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Brief Communication

Two-Allergen Model Reveals Complex Relationship between IgE Crosslinking and Degranulation

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SUMMARY

Allergy is an immune response to complex mixtures of multiple allergens, yet current models use a single synthetic allergen. Multiple allergens were modeled using two well-defined tetravalent allergens, each specific for a distinct IgE, thus enabling a systematic approach to evaluate the effect of each allergen and percentage of allergen-specific IgE on mast cell degranulation. We found the overall degranulation response caused by two allergens is additive for low allergen concentrations or low percent specific IgE, does not change for moderate allergen concentrations with moderate to high percent specific IgE, and is reduced for high allergen concentrations with moderate to high percent specific IgE. These results provide further evidence that supraoptimal IgE crosslinking decreases the degranulation response and establishes the two-allergen model as a relevant experimental system to elucidate mast cell degranulation mechanisms.

INTRODUCTION

Common allergen sources such as peanuts contain complex mixtures of allergens. Recent efforts have been made to identify each allergen present in the mixtures to determine the major allergens that are capable of eliciting a degranulation response in the majority of patients who possess the specific allergy. For example, of the 11 different proteins present in peanuts that are capable of inducing immunoglobulin E (IgE) antibody production, 4 have been identified as major allergens (Zhuang and Dreskin, 2013). Of the four major peanut allergens, Ara h 2 and Ara h 6 are responsible for the majority of the degranulation response initiated by crude peanut extract (CPE) (Porterfield et al., 2009). Interestingly, removal of either Ara h 2 or Ara h 6 from CPE does not affect the activity of the crude extract (Chen et al., 2011). However, if both are removed, the potency of the CPE is significantly diminished, indicating that the presence of the second major allergen has negligible effects on the degranulation response (Chen et al., 2011). Despite the presence of multiple major allergens capable of inducing a degranulation response in a natural system, current models use only a single synthetic allergen paired with a single monoclonal IgE to stimulate a degranulation response (Passante and Frankish, 2009). Even with their limitations, the current models have helped to discover critical aspects of mast cell signaling. Of particular interest, recent work has identified the Src homology 2-containing inositol polyphosphate 5′-phosphatase (SHIP1) as a critical regulator in the suppression of mast cell degranulation in response to supraoptimal allergen-IgE crosslinking (Huber, 2013). In this study, using a two-allergen model, we sought to investigate further the conditions where supraoptimal allergen-IgE crosslinking results in reduced mast cell degranulation. This was accomplished by using two allergens, each specific for a different monoclonal IgE, at varying concentrations to assess the conditions where the effect of the second allergen resulted in increased degranulation, no change in degranulation, and decreased degranulation associated with supraoptimal IgE crosslinking. Additionally, we evaluated how the degranulation response changes with decreasing percentage of allergen-specific IgE.

RESULTS

Design of the Two-Allergen Model

In our previous work, we described the design, synthesis, and characterization of synthetic tetravalent allergens (Handlogten et al., 2012, 2013a, 2013b). These well-defined tetravalent allergens have several advantages compared to the widely used haptenated proteins, such as 2,4-Dinitrophenyl hapten (DNP) conjugated to BSA (DNP-BSA), as model allergens. The method used to synthesize these widely used allergens relies on the nonspecific conjugation of DNP to the ε-amine of lysine residues of BSA. This process results in poorly defined allergens with significant heterogeneity in both the number of haptens per protein and the sites of hapten conjugation, resulting in variable potency from batch to batch. This complicates results as only the average number of haptens per carrier can be determined, with many of the haptens likely unavailable to bind to surface-bound IgE due to steric constraints (Hlavacek et al., 1999; Xu et al., 1998). As a result, a small subpopulation of the synthetic allergen, with properties distinct from the average, may be responsible for the majority of the degranulation response. In the design of the tetravalent allergens, we ensured that each hapten is available to bind to a distinct IgE antibody without the capacity to bind bivalently to a single IgE. In addition, the tetravalency models the valency of several common allergens, including Ara h 3 from peanuts, Tri a 14 from wheat, and Cuc m 2 from melon, each of which have four immunodominant epitopes (Denery-Papini et al., 2011; Rabjohn et al., 1999; Tordesillas et al., 2010). Finally, the synthetic scheme used for the tetravalent allergens ensures that each

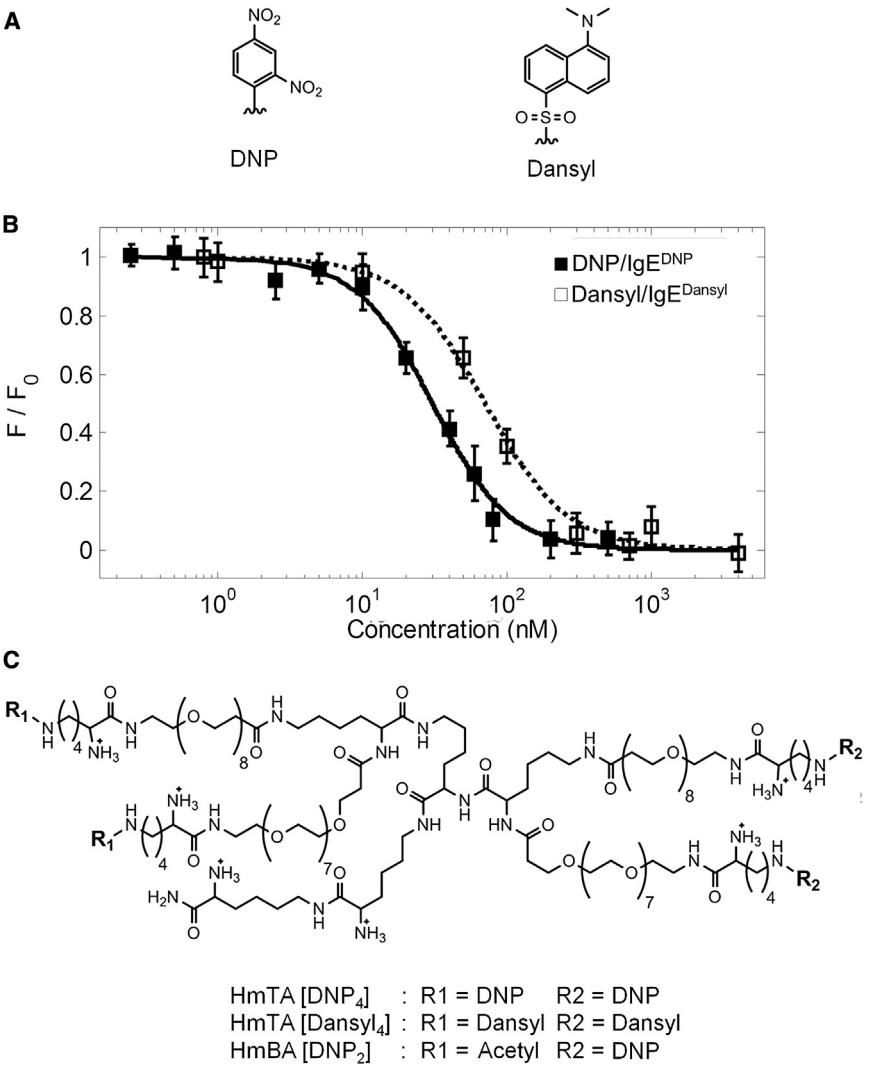

Figure 1. Characterization and Structure of the HmTAs

(A) Structures of the haptens DNP and dansyl used in the tetravalent allergens.

(B) The affinity of each hapten was determined for its specific IgE using a fluorescence-quenching technique (Supplemental Information). The K_D of DNP for IgE^{DNP} was 22 ± 2 nM, and the K_D of dansyl for IgE^{dansyl} was 54 ± 4 nM. Data represent the means ± SD of triplicate experiments.

(C) Structures of the synthetic allergens HmTA [DNP_4], HmTA [$dansyl_4$], and HmBA [DNP_2].

allergen is identical, thus allowing for direct analysis of the allergen properties on the stimulation of mast cell degranulation. In the present study, we used two homotetravalent allergens (HmTAs), each specific for a different IgE, to model the multiple major allergens found in typical allergen sources. In addition, we used a third, non-allergen-specific IgE to better represent the heterogeneity of IgE present on mast cell surfaces. The two-allergen model provides a more physiologically relevant allergy system to evaluate the effect of multiple allergy-inducing proteins present in typical allergen sources on mast cell degranulation.

Evaluation of the Tetravalent Allergens

The two-allergen model required two hapten/IgE pairs and a third non-allergen-specific IgE. The DNP/IgE^{DNP} pair is the most commonly used system to study mast cell degranulation; consequently, it was selected as the first hapten/IgE pair (Andrews et al., 2009; Passante and Frankish, 2009). The second hapten/IgE pair selected was dansyl/IgE^{dansyl}. This hapten/IgE pair was selected because dansyl, similar to DNP, is a small molecule that is easily incorporated into multivalent designs (Figure 1A). Finally, $IgE^{cyclin\ A}$ was selected as the third IgE to represent IgE of other specificity present on the surface of mast cells. The first step in characterizing the two-allergen model was to determine the affinity of DNP and dansyl for their respective IgE antibodies using a fluorescence-quenching assay as described previously (Handlogten et al., 2011). Using this technique, we determined that the dissociation constant, K_D, of DNP for IgE^{DNP} was 22 ± 2 nM and that the K_D of dansyl for IgE^{dansyl} was 54 ± 4 nM (Figure 1B). With the same technique, we also determined there was no cross-reactivity between the hapten/IgE pairs (Figure S1 available online).

Next, tetravalent versions of both DNP and dansyl were synthesized by conjugating each hapten to the tetravalent scaffold resulting in HmTA DNP (HmTA [DNP_4]) and HmTA dansyl (HmTA [$dansyl_4$]; Figure 1C). In addition, a homobivalent allergen DNP (HmBA [DNP_2]) with a structure identical to that of HmTA [DNP_4], except with two acetylated arms, was also synthesized to evaluate the effect of allergen valency on mast cell degranulation (Figure 1C). To further establish that there was no cross-reactivity in the IgE-allergen pairs, mast cells were primed with IgE^{DNP}, IgE^{dansyl}, or $IgE^{cyclin\ A}$ and exposed to increasing concentrations of the synthetic allergens. As expected, the tetravalent allergens only stimulated a response when the mast cells were primed with the allergen-specific IgE, IgE^{DNP} for HmTA[DNP_4], or IgE^{dansyl} for HmTA [$dansyl_4$] (Figure 2). Both HmTA [DNP_4] and HmTA [$dansyl_4$] stimulated a similar percent degranulation; however, the HmTA [DNP_4] allergen proved to be more potent, stimulating a maximum response at 10 nM compared to 100 nM for HmTA [$dansyl_4$]. This is likely a reflection of the difference in affinities of DNP for IgE^{DNP} (K_D = 22 nM) compared to dansyl for IgE^{dansyl} (K_D = 54 nM). In line with previous work, the bivalent allergen HmBA [DNP_2] failed to stimulate a response under any condition due to insufficient valency (Figure 2) (Posner et al., 2007; Sil et al., 2007). Combined, these results validate that there is no cross-reactivity between the IgE-hapten pairs and demonstrate the suitability of the two-allergen model for investigating the effect of multiple unique allergens on mast cell degranulation.

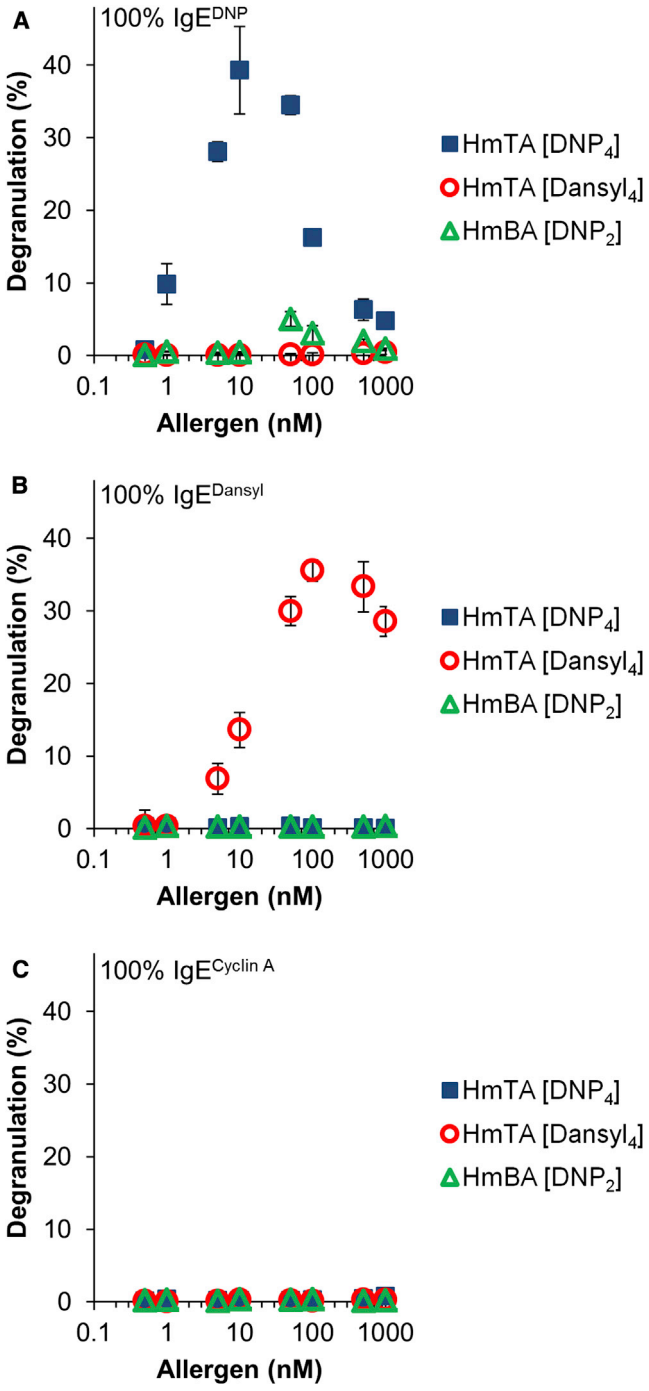

Figure 2. Specificity of the Tetravalent Allergens

RBL cells were primed with (A) IgE^{DNP}, (B) IgE^{dansyl}, and (C) $IgE^{cyclin\ A}$ and then exposed to increasing concentrations of the synthetic allergens HmTA [DNP_4], HmTA [$dansyl_4$], and HmBA [DNP_2] to determine maximum degranulation response. HmTA [DNP_4] and HmTA [$dansyl_4$] only stimulated a response when the RBL cells were primed with IgE^{DNP} and IgE^{dansyl}, respectively. HmBA [DNP_2] did not stimulate a response under any condition. Data represent the means ± SD of triplicate experiments.

Evaluation of the Two-Allergy Model System

Next, rat basophilic leukemia (RBL) cells were primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl}. We previously established that the relative concentration of each IgE in solution is preserved on the surface of the mast cells (Handlogten et al., 2012). The mast cells were then exposed to mixtures of HmTA [DNP_4] and HmTA [$dansyl_4$], with concentrations ranging from 0.5 nM to 1,000 nM (Figure 3; Figure S2). These results revealed a complex relationship between allergen concentration and the respective degranulation response. For low concentrations of HmTA [DNP_4] and HmTA [$dansyl_4$], the presence of both allergens resulted in increased degranulation; for moderate allergen concentrations, the presence of a second allergen did not change the degranulation response; and for high allergen concentrations, the presence of a second allergen decreased the degranulation response. Interestingly, at the highest allergen concentrations, the overall degranulation response began to increase again as observed with HmTA [$dansyl_4$] concentrations of 50 and 100 nM (Figure 3). The bell-shaped dose-response curve has traditionally been attributed to the degree of IgE aggregation (Huber, 2013; Posner et al., 2007; Sil et al., 2007). As the allergen concentration increases, the degree of IgE crosslinking increases up to a point when monovalent allergen-IgE interactions begin to dominate due to excess allergen. According to this model, the presence of the second allergen could result in either increased degranulation when administered at moderate concentrations or no change in degranulation when administered at very high or very low concentrations. Instead, we observed that, at HmTA [$dansyl_4$] concentrations above 50 nM when the concentration of HmTA [DNP_4] exceeded 10 nM, the degranulation response decreased (Figure 3; Figure S2). Since these allergens each bind to a separate IgE, the decrease in the degranulation response cannot be attributed to competitive inhibition of IgE crosslinking caused by excess allergen. Instead, there is an optimal degree of IgE crosslinking on the surface of mast cells, above which there are inhibitory pathways that become active to limit the degranulation response (Huber, 2013). Accordingly, the degranulation response continues to increase with allergen concentration until the optimal degree of IgE crosslinking is reached, as observed by the increased degranulation response with low concentrations of HmTA [DNP_4] and HmTA [$dansyl_4$] each. As the degree of crosslinking approaches the optimal amount, the additive effect of the second allergen decreases, as observed by the degranulation response with moderate concentrations of both HmTA [DNP_4] and HmTA [$dansyl_4$]. Finally, as the degree of crosslinking increases beyond the optimal level, the degranulation response decreases regardless of which allergen is causing the increased IgE crosslinking, as demonstrated by a decrease in degranulation observed at high concentrations of HmTA [DNP_4] and HmTA [$dansyl_4$].

Next, we investigated how the degranulation response changed with decreasing percent specific IgE by incorporating $IgE^{cyclin\ A}$. As can be seen in Figures 3 and S2, the trends observed with RBL cells primed with the 50/50 mix of IgE^{DNP} and IgE^{dansyl} are similar to those primed with the 25/25/50 and 15/15/70 mixes of IgE^{DNP}/IgE^{dansyl}/$IgE^{Cyclin\ A}$. However, as the percent specific IgE is further decreased to 10/10/80 and 5/5/90: IgE^{DNP}/IgE^{dansyl}/$IgE^{cyclin\ A}$, the degranulation response exhibited a subtle change (Figure 3). Maximum degranulation still

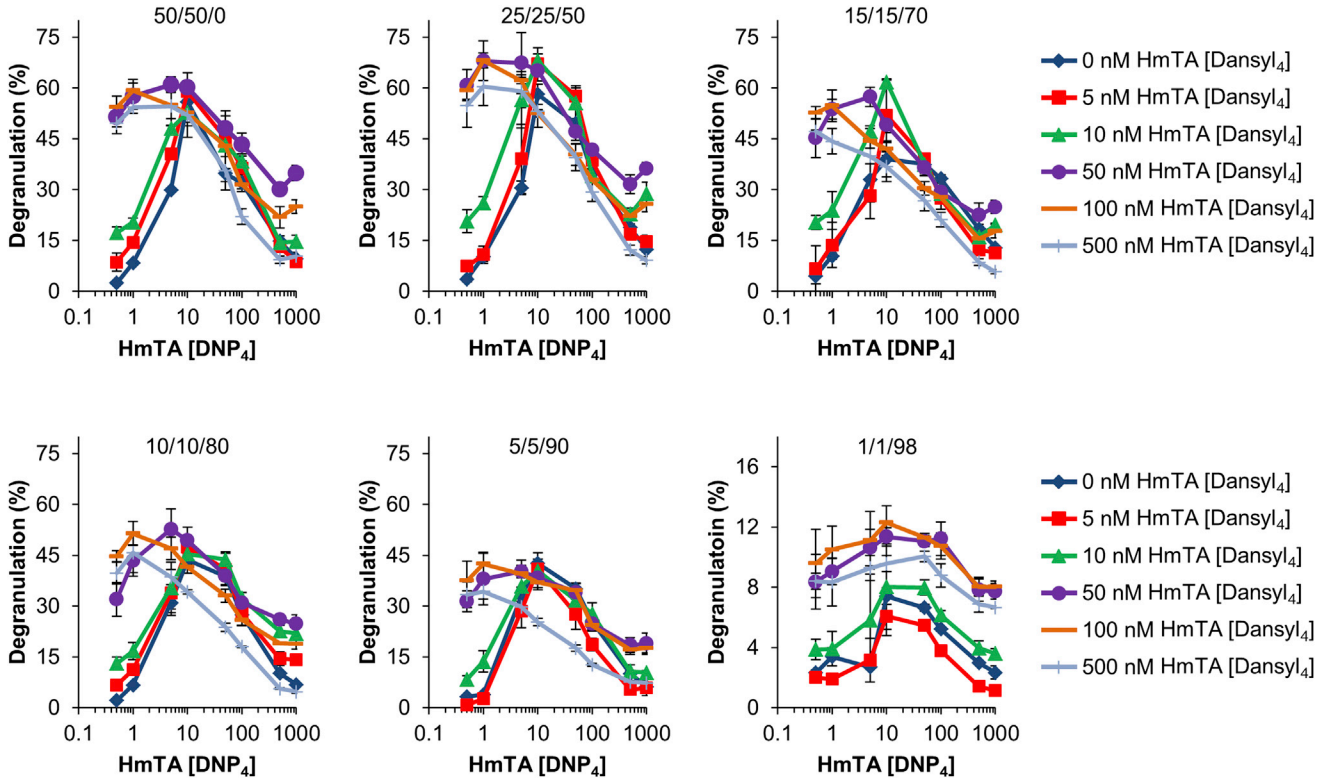

Figure 3. Two-Allergen Model Reveals Complex Relationship between IgE Crosslinking and Degranulation

RBL cells were primed with mixtures of IgE (50/50/0, 25/25/50, 15/15/70, 10/10/80, 5/5/90, and 1/1/98: $IgE^{DNP}/IgE^{dansyl}/IgE^{cyclin\ A}$) at a saturating total IgE concentration of 1 μg/ml. The cells were washed and then exposed to mixtures of HmTA [DNP_4] with HmTA [$dansyl_4$], ranging from 0.5 to 1,000 nM concentration of each allergen. Data represents the means ± SD of triplicate experiments.

See also Figure S2.

occurred at 10 nM HmTA [DNP_4]; however, the decrease in degranulation previously observed with an increasing HmTA [DNP_4] concentration to 50 nM did not occur. Instead, the degranulation response reached a plateau at 10 nM HmTA [DNP_4] and did not begin to decrease significantly until a concentration of 100 nM HmTA [DNP_4]. This suggests that, since there was a lower percentage of allergen-specific IgE on the surface of the mast cells, a higher concentration of the allergen was required to reach the same degree of IgE crosslinking. The binding curve of DNP to IgE^{DNP} (Figure 1B) supports this theory. While the DNP-IgE^{DNP} K_D is 22 nM (50% bound), 100% binding does not occur until ~200 nM, demonstrating that there is increased binding of allergen to the surface-bound IgE until a concentration of ~200 nM. Furthermore, as the percentage of allergen-specific IgE was decreased to 1/1/98: $IgE^{DNP}/IgE^{dansyl}/IgE^{cyclin\ A}$, the degranulation response stimulated by the two allergens was approximately additive at all allergen concentrations. This result suggests that, with only 1% specific IgE for each allergen, there is not sufficient IgE to attain supraoptimal IgE crosslinking.

To further demonstrate that the changes in degranulation observed in Figure 3 were due to changes in IgE crosslinking and not due to some other property of DNP, we used a monovalent DNP ligand in place of HmTA [DNP_4]. The RBL cells were primed with 25/25/50: $IgE^{DNP}/IgE^{dansyl}/IgE^{cyclin\ A}$, which was the condition where maximum degranulation was observed (Figure 3). Then, the RBL cells were exposed to mixtures of HmTA [$dansyl_4$] and monovalent DNP, with the concentration of each ranging from 0.5 to 1,000 nM (Figure 4A). As expected, the monovalent DNP ligand did not cause any significant change in the degranulation response, demonstrating that the differences in observed degranulation with increasing concentrations of HmTA [DNP_4] in Figure 3 were due to changes in the crosslinking of the IgE antibodies on the RBL cell surface.

Several molecules have recently been identified as having a role in inhibitory pathways that limit degranulation in response to supraoptimal IgE crosslinking. Among these molecules, SHIP1 has been identified as a critical negative regulator of mast cell degranulation as demonstrated using SHIP1-deficient mast cells. SHIP1-deficient mast cells do not exhibit reduced degranulation in response to supraoptimal allergen concentrations (Gimborn et al., 2005; Kraft and Kinet, 2007; Molfetta et al., 2007). Therefore, we investigated the phosphorylation of SHIP1 using the two-allergen model. RBL cells were stimulated with 100 nM HmTA [$Dansyl_4$], the concentration that elicited the highest response (Figure 2B), with HmTA [DNP_4] increasing from 0.5 to 1,000 nM. Based on the results in Figure 3, we anticipated that there would be little phosphorylation of SHIP1 at the lowest concentrations of HmTA [DNP_4], as these were the conditions where maximum degranulation occurred, and that the

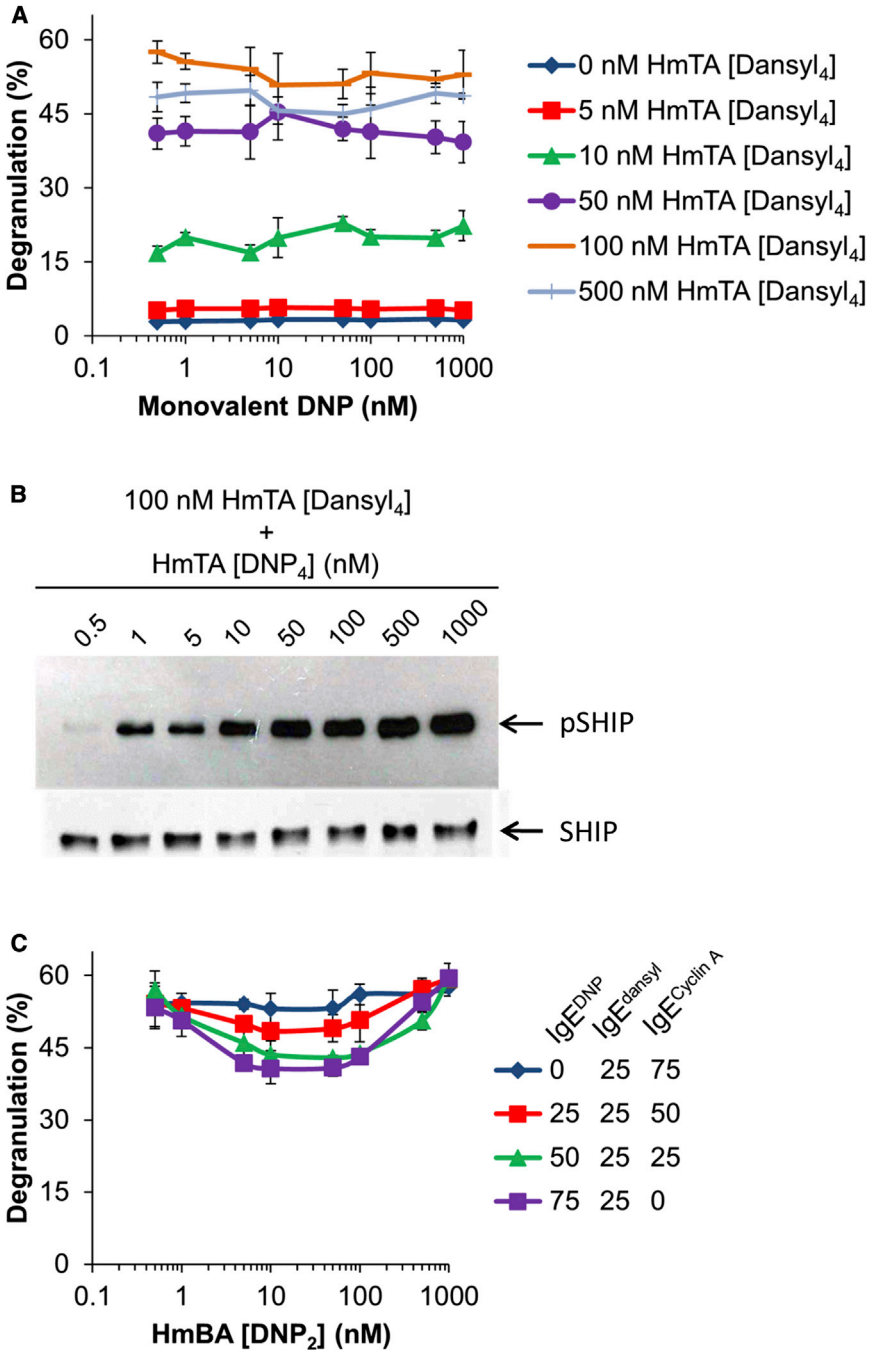

Figure 4. Effect of Valency on Degranulation in Two-Allergen Model

(A) RBL cells were primed with 25/25/50: IgE^{DNP}/IgE^{dansyl}/$IgE^{cyclin\ A}$ and then exposed to mixtures of HmTA [$dansyl_4$] and monovalent DNP ranging from 0.5 to 1,000 nM.

(B) RBL cells were primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl} and then stimulated with 100 nM of HmTA [$dansyl_4$] with 0.5–1,000 nM of HmTA [DNP_4]. The postnuclear supernatants were subjected to anti-SHIP immunoprecipitation and then anti-phospho-tyrosine and anti-SHIP immunoblotting to demonstrate equal loading.

(C) RBL cells were primed with mixtures of IgEs (0/25/75, 25/25/50, 50/25/25, and 75/25/0: IgE^{DNP}/IgE^{dansyl}/$IgE^{cyclin\ A}$) at a total IgE concentration of 1 μg/ml, followed by exposure to 100 nM HmTA [$dansyl_4$] with HmBA [DNP_2] ranging from 0.5 to 1,000 nM. Data represent the means ± SD of triplicate experiments.

phosphorylation of SHIP1 would increase with HmTA [DNP_4] concentration to account for the decreased degranulation observed in Figure 3. Indeed, as observed in Figure 4B, the phosphorylation of SHIP1 increased with the HmTA [DNP_4] concentration, confirming the role of SHIP1 as a negative regulator of mast cell degranulation that is activated in response to supraoptimal IgE crosslinking.

Bivalent allergens have been previously shown to form cyclic dimers with IgE, and these clusters of cyclic dimers typically do not stimulate a degranulation response (Posner et al., 2007; Sil et al., 2007). We next investigated if the bivalent DNP allergen HmBA [DNP_2], which was unable to stimulate mast cell degranulation (Figure 2), was able to decrease the degranulation response caused by HmTA [$dansyl_4$]. For these experiments, the RBL cells were primed with four different IgE mixtures that had the same percent IgE^{dansyl} with increasing percent IgE^{DNP}. The mixtures were 0/25/75, 25/25/50, 50/25/25, and 75/25/0: IgE^{DNP}/IgE^{dansyl}/$IgE^{cyclin\ A}$. The RBL cells were then exposed to 100 nM HmTA [$dansyl_4$], with increasing concentrations of HmBA [DNP_2]. As observed in Figure 4B, in the absence of IgE^{DNP}, the bivalent allergen HmBA [DNP_2] had no effect on mast cell degranulation. However, as the percentage of IgE^{DNP} was increased, there was a small reduction in the degranulation response observed from 5 to 100 nM HmBA [DNP_2]. The reduction in degranulation response disappeared by 1,000 nM HmBA [DNP_2], presumably as IgE^{DNP} crosslinking was inhibited due to excess HmBA [DNP_2] in solution. The partial inhibition of degranulation observed with HmBA [DNP_2] suggests that only large, signaling-competent clusters of IgE are capable of activating the pathways responsible for limiting the degranulation response.

DISCUSSION

Mast cell degranulation experiences a bell-shaped dose-response behavior with increasing allergen concentration. This response has traditionally been attributed to excess allergen competitively inhibiting IgE crosslinking on the mast cell surface, such that, at high allergen concentrations, each IgE is bound monovalently to a different allergen, preventing the formation

of signaling-competent clusters. Using the two-allergen model, we were able to demonstrate that the decrease in degranulation cannot be due to a decrease in IgE crosslinking but, to the contrary, is due to a further increase in IgE crosslinking. The specificity of the hapten-IgE pairs ensured that IgE crosslinking caused by the first allergen-IgE interactions on the RBL cells was not inhibited by the second allergen. Consequently, the decreased degranulation observed at high concentrations of both HmTA [DNP_4] and HmTA [$dansyl_4$] was due to increased IgE crosslinking. This confirms recent evidence suggesting that the decrease in degranulation at high allergen concentrations is caused by active inhibitory pathways (Huber, 2013). In particular, SHIP1 has been identified as a key regulator of mast cell degranulation, which we confirmed using our two-allergen model. However, the results in Figure 3 demonstrate that when mast cells were primed with high levels of allergen-specific IgE and stimulated 50 or 100 nM HmTA [$dansyl_4$], the degranulation response increased as the concentration of HmTA [DNP_4] increased from 500 to 1,000 nM, despite increased SHIP1 phosphorylation (Figure 4B). The mechanisms through which SHIP1 and other regulatory molecules limit the degranulation response have yet to be fully elucidated. Further investigation and identification of the molecules involved in the suppression of degranulation will likely lead to the identification of novel molecular targets that could be exploited to treat patients with acute and chronic allergic diseases. The two-allergen model is uniquely suited to analyze the effect of supraoptimal IgE crosslinking on mast cell degranulation and to elucidate these critical aspects regulating mast cell degranulation.

SIGNIFICANCE

Most allergen sources contain a complex mixture of allergens, many of which are capable of stimulating a strong allergic response on their own, yet current allergen model systems use a single allergen specific for a single monoclonal IgE antibody. To investigate the influence of each allergen in these complex mixtures, we used two well-defined tetravalent allergens, each specific for a distinct IgE, to model an allergen source containing multiple allergens. In addition, we used a third, non-allergen-specific IgE to represent the heterogeneity of IgE present on mast cells. This system allowed us to investigate how the presence of two allergens effect the overall degranulation response and how the response stimulated by the two allergens changes as the percent IgE specific for each allergen is varied. Using this system, we demonstrated that, at low allergen concentrations, the presence of a second allergen increases the degranulation response; at moderate allergen concentrations, the second allergen does not change the degranulation response; and at high allergen concentrations, the degranulation response decreases. Traditionally, the decreased degranulation observed at high allergen concentrations has been attributed to a decrease in IgE crosslinking caused by excess allergen in solution competitively inhibiting allergen-IgE interactions. However, each of the two allergens is specific for a different IgE, so the decreased degranulation cannot be attributed to decreased IgE crosslinking. Therefore, our results provide further evidence of recently identified active inhibitory pathways that regulate mast cell degranulation. The mechanisms through which supraoptimal IgE crosslinking decreases the degranulation response remain to be fully elucidated, and it is likely that further study of these pathways, using more physiologically relevant systems such as the two-allergen model, will lead to novel molecular targets for treating allergy and asthma.

EXPERIMENTAL PROCEDURES

Fluorescence Quenching Binding Assay

The binding constants of the monovalent haptens to the respective IgEs were determined using a previously described fluorescence quenching assay (Handlogten et al., 2011).

Synthesis of the Tetravalent and Bivalent Synthetic Allergens

All molecules were synthesized using standard Fmoc chemistry on a solid support as described in the Supplemental Information.

Degranulation Assays

IgE^{dansyl} (clone 27-74) and $IgE^{cyclin\ A}$ (clone BF683) were purchased from BD Biosciences. Degranulation assays were carried out as previously described (Handlogten et al., 2011).

Immunoprecipitation and Western Blotting

RBL cells were incubated overnight with an equimolar solution of IgE^{DNP} and IgE^{dansyl}. Cells were washed twice and re-equilibrated to 37°C for 30 min prior to stimulation with the indicated allergen mixtures for 3 min. After stimulation, cells were scraped on ice, pelleted, and solubilized with 0.5% NP-40 and 0.5% deoxycholate in 4°C phosphorylation solubilization buffer. Samples were normalized for protein content and then subjected to immunoprecipitation using agarose-conjugated monoclonal anti-SHIP antibody (P1C1) from Santa Cruz Biotechnology with three subsequent washing steps with phosphorylation buffer containing 0.5% NP-40. The precipitate was separated by SDS-PAGE and analyzed using western blotting using an anti-p-Tyr antibody (PY99) from Santa Cruz Biotechnology or monoclonal anti-SHIP antibody (P1C1) from Santa Cruz Biotechnology as previously described (Gimborn et al., 2005).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.08.019.

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