of culture in IL-3, the cells were recovered, counted and re-established in a 24 hour culture (with IL-3) with various levels of stimulation. *Panel A*: Concentration-dependence of histamine release (□) and loss of syk expression (●) in 24-hour cultures (n=3). Histamine release was sampled from the supernatants after 24 hours and the cells analyzed by cell lysis and Western blotting. The EC₅₀ for histamine release is approximately 0.00018 $\mu\text{g/ml}$ and EC₅₀ for loss of syk expression is approximately 0.06 $\mu\text{g/ml}$. *Panel B*: Concentration-dependence of histamine release (□) (which is the same data plotted in panel A) and loss of FcεRI expression (●) in 24-hour cultures (n=3). Histamine release was sampled from the supernatants after 24 hours and the cells analyzed by flow cytometry for the presence of the probe IgE, biotinylated gp120-specific IgE (see methods). The EC₅₀ for histamine release is approximately 0.00018 $\mu\text{g/ml}$ and EC₅₀ for loss of FcεRI

expression is approximately 0.00008 $\mu\text{g/ml}$. *Panel C*: Run in parallel with the results shown in panels A and B, panel C shows the loss of syk expression, Fc ϵ RI expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.00006, 0.0002, 0.0006, 0.002, 0.006 $\mu\text{g/ml}$ with a final histamine release test concentration of 0.02 $\mu\text{g/ml}$) over a 24 hours period. Histamine release was sampled from the supernatants after 24 hours and the cells analyzed by flow cytometry or by Western blotting (n=3).

Figure 4.

Concentration-dependence of IgE-mediated signaling in basophils first cultured for 3 days in 10 ng/ml IL-3. Purified basophils were stimulated with the concentrations of anti-IgE Ab shown in the figure and cell pellets lysed after 20 minutes for a multiplex Western blotting (see methods). (◆) histamine release, (■) Erk phosphorylation, (●) c-cbl phosphorylation, (□) SHIP1 phosphorylation, (○) lyn phosphorylation (using a anti-phospho Src Ab), (▲) syk phosphorylation (using immunoprecipitation of syk followed by Western blotting with anti-phosphotyrosine Ab). Cytosolic calcium was analyzed at two concentrations (0.5 µg/ml vs. 0.006 µg/ml) and the time-average of the kinetic curve plotted as a gray histogram. The heavy dotted line is placed in the figure to provide context for the concentration-dependence of day-0 histamine release.

Figure 5.

Relationship between syk loss induced by overnight culture of BPO-sensitized basophils with either BPO(12)-HSA or BPO2 (penicillin linked by 1,8 diamino-octane). The concentration for BPO-HSA was chosen to be optimal for induction of histamine release ($0.5 \mu\text{g/ml}$) and slightly sub-optimal for BPO2 ($5 \times 10^{-9} \text{ M}$) in order to skew aggregate size towards dimers [29]. Previous studies have demonstrated that the EC50 for these two antigens show an approximately 4 fold difference in ability to induce histamine release or a 10-fold difference for equivalent histamine release (since BPO2 doesn't induce the same maximum release as BPO -HSA) [5]. Basophils were sensitized with different amounts of BPO-specific IgE and ratio of stimulated/control plotted for BPO-HSA vs. BPO2. A 4-fold difference in efficacy would have appeared to have a y-intercept that was significantly negative. The slope of the curve might also not be near one. The slope of the experimental results was essentially one with an intercept of zero.