

Rapid IgE desensitization is antigen specific and impairs early and late mast cell responses targeting Fc ϵ RI internalization

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Rapid IgE desensitization provides temporary tolerization for patients who have presented severe hypersensitivity reactions to food and drugs, protecting them from anaphylaxis, but the underlying mechanisms are still incompletely understood. Thus, here we develop an effective and reproducible in vitro model of rapid IgE desensitization for mouse BM-derived mast cells (BMMCs) under physiologic calcium conditions, and we characterize its antigen specificity and primary events. BMMCs were challenged with DNP-human serum albumin conjugated (DNP-HSA) and/or OVA antigens, delivered either as a single dose (activation) or as increasing sequential doses (desensitization). Compared to activated cells, desensitized BMMCs had impaired degranulation, calcium flux, secretion of arachidonic acid products, early and late TNF- α production, IL-6 production, and phosphorylation of STAT6 and p38 mitogen-activated protein kinase (p38 MAPK). OVA-desensitized cells responded to DNP and DNP-desensitized cells responded to OVA, proving specificity. Internalization of specific antigen, IgE and high-affinity receptor for IgE (Fc ϵ RI) were impaired in desensitized BMMCs. Our results demonstrate that rapid IgE desensitization is antigen specific and inhibits early and late mast cell activation responses and internalization of the antigen/IgE/Fc ϵ RI complexes.

Keywords: Desensitization · DNP/OVA · Fc ϵ RI · IgE · Mast cells

Introduction

Exposure of IgE-sensitized patients to medication or food allergens can cause the sudden systemic release of inflammatory mediators from activated mast cells, leading to anaphylaxis [1, 2]. Avoidance may be difficult for food-sensitized patients due to cross-reactive food allergens. For medication-sensitized patients, avoidance may lead to significant morbidity and mortality if treatment for cancer or severe infection becomes

necessary, and may decrease the quality of life among patients with chronic inflammatory diseases sensitized to monoclonal antibodies. Desensitization protocols have been developed to help deliver full therapeutic doses of drug allergens, in an incremental, stepwise fashion without eliciting life-threatening symptoms [3–5]. More recently, food desensitization protocols have been generated to protect children and adults from accidental exposures to allergenic foods [6, 7]. Most IgE-sensitized patients present a positive skin test to the offending food or medication, indicating that mast cells and IgE are the main targets of these reactions. After rapid desensitization, specific skin test reactivity is abolished, implying a complete inhibition of the mechanisms that induce mast cell activation [8].

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Mast cells are activated by antigen crosslinking of IgE-bound high-affinity receptor for IgE (Fc ϵ RI) receptors, and aggregation of these receptors results in rapid phosphorylation of tyrosine residues in the ITAMs of β and γ chains by lyn kinase, which leads to recruitment and activation of spleen tyrosine kinase (syk) and fyn. Both fyn and syk phosphorylate downstream targets, leading to calcium mobilization, degranulation, arachidonic acid metabolism, and cytokine and chemokine gene transcription [9, 10]. As opposed to activation, desensitization is a process in which mast cells are rendered hypo-responsive to an activating challenge, either by exposure to low antigen doses in calcium-depleted conditions [11] or by exposure to incremental doses of antigen, in the presence of calcium [12, 13]. Calcium-depleted conditions cannot be applied to human desensitizations, and few studies have addressed physiological desensitizations, since events occurring in the absence of extracellular calcium may not reflect the same pathways as those occurring in the presence of calcium [14]. Internalization of Fc ϵ RI through progressive crosslinking at low levels of antigen has been postulated as the likely mechanism for cell-surface depletion of IgE and cellular unresponsiveness to specific activating doses of allergen [12]. Depletion of molecular targets of activation such as syk has been shown in prolonged antigen desensitization, indicating a universal rather than specific desensitization [15].

Based on our previous study [16], we report here a model of mouse BM-derived mast cell (BMMC) specific rapid desensitization to DNP and OVA antigens in the presence of physiologic levels of calcium. Increasing doses of antigen delivered at fixed time intervals induced a highly specific and prolonged hypo-responsiveness to triggering doses of the desensitizing antigen. Mast cells desensitized to DNP or OVA demonstrated almost complete inhibition of β -hexosaminidase and pre-formed TNF- α release, calcium flux and arachidonic acid metabolism. They did not release significant amounts of newly generated IL-6 or TNF- α and failed to phosphorylate STAT6 and p38 MAPK. When sensitized to both DNP and OVA antigens, DNP-desensitized cells responded fully to OVA and vice versa. Most importantly, specific rapid desensitization targeted the internalization of antigen/IgE/Fc ϵ RI complexes since antigen-specific IgE bound to the α chain of the Fc ϵ RI remained at the membrane level. This model may provide support for the specificity and effectiveness of human desensitizations.

Results

Inhibition of BMMC responses to sequential versus single-dose DNP antigen delivery

In order to compare single-dose antigen delivery (activation) with sequential cumulative doses (rapid desensitization), we first assessed the dose response curve to DNP-human serum albumin conjugated (DNP-HSA) antigen, by β -hexosaminidase release, with cells sensitized with anti-DNP IgE (see Fig. 1A). DNP-HSA doses of 1, 5 and 10 pg (DNP in figure) were non-activating as

single doses, since the percentage of β -hexosaminidase release was comparable to that of the control (1 ng HSA). Cells challenged with higher doses of antigen (> 10 pg DNP-HSA) delivered as single doses achieved significant β -hexosaminidase release. The black bar in Fig. 1A (1 ng DNP) represents the optimal triggering dose of 1 ng DNP-HSA used as target dose for rapid desensitization to 1 ng of DNP-HSA (DNP Des). The release obtained with single-dose additions in Fig. 1A was compared to that obtained with doses added sequentially, following every step of the desensitization protocol (see Fig. 1B, white bars). White bars represent β -hexosaminidase release at each particular point in the cumulative sequence of antigen additions. A maximum of 10% β -hexosaminidase release was achieved at all points in the sequence, showing that the desensitization process did not induce a slow release of mediators.

To determine whether there was a threshold dose that initiated hypo-responsiveness, replicate samples were used, and at each particular point in the sequence of antigen additions, cells were also challenged with a triggering dose of 1 ng DNP-HSA (see Fig. 1B, gray bars). Response to the triggering dose declined with increasing number of sequential doses. The greatest hypo-responsiveness was achieved with the highest number of sequential additions (11, in Fig. 1B), indicating that hypo-responsiveness was not stabilized until the end of the desensitization protocol.

To test whether cells' hypo-responsiveness achieved with rapid desensitization to 1 ng DNP-HSA could be overcome with higher challenging doses, we analyzed the response of desensitized cells to activating doses of 1, 2, 3, 4 and 5 ng of DNP-HSA. Up to five-fold increase in challenging dose did not reverse desensitization (see Fig. 1C).

The protocol was effective when increasing the target dose to 5 and 10 ng, with the same number of steps, time between steps and starting dose (1/1000 the target dose), but less inhibition of β -hexosaminidase release was observed (see Fig. 1D). Cells desensitized to 1 ng DNP-HSA showed a 75% inhibition whereas cells desensitized to 5 and 10 ng DNP-HSA had a 65 and 41% inhibition of β -hexosaminidase release, respectively.

BMMC rapid desensitization impairs early and late phase cell responses

BMMCs sensitized with anti-DNP IgE or anti-OVA IgE were rapid-desensitized as per the protocol presented in Table 1. In both DNP and OVA systems, we measured the release of β -hexosaminidase when antigen was delivered as a single dose (1 ng DNP-HSA/10 ng OVA, black bars in Fig. 2A) or when antigen was delivered following the rapid desensitization protocol (white bars in Fig. 2A). Cells desensitized to 1 ng DNP-HSA and 10 ng OVA showed a 78 and 71% inhibition of β -hexosaminidase, respectively.

Exocytosis of pre-formed mediators from granules cannot occur without external calcium entry. During mast cell activation, the release of calcium from the endoplasmic reticulum provides

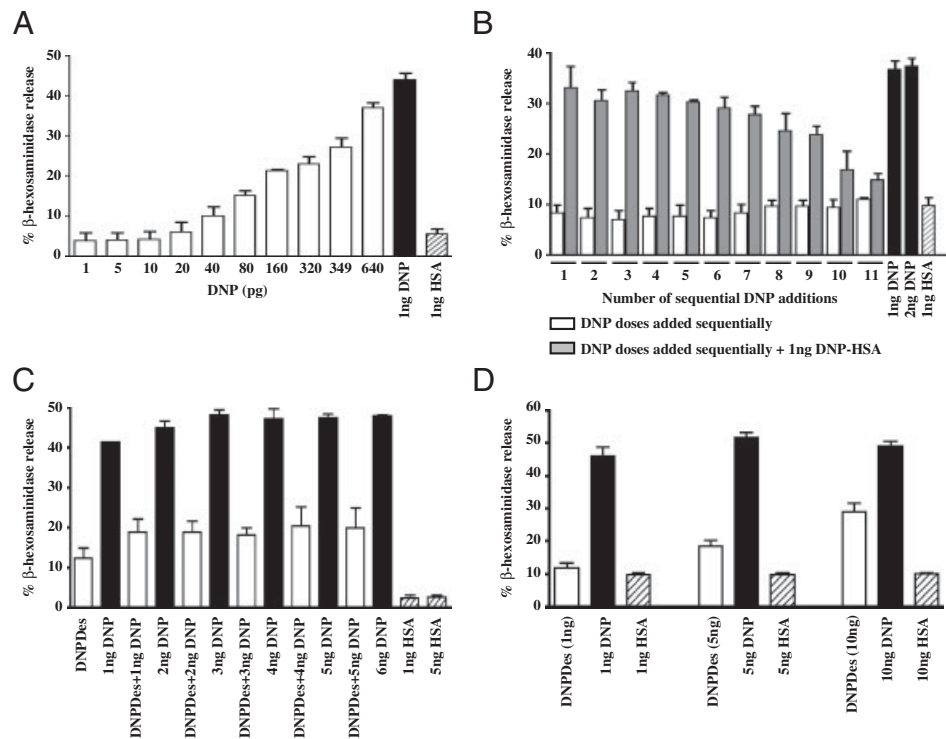


Figure 1. Inhibition of BMMC responses to sequential versus single-dose DNP antigen delivery. Percentage of β -hexosaminidase release assay in cells sensitized overnight with anti-DNP IgE. (A) Dose response to DNP-HSA. (B) DNP-HSA doses sequentially added. White bars show accumulation of β -hexosaminidase at each particular point in the sequence of DNP-HSA additions (DNP in the graph) as per protocol in Table 1. Grey bars show replicate samples in which 1 ng DNP-HSA was added 10 min after the last DNP-HSA addition in the sequence of DNP-HSA additions as per protocol in Table 1. (C) Responsiveness of desensitized BMMCs to different DNP-HSA challenge doses. (D) Rapid desensitizations to 1, 5 and 10 ng of DNP-HSA. All data are expressed as mean+SEM of three independent experiments.

Table 1. Rapid desensitization protocol^{a)}

Steps	Time (min)	Volume (μ L)		Concentration (pg/ μ L)			Dose (pg)	
		DNP-HSA/OVA		DNP-HSA	OVA		DNP-HSA	OVA
1	0	1	1	1	10		1	10
2	10	1	1	5	50		5	50
3	20	1	1	5	50		5	50
4	30	1	1	10	100		10	100
5	40	1	1	10	100		10	100
6	50	2	2	10	100		20	200
7	60	2	2	20	200		40	400
8	70	4	4	20	200		80	800
9	80	8	8	20	200		160	1600
10	90	16	16	20	200		320	3200
11	100	17.5	17.5	20	200		350	3500
11 Steps	100 min	54.5 μ L ^{b)}					1 ng	10 ng

^{a)} Eleven incremental doses of antigen DNP-HSA or OVA were delivered to BMMCs at fixed time intervals until the target dose (1 ng DNP or 10 ng OVA) was reached.
^{b)} Added to 50 μ L of cells.

the signal for calcium-release-activated calcium (CRAC) channels to open, allowing extracellular calcium flux [17]. We compared changes in fluorescence ratios when a triggering dose of 1 ng DNP-HSA was added to non-desensitized cells, to desensitized

cells and to cells that had not been sensitized with anti-DNP IgE. DNP-desensitized cells showed 90% inhibition of calcium mobilization (see Fig. 2B), indicating that calcium-dependent events are impaired during desensitization.

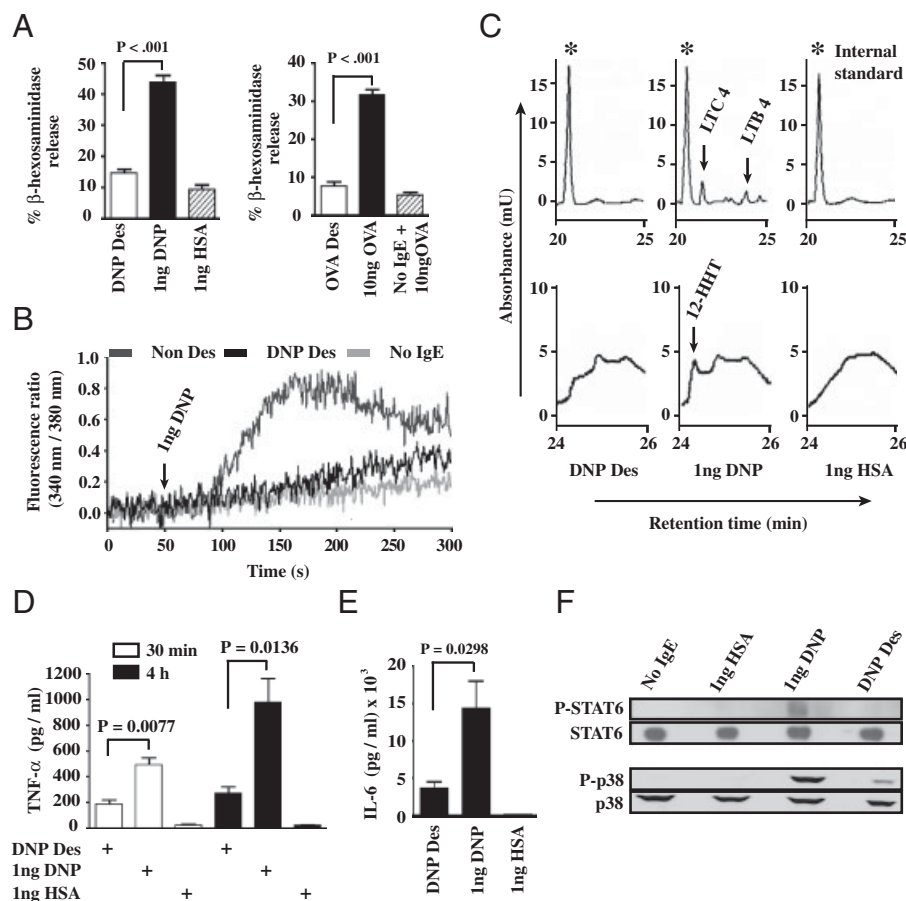


Figure 2. Rapid desensitization impairs early- and late-phase activation responses in BMMCs. (A) Percentage of β -hexosaminidase release after desensitization (DNPDes or OVADes) or DNP-HSA or OVA challenge (1 ng DNP or 10 ng OVA). Negative controls were 1 ng HSA or No IgE + 10 ng OVA. (B) Calcium flux when 1 ng DNP-HSA is added to cells treated as indicated. (C) RP-HPLC analysis of arachidonic acid products in supernatants of cells treated as indicated, with retention times for PGB₂ (internal standard), LTC₄, LTB₄ and 12-HHT of 20.6, 21.4, 23.7 and 24.4 min, respectively. (D) TNF- α secretion (E) IL-6 secretion and (F) phospho-STAT6 and phospho-p38 MAP kinase Western blots of cells treated as indicated. (A, D and E) data are expressed as mean $\pm$ SEM of three independent experiments, $p < 0.05$ was considered to be significant. (B, C and F) Data are representative of three independent experiments. DNPDes: rapid desensitization to DNP; OVADes: rapid desensitization to OVA.

Because calcium mobilization is key to arachidonic acid metabolism and generation of prostaglandins and leukotrienes, we studied arachidonic acid products. Thirty minutes after 1 ng DNP-HSA challenge, cell supernatant was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC); cysteinyl leukotriene C₄ (LTC₄), leucotriene B₄ (LTB₄), and 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid (12-HHT) were identified with retention times of 21.4, 23.7 and 24.4 min, respectively, with prostaglandin B₂ (PGB₂) as an internal standard. In contrast, LTB₄, LTC₄ and 12-HHT were not detected in rapidly desensitized cell supernatants or in cells treated with 1 ng HSA (see Fig. 2C). This result indicates a lack of arachidonic acid metabolism with desensitization.

Other proinflammatory mediators are released from mast cells upon activation, such as TNF- α and IL-6 cytokines. Pre-formed TNF- α is released upon IgE stimulation in the early-phase response, while secretion of de novo synthesized TNF- α and IL-6 production occurs several hours post-stimulation, in the late-phase response. Because early-phase activation events may

influence late-phase responses, and because desensitization may affect early and late-phase responses differently, we studied TNF- α , a product of mast cell responses in both phases, and IL-6, a cytokine not typically stored but produced in the late phase. Pre-formed TNF- α released with 1 ng DNP-HSA challenge was $490 \text{ pg} \pm 15\%$, while in rapid-desensitized cells the release was $185 \text{ pg} \pm 23\%$, a significant 62% reduction (see Fig. 2D, white bars). During the late-phase response, 4 h after activation or desensitization, the release of newly generated TNF- α from DNP-activated cells was $978 \text{ pg} \pm 23\%$, while rapid-desensitized cells released $272 \text{ pg} \pm 22\%$, a significant 72% reduction (see Fig. 2D, black bars). The production of IL-6 assessed 4 h after activation or desensitization (see Fig. 2E) was $14362 \text{ pg} \pm 42\%$ and $3665 \text{ pg} \pm 35\%$, respectively, showing a 75% reduction. Those results indicate that desensitization impaired early- and late-phase mast cell responses.

It has been reported that STAT6 plays a pivotal role in antigen/IgE/Fc ϵ R1-mediated cytokine release from mast cells and that STAT6 phosphorylation occurs not only through the JAK-STAT

pathway after IL-4 receptor activation but also after antigen crosslinking of Fc ϵ RI/IgE [18]. Since our previous studies showed that STAT6-null BMMCs from BALB/c and C57BL/6 mice could not be desensitized [16], we explored how rapid desensitization targeted STAT6. We evaluated STAT6 phosphorylation in DNP-HSA-activated and desensitized cells and observed that STAT6 was not phosphorylated with rapid desensitization (see Fig. 2F).

Since Fc ϵ RI-mediated mitogen-activated protein kinases (MAPKs) activation leads to gene transcription of several cytokines [19, 20], we next examined the levels of phosphorylation of p38 MAPK in DNP-HSA-activated and desensitized cells (see Fig. 2F). As expected by the low levels of TNF- α and IL-6 production, p38 MAPK phosphorylation was inhibited by rapid desensitization, indicating that molecular events leading to cytokine gene transcription were inhibited during rapid desensitization.

Duration of, and antigen requirements for, hypo-responsiveness after rapid desensitization

Because the duration of desensitization may depend on the presence of bound and soluble antigen, we determined the duration of, and antigen requirements for, maintaining hypo-responsiveness after desensitization.

Cells challenged with 1 ng DNP-HSA at 10 min, 2 h and 4 h after desensitization, remained hypo-responsive with a 20% β -hexosaminidase release (see Fig. 3A, first bar of each time group of bars). Treatment of desensitized cells with ionomycin at 10 min, 2 h or 4 h after desensitization, resulted in high levels of β -hexosaminidase release (see Fig. 3A, second bar of each time group of bars), indicating that desensitized cells were not mediator-depleted. Further time points were not pursued due to diminishing cell viability after 6 h (from 91 to 83% viability 4 h after desensitization (100 min)). This decrease in cell viability was attributed to low volume (10^6 cells in 50–100 μ L) and IL-3 and CO₂ depletion.

We then considered the possibility that desensitized BMMCs could remain hypo-responsive to further stimulation due to the

excess of soluble antigen. Washed and non-washed desensitized cells responded similarly to challenge (see Fig. 3B), indicating that once hypo-responsiveness was achieved the presence of soluble antigen was not required for maintaining desensitization.

Rapid desensitization inhibits Fc ϵ RI internalization but does not impair specific activation

Internalization of antigen/IgE/Fc ϵ RI complexes has been demonstrated after cell activation [21, 22], and it has been suggested that mast cell hypo-responsiveness to low antigen doses is due to internalization of antigen-bound receptors [12]. We wanted to determine the fate of the antigen/IgE/Fc ϵ RI complex with desensitization.

We analyzed surface expression of Fc ϵ RI α and IgE in rapid-desensitized cells, in cells challenged with 1 ng DNP-HSA or with 1 ng HSA, and in non-sensitized cells. Surface expression levels of Fc ϵ RI α and IgE in desensitized cells were similar to those of cells challenged with 1 ng HSA and significantly higher than in activated cells (see Fig. 4A), indicating the impairment of internalization of IgE and Fc ϵ RI α .

Since most of the IgE/Fc ϵ RI complexes remained on the cell surface, we sought to determine whether anti-IgE could crosslink free IgE on desensitized cells. DNP-desensitized cells released β -hexosaminidase when treated with anti-IgE (see Fig. 4B), indicating that unbound IgE was available for crosslinking and remained accessible.

To assess the fate of the desensitizing antigen, we used Alexa Fluor 488-conjugated OVA and followed its localization in activated and desensitized cells (see Fig. 4C). A cross-sectional view of the intracellular compartment revealed that cells challenged with 50 ng of fluorescently labeled OVA showed large internalized aggregates, as confirmed by other researchers [23]. In contrast, OVA-desensitized cells showed fewer and smaller fluorescent aggregates, and their visual appearance was similar to that of cells challenged at 4°C, in which crosslinked receptors were not internalized and appeared with small aggregates bound to the membrane.

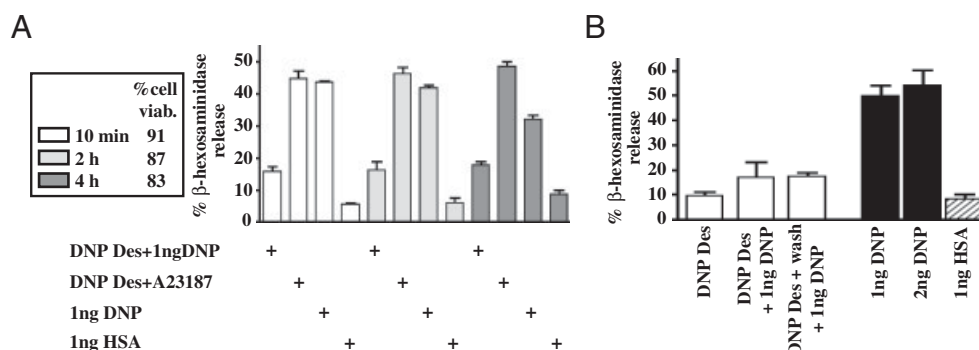


Figure 3. Duration of, and antigen requirements for, hypo-responsiveness after rapid desensitization. (A) Percentage of β -hexosaminidase release from cells sensitized overnight with anti-DNP IgE in response to the indicated treatments at various time points after desensitization. (B) Maintenance of desensitization with or without washing before challenge with 1 ng DNP-HSA. Data are expressed as mean $\pm$ SEM of three independent experiments.

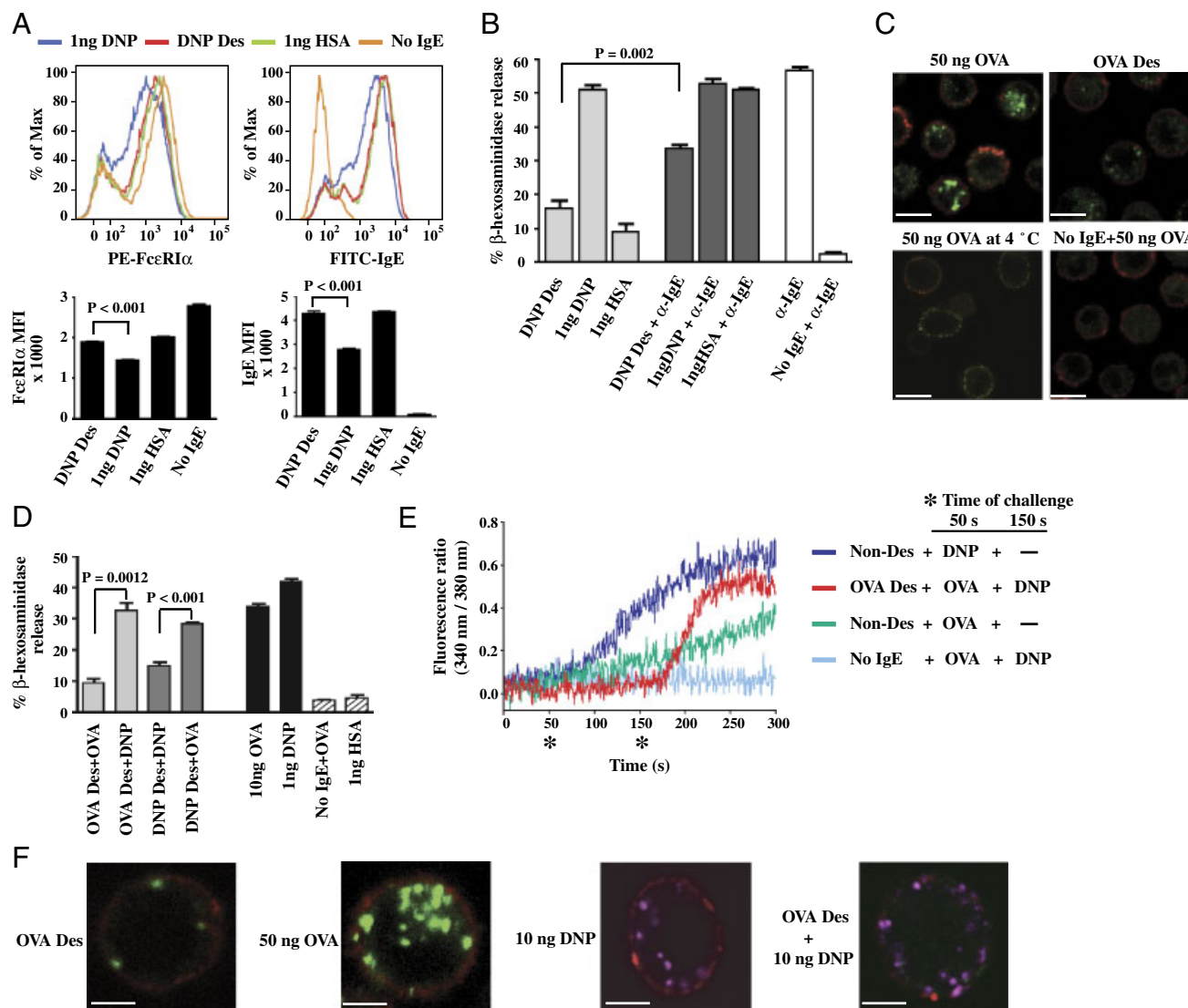


Figure 4. Antigen/IgE/Fc ϵ RI complex internalization is inhibited during BMMC rapid desensitization but does not impair specific activation. (A) Cells sensitized overnight with anti-DNP IgE or non-sensitized cells used as a control (No IgE) were treated as indicated. Representative histograms with Fc ϵ RI α and IgE surface expression (upper panel) and mean fluorescence intensities are shown (lower panel). (B) Percentage of β -hexosaminidase release in response to the indicated treatments. (C) Anti-OVA IgE sensitized cells or non-sensitized cells (No IgE) were treated as indicated and visualized by confocal microscopy. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green). Fields were obtained from one experiment and are representative of four independent experiments. Scale bar = 8.5 μ m, original magnification 630 $\times$. In (D, E and F) cells were sensitized with both anti-DNP IgE and anti-OVA IgE and non-sensitized cells were used as negative control for OVA activation (No IgE). Percentage of β -hexosaminidase release (D) and calcium flux (E) of cells in response to the indicated treatments. Data in (A, lower panel, B and D) are expressed as mean $\pm$ SEM of three independent experiments. Statistical significance was determined using Student's unpaired two-tailed *t* test, $p < 0.05$ was considered to be significant. (F) Confocal microscopy of cells treated as indicated. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green) and DNP-DyLight 649 (purple). Fields were obtained from one experiment and are representative of three independent experiments. Scale bar = 3 μ m, original magnification 630 $\times$.

Since desensitized cells were hypo-responsive to further triggering doses of the same antigen, we studied the response to a second triggering antigen. Cells sensitized with anti-DNP IgE and anti-OVA IgE were desensitized to OVA or to DNP and then challenged with triggering doses of DNP-HSA or OVA, respectively. Cells desensitized to OVA responded (β -hexosaminidase release) to a triggering dose of 1 ng DNP-HSA, and cells desensitized to DNP responded to a triggering dose of 10 ng OVA (see Fig. 4D), indicating that

mediators were not depleted after desensitization to one antigen and that desensitization disabled the specific response only to the desensitizing antigen.

We then analyzed the specificity of the calcium responses. Cells desensitized to OVA had impaired calcium influx when triggered with 10 ng OVA, but the influx was restored by a triggering dose of 1 ng DNP-HSA (see Fig. 4E, red line), indicating that the calcium response was compartmentalized by specific antigen.

We then analyzed specificity using confocal microscopy (see Fig. 4F). OVA-desensitized cells showed low internalization of labeled OVA antigen (green) as compared to the larger aggregates seen in OVA-activated cells. When OVA-desensitized cells were challenged with DNP-HSA (purple), the amount of internalization was comparable to that of DNP-HSA activated cells, indicating that desensitization left unaffected the specific mechanisms of cell activation and receptor internalization.

Discussion

Our understanding of IgE desensitizations has been limited by the paucity of *in vitro* mast cell models providing quantitative and qualitative insight into the early and late cell responses. Here, we present an *in vitro* 11-step model of mouse BMMC rapid IgE desensitization under physiologic calcium conditions and characterize its kinetics, effectiveness, antigen specificity and receptor internalization-associated events.

We showed that desensitization is a dynamic process in which each step provides a platform for the next level of response reduction and that once desensitized, mast cells remain hypo-responsive to further antigen challenges. Increasing the target antigen dose for desensitization decreased the hypo-responsiveness, suggesting that additional steps and/or time between steps might help to achieve better desensitization, thus underscoring the critical relationship between IgE sensitization and the target antigen dose for desensitization. In human desensitizations the level of IgE sensitization varies and is unknown for each patient and the target dose used for desensitization is empirical, which impacts its safety [4, 5, 8].

The mechanism of desensitization is not fully understood and we have observed that low antigen doses induce small amounts of extracellular calcium flux, indicating the mobilization of endoplasmic reticulum stores, enabling functional CRAC channels to open [17]. The sequential delivery of low antigen doses during desensitization may provide continued low levels of calcium entry with conformational changes of CRAC and other calcium-related channels locking further calcium entry and blocking signal transduction. Because calcium entry is clearly specifically impaired in our model, since a second non-desensitizing antigen allowed restoration of calcium flux, membrane compartmentalization may be required to exclude signal transduction molecules around desensitized receptors.

We observed that in desensitized cells, phosphorylation of STAT6 and p38 MAP kinase was impaired and consequently TNF- α and IL-6 production was diminished. Since earlier studies indicated that STAT6-null BMDCs could not be desensitized [16], it is possible that STAT6 activity is required for desensitization, via a pathway different from the one leading to the acute and late activating responses. Our system is limited by the fact that BMDCs are cultured in IL-3, which may affect cytokine production [24]. Nonetheless, this may have an important correlate in human desensitizations since our group has not observed delayed reactions in desensitized patients, confirming that the inhibition

of mast cell activation during desensitization prevented later hypersensitivity reactions [4, 5].

Maintenance of hypo-responsiveness in desensitized cells was not sustained by the presence of an excess of soluble antigen since washed cells remained desensitized. It is possible that bound antigen is equilibrated in desensitized cells. Earlier studies [12, 13] suggested that the hypo-responsiveness induced by desensitization was due to internalization of antigen/IgE/Fc ϵ RI complexes and that the lack of available IgE renders the cells refractory to further stimulation. In contrast, we show here that, unlike activation, internalization of IgE and Fc ϵ RI is impaired during specific desensitization (Fig. 4A) and that desensitized cells can be triggered by anti-IgE, since unbound IgE remains accessible and is available for crosslinking (Fig. 4B). Saturating doses of IgE in a co-culture system and the use of higher antigen doses [12] may promote internalization while low doses may redistribute antigen-bound receptor at the membrane level. Moreover, others have shown that low doses of antigen induce antigen-crosslinked receptors to remain mobile on the cell surface [25]. In addition, microscopy studies gave us the opportunity to directly look into antigen localization after desensitization.

Previous studies in calcium-depleted conditions have shown that cells desensitized to one antigen could not be triggered with a non-desensitizing second antigen [26], possibly through a disabling mechanism involving syk. Due to the amount of IgE sensitization and low antigen doses used in our model, we could not detect syk phosphorylation. Our findings indicate that the mast cell-activating machinery was intact for a non-desensitizing antigen action, since no mediator depletion occurred with desensitization, calcium flux was restored in desensitized cells when challenged with a non-desensitizing antigen and microscopic analysis confirmed that rapid desensitization is antigen specific and does not induce anergy [27].

While we do not know the exact mechanism that could explain this inhibition of receptor internalization during desensitization, it is possible that the mobility of antigen/IgE/Fc ϵ RI complexes and membrane re-arrangement could prevent their internalization, as shown by others with low doses of multivalent antigen [25]. In addition, receptors engaged with low doses of antigen could be segregated into different compartments, preventing access to phosphorylating molecules. Inhibitory phosphatases such as SHP-1 may not be excluded from those compartments, thus preventing phosphorylation of key molecules required for signal transduction. A time course study of SHP-1 phosphorylation in RBL-2H3 cells [28] has shown a peak at 1 min of Fc ϵ RI cross-linking and a gradually decline within 10 min. Our initial results indicated a lack of phosphorylation at 100 min. (data not shown). Further studies are planned to look for phosphorylation of SHP-1 and other ITIM-bearing molecules [29, 30] at each step of the desensitization protocol since it may be transient.

In conclusion, this model of rapid IgE desensitization is effective and reproducible and provides an optimal dose–time relationship, leading to almost complete abrogation of early- and late-phase activation events. This model of antigen-specific desensitization disables the specific response to one antigen

but keeps the cell machinery unaffected, unlike non-specific desensitization. Most importantly, we show here that specific rapid desensitization inhibits internalization of the antigen/IgE/FcεRI complexes.

The lack of severe anaphylactic reactions in our previous clinical reports [4, 5], including hundreds of desensitizations using a modified protocol, illustrates a profound inhibition of acute and delayed mast cell activation. These studies provide proof of concept for the effectiveness and specificity of human desensitizations.

Materials and methods

Cell culture

BMMCs derived from femurs of male BALB/c mice 8–12 wk old (Jackson Laboratory) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1% Penicillin-Streptomycin, 0.1 mM MEM nonessential amino acids (all from Sigma-Aldrich) and 10 ng/mL of IL-3. IL-3 was obtained from supernatants of 293T cells expressing mouse IL-3 [31, 32].

Activation and rapid desensitization of BMMCs to DNP and OVA antigens

DNP antigen: Cells were sensitized overnight with anti-DNP IgE (0.25 µg/10⁶ cells/mL). The next day, cells were washed to eliminate possible excess of unbound IgE, resuspended in 50 µL of fresh medium without IL-3 and placed at 37°C. For desensitization, cells were treated as per Table 1 (rapid desensitization protocol), and 10 min after the last DNP-HSA addition, placed on ice for β-hexosaminidase release assay. For activation, cells were challenged with 50 µL of DNP-HSA at 20 pg/µL (1 ng DNP) and for control, with 50 µL of HSA at 20 pg/µL (1 ng HSA), and after 10 min, placed on ice for β-hexosaminidase release assay. β-Hexosaminidase release assay was performed as previously described [16].

OVA antigen: Same described method used for DNP antigen, but with overnight sensitization performed with murine post-immunization serum with OVA-specific IgE (0.25 µg/10⁶ cells/mL) (anti-OVA IgE). For activation, 50 µL of OVA at 200 pg/µL (10 ng OVA) was used. For control, 50 µL of OVA at 200 pg/µL was added to cells without anti-OVA IgE overnight incubation.

For specificity experiments, cells were sensitized overnight with 0.25 µg/10⁶ cells/mL of both anti-DNP IgE and anti-OVA IgE.

Challenge with anti-IgE

After cells were desensitized or challenged with DNP or HSA, we treated them with 100 ng of rat anti-mouse IgE (clone R35-72

from BD Pharmingen). For control, cells incubated overnight with or without anti-DNP IgE were also treated with 100 ng of rat anti-mouse IgE.

Measurement of intracellular calcium

Desensitized, non-desensitized and non-IgE treated cells were washed and resuspended in HBSS containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.1% BSA (Buffer A). Cells were then loaded with 2.5 µM Fura-2AM (Molecular Probes) in the presence of 2.5 mM probenecid for 30 min at 37°C. After being labeled, cells were washed and resuspended in cold Buffer A (0.5 × 10⁶/mL). Fluorescence output was measured with excitation at 340 and 380 nm in the F-4500 Fluorescence Spectrophotometer (Hitachi), and the relative ratio (*R*) of fluorescence emitted at 510 nm was recorded. For all fluorescence ratios to start at zero, the first fluorescence value of each sample was subtracted from all its subsequent fluorescence values.

RP-HPLC

After desensitization or challenge, cell supernatants were collected and LTB₄, LTC₄ and 12-HHT were measured by RP-HPLC following a published protocol [33]. Briefly, samples were applied to a C18 Ultrasphere RP column (Beckman Instruments) equilibrated with a solvent consisting of methanol/ACN/water/acetic acid (10:15:100:0.2, v/v), pH 6.0 (Solvent A). After injection of the sample, the column was eluted at a flow rate of 1 mL/min with a programmed concave gradient to 55% of the equilibrated Solvent A and 45% of Solvent B (100% methanol) over 2.5 min. After 5 min, Solvent B was increased linearly to 75% over 15 min and maintained at this level for an additional 15 min. The UV absorbance at 280 and 235 nm and the UV spectra were recorded simultaneously. PGB₂ was used as an internal standard.

TNF-α and IL-6 measurement

After desensitization or challenge, TNF-α and IL-6 contents in cell-free supernatants were estimated using a mouse TNF-α or IL-6 ELISA kits (eBioscience), either 30 min or 4 h after activation or desensitization, according to the manufacturer's protocol.

Duration of rapid desensitization

Cells were rapid desensitized as per Table 1. After desensitization (nearly 2 h) cells were maintained for 10 min, 2 hours, or 4 hours at 37°C. After each time period, 1 ng of DNP-HSA or 25 µL of calcium ionophore A23187 (Sigma-Aldrich) 10 µM was added. Non-desensitized cells were kept at 37°C and challenged with 1 ng of DNP-HSA or 1 ng HSA at the same time points as for

desensitized cells. The total time for all cells at 37°C, since rapid desensitization protocol lasts nearly 2 h, was 6 h. Cell viability was assessed by trypan blue dye exclusion.

Immunoblot analysis

After desensitization or challenge, cells were collected and washed with cold PBS. Pellets were lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). Total protein lysates were subjected to SDS-PAGE on a 4–12% polyacrylamide gel and transferred to a nitrocellulose membrane (both from Invitrogen). Membranes were blotted with anti-Phospho-STAT6 (phosphotyrosine 641) and anti-STAT6 from Sigma-Aldrich or with anti-Phospho-p38MAP kinase and anti-p38 α MAP kinase from Cell Signaling. Signal detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Flow cytometry analysis

After desensitization or challenge, cells were placed at 4°C, then washed and resuspended in PBS containing 0.5% BSA and 0.05% sodium azide at 4°C and incubated with anti-Fc γ RI/II mAb (eBioscience) for 20 min on ice to block Fc γ receptors. Cells were then incubated with 5 μ g/mL FITC rat anti-mouse IgE (BD Biosciences) or 2 μ g/mL PE Armenian hamster anti-mouse Fc ϵ RI α (eBioscience) or with the recommended isotype controls. Cells were analyzed on a BD Biosciences FACSCanto flow cytometer, using FACSDiva acquisition software and FlowJo analysis software.

Confocal microscopy

Antigens used were Alexa Fluor 488-conjugated OVA (Molecular Probes) and DyLight Fluor 649-conjugated DNP, labeled with DyLight 649 NHS Ester (Thermo Scientific). Due to detection limitations, OVA activation dose was 50 ng, DNP activation dose was 5 ng and the rapid OVA desensitization protocol was consequently adjusted based on the volumes used in the protocol in Table 1 but at higher concentrations. After desensitization or challenge, cells were washed and resuspended in cold PBS. Cells were transferred onto poly-L-lysine-coated round cover slips for 20 min at 4°C and then fixed with 4% paraformaldehyde in PBS for 10 min at 4°C. After three washes with PBS, cells were incubated with cholera toxin subunit B-Alexa Fluor 555 conjugate (Molecular Probes) 1:500 in PBS for 10 min at 4°C, washed three times with PBS and mounted using an aqueous mounting medium (15% wt/v polyvinyl alcohol, 33% v/v glycerol, 0.1% azide). Images were collected sequentially using a 63 $\times$ plan Apo NA 1.4 objective on Leica SP5X laser scanning confocal system attached to an inverted Leica DMI6000 microscope.

Statistical analyses

Data are expressed as mean $\pm$ SEM using Prism4. Statistical significance was determined using Student unpaired two-tailed *t* test. *p* < 0.05 was considered to be significant.

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Abbreviations: 12-HHT: 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid · BMMCs: BM-derived mast cells · CRAC: calcium-release-activated calcium · FcεRI: high-affinity receptor for IgE · HSA: human serum albumin · LTB₄: leucotriene B₄ · LTC₄: cysteinyl leukotriene C₄ · PGB₂: prostaglandin B₂ · syk: spleen tyrosine kinase

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