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Inhibition of weak-affinity epitope-IgE interactions prevents mast cell degranulation

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Abstract

Development of specific inhibitors of allergy has had limited success, in part, owing to a lack of experimental models that reflect the complexity of allergen-IgE interactions. We designed a heterotetravalent allergen (HtTA) system, which reflects epitope heterogeneity, polyclonal response and number of immunodominant epitopes observed in natural allergens, thereby providing a physiologically relevant experimental model to study mast cell degranulation. The HtTA design revealed the importance of weak-affinity epitopes in allergy, particularly when presented with high-affinity epitopes. The effect of selective inhibition of weak-affinity epitope-IgE interactions was investigated with heterobivalent inhibitors (HBIs) designed to simultaneously target the antigen- and nucleotide-binding sites on the IgE Fab. HBI demonstrated enhanced avidity for the target IgE and was a potent inhibitor of degranulation *in vitro* and *in vivo*. These results demonstrate that partial inhibition of allergen-IgE interactions was sufficient to prevent mast cell degranulation, thus establishing the therapeutic potential of the HBI design.

Type I hypersensitivity (allergy) is a disorder of the immune system that occurs when the adaptive immune system is directed against substances in the environment that would otherwise be harmless. Immediate hypersensitivity initiates when IgE antibodies bound to their high-affinity receptor (FcεRI) on mast cells are cross-linked by multivalent allergens, leading to mast cell degranulation^{1,2}. Naturally occurring allergens are typically complex, structurally heterogeneous proteins, with multiple allergy-inducing epitopes. Accordingly, the IgE antibodies that are generated against these proteins are polyclonal in nature and bind their respective epitopes with a range of affinities^{3,4}. Typical allergens have two to twelve epitopes that are recognized by polyclonal IgE antibodies^{5–8}. Recent evidence suggests that

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among the identified epitopes on a given allergen, only one to five are immunodominant, meaning they are involved in the degranulation response observed in the majority of patients with that particular allergy^{7,9–11}. For example, the peanut allergen Ara h 3, the wheat allergen Tri a 14 and the melon allergen Cuc m 2 were each found to have four distinct epitopes triggering the allergic reaction^{6,12,13}. Additionally, the melon allergen was found to have two high-binding and two low-binding IgE epitopes (Fig. 1a)^{4,14}.

Owing to the complexity of natural allergens, it has been a challenge to develop experimental models that mimic natural allergic responses. As a result, most allergy studies are performed using dinitrophenyl (DNP) with DNP-specific IgE (IgE^{DNP}) as the hapten-antibody pair^{15–19}. The DNP-IgE^{DNP} system uses monoclonal IgE^{DNP} to bind the IgE receptor, FcεRI, on mast cells. After the mast cells are primed with IgE^{DNP}, cross-linking is induced with DNP-conjugated synthetic allergens typically synthesized using BSA, human serum albumin or ovalbumin as the scaffold. Despite the prevalence of this model, it has several shortcomings. First, DNP binds IgE^{DNP} with an atypically high affinity, which is not representative of the broad range of affinities IgEs have for natural allergy epitopes^{10,13,20}. Second, the hapten conjugation methods used are nonspecific, resulting in heterogeneous synthetic allergens containing 2 to 25 haptens, which is far more than the number of allergy-inducing epitopes found in natural allergens^{16–18}. Furthermore, the heterogeneity of these synthetic allergens complicates interpretation of results as the precise number and orientation of the haptens are unknown, and some haptens may be unavailable to bind surface-bound IgE owing to steric constraints^{21,22}. Finally, multiple presentations of the same hapten on a synthetic allergen do not accurately represent the multiple distinct epitopes on a natural allergen^{3,4}. Although previous studies using the DNP-IgE^{DNP} model have provided important insight into mast cell degranulation, they fall short in modeling the complexity of natural allergen-IgE interactions.

In this study, we developed a well-defined, multicomponent experimental system that enabled an integrative approach to study mast cell degranulation (Fig. 1b,c). Specifically, in our design, we incorporated (i) epitope heterogeneity, (ii) IgE antibody variability, (iii) range of epitope-IgE affinities and (iv) relevant epitope valency observed in natural allergens to better reflect the complexity of allergen-IgE interactions. This was accomplished through the design of a tetravalent scaffold that allowed four haptens to bind simultaneously to four different IgE antibodies. The flexibility of the design allowed for the simultaneous presentation of four identical haptens, homotetravalent allergens (HmTAs), or two sets of two haptens, HtTAs. In our design, we also controlled the affinities of the IgE-hapten interactions by using multiple IgE-hapten pairs and by chemically modifying the haptens to reflect the range of the epitope-IgE affinities present in physiological systems. The tetravalent allergen design provided a more realistic representation of natural allergens and allowed us to investigate the role of weak-affinity epitopes when presented alone or with high-affinity epitopes on a single allergen. Our results demonstrated that low-affinity epitopes have a major role in eliciting an allergic response when presented in combination with high-affinity epitopes.

Next, we evaluated the therapeutic potential of selective inhibition of weak-affinity epitope-IgE interactions by taking an engineering approach where we designed selective and targeted inhibitors (HBIs) of weak-affinity interactions by using the conserved nucleotide-binding site found on the Fab domain of IgE antibodies (Fig. 1d). Through a combination of molecular modeling and experimental approaches, we have extensively characterized this underused site and demonstrated that this binding site is conserved across all immunoglobulins^{17,23}. In addition, our analysis demonstrated that the conformation of the four residues that make up this binding site, two tyrosine residues on the light chain and one tyrosine and one tryptophan on the heavy chain, are also highly conserved^{17,24,25}.

Previously, we described in detail the design, synthesis and characterization of HBIs that selectively inhibited allergen binding to IgE antibody, thereby inhibiting mast cell degranulation in a variant of the commonly used DNP-IgE^{DNP} model¹⁷. HBIs were composed of a hapten molecule conjugated to a nucleotide-binding site ligand, which enabled simultaneous targeting of the antigen-binding site as well the nucleotide-binding site located on the Fab domain of all antibodies (Fig. 1d). Simultaneous bivalent binding to both of these sites provided the HBIs with enhanced avidity and selectivity for the target IgE and enabled competitive inhibition of allergen binding. In this study, we demonstrated that an HBI designed to selectively inhibit only the weak-affinity epitope-IgE interactions of a HtTA consisting of high- and low-affinity haptens was sufficient to prevent degranulation in both *in vitro* and *in vivo* allergy models (Fig. 1e). Taken together, the HtTA design provided an experimental tool to elucidate formerly unrevealed aspects of mast cell degranulation, and the HBI design provided us with a new antibody-targeting approach with therapeutic potential to selectively inhibit allergic responses.

Results

Design and characterization of tetravalent allergens

Previous methods of synthesizing allergens use nonspecific chemical methods to conjugate haptens to protein scaffolds, resulting in poorly defined allergens that complicate interpretation of results^{15–18,21,22}. To address this problem, we synthesized well-defined and well-characterized tetravalent allergens with the criteria that each of the four haptens bound a different IgE. Through a combination of experimental approaches and molecular modeling, it has been demonstrated that the average distance between the two Fab domains of IgE is 11–13 nm and that, owing to the differences between the extended and in-solution length of ethylene glycol, a PEG₃₃₅₀ linker (extended length of 29 nm) is required to span the two antigen-binding sites on a single IgE^{26–28}. Previously, we identified that ethylene glycol with an extended length of ~6 nm is optimal for haptens to bind multiple antibodies without bridging the two antigen-binding sites on a single antibody^{29–33}. Consequently, in our tetravalent allergen design, the four hapten moieties were conjugated to the core of the molecule with 8 units of ethylene glycol, which provided an extended length of 3.2 nm, yielding a maximum separation of 6.4 nm between haptens (Fig. 2a,b). The resulting separation distance between haptens was substantially shorter than the length required for bivalent binding to a single IgE, ensuring that the tetravalent allergen cross-linked the neighboring IgE molecules on mast cells rather than the two Fab arms of a single IgE²⁸. Lysine residues were incorporated into the scaffold to provide a means of conjugating each moiety to the ethylene glycol linker as well as to provide a charge to increase the solubility of the synthetic allergens. The flexibility and solubility of the tetravalent scaffold ensured that each hapten was available to bind an IgE antibody, yet the length of the ethylene glycol linker made it sterically unfavorable for a single IgE to bind bivalently to a single tetravalent allergen.

The next step was the identification of haptens with a broad range of affinities for IgE antibodies to reflect the range of affinities found in natural allergy systems. To identify the high-affinity and low-affinity haptens, we determined the monovalent binding affinities of several hapten-IgE pairs using a previously described fluorescence quenching method¹⁷. Out of the screened candidates, dansyl-IgE^{dansyl} was identified as a high-affinity pair with a monovalent $K_d^{\text{dansyl}} = 54 \pm 4$ nM (Fig. 3a). To identify a hapten-IgE pair that could be used to represent the weak-affinity interactions, we modified the commonly used DNP-IgE^{DNP} pair. Although DNP binds IgE^{DNP} with high affinity, we established that nitrofurane (NF) binds IgE^{DNP} with a monovalent $K_d^{\text{NF}} = 41 \pm 4$ μ M (Fig. 3a). The dansyl-IgE^{dansyl} and NF-IgE^{DNP} pairs provided a 760-fold difference in affinity, allowing us to model both the high-

and low-affinity epitopes found in natural allergy systems. Notably, inclusion of two different IgE-hapten pairs in our design provided a closer approximation to physiological conditions, where multiple clones of IgEs are involved in mast cell degranulation (Fig. 1b,c). The HtTA design provided that a degranulation response will only occur if both hapten-specific IgEs are present on the mast cell surface; if only one of the respective IgEs is present, the HtTA will essentially behave as a bivalent ligand, which is insufficient for triggering degranulation^{34,35}. As a first step in demonstrating that both hapten-IgE pairs are necessary for degranulation, we assessed any cross-reactivity between the two haptens; NF did not cross-react with IgE^{dansyl}, and, similarly, dansyl did not cross-react with IgE^{DNP} (**Supplementary Results**, Supplementary Fig. 1).

Next, we synthesized a series of tetravalent synthetic allergens using Fmoc chemistry on a solid support (Fig. 2b). Conjugation of dansyl to the tetravalent scaffold, with a valency of 4, provided us with a high-affinity HmTA (HmTA [dansyl₄]; **1**), whereas conjugation of NF to the tetravalent scaffold, with a valency of 4, provided us with a low-affinity HmTA (HmTA [NF₄]; **2**). Notably, conjugation of both dansyl and NF to the tetravalent scaffold, each with a valency of 2, provided us with HtTA [dansyl₂NF₂] (**3**) to elucidate the role weak-affinity epitopes have in mast cell degranulation when presented in combination with high-affinity epitopes on the same allergen. Homobivalent allergen dansyl (HmBA [dansyl₂]; **4**), which has the same structure as HtTA [dansyl₂NF₂] except for two acetylated arms instead of NF, was also synthesized for use as a control.

After synthesizing the tetravalent allergens, it was necessary to characterize the molecules to ensure that each hapten was available for simultaneous binding to a separate IgE antibody. This was accomplished by analyzing the complexes formed by a stoichiometric mixture of IgE antibodies to tetravalent allergens (2:1) using dynamic light scattering. The results of a representative experiment performed by mixing HtTA [dansyl₂NF₂] with 2 molar equivalents of IgE (50:50 mixture of IgE^{DNP} and IgE^{dansyl}) are shown in Figure 3b. Although IgE antibodies had an average hydrodynamic diameter of 10 nm (Fig. 3b), addition of HtTA [dansyl₂NF₂] to the antibody mixture rapidly resulted in the formation of complexes with a hydrodynamic diameter of 20 nm (Fig. 3b). Markedly, the signal at 10 nm completely disappeared, indicating that all of the monomeric IgEs were sequestered by the tetravalent allergen. Thermodynamic equilibrium was reached in less than 1 min, and complexes were stable over several hours. Similarly, HmTA [dansyl₄] formed complexes with a hydrodynamic diameter of 20 nm with IgE^{dansyl} and, as expected, did not form complexes with IgE^{DNP} (Supplementary Fig. 2). The allergen HmTA [NF₄] did not form complexes with IgE^{DNP} owing to the low affinity of the NF-IgE^{DNP} interaction (Supplementary Fig. 2). On the basis of these results and the design of the tetravalent scaffold, we concluded that the structure formed was a bicyclic tetramer consisting of four IgEs and two tetravalent allergens (IgE₄HtTA₂ or IgE₄HmTA₂). The only other possible structure with a hydrodynamic diameter of 20 nm with complete sequestration of IgE is two IgEs binding a single tetravalent allergen (IgE₂HtTA or IgE₂HmTA); however, this was eliminated as a possibility on the basis of the design of the tetravalent allergens. Efficient cross-linking of two Fabs on a single IgE requires a hapten separation distance of 11–13 nm, which is substantially longer than the maximum separation distance between haptens in our design^{28,33,36}. Additionally, the binding of HtTA [dansyl₂NF₂] to IgE^{dansyl}, IgE^{DNP} and an equimolar solution of both IgEs was evaluated using the previously described fluorescence quenching technique¹⁷. There was a 3.3-fold increase in the affinity of HtTA [dansyl₂NF₂] for IgE^{DNP} in the presence of IgE^{dansyl} compared to IgE^{DNP} alone, thus providing further evidence for the formation of a bicyclic tetramer consisting of four IgEs and two tetravalent allergens (Supplementary Fig. 3). The complexes were not stable for the ~10 min required for size-exclusion chromatography analysis owing to the weak-affinity haptens. Combined, these results indicated that each hapten on the tetravalent allergen was simultaneously bound

to a different IgE, demonstrating that binding of four IgE molecules to the tetravalent allergen was not sterically hindered.

Tetravalent allergens induce degranulation *in vitro*

Hapten-conjugated BSA is a poor model of natural allergens. However, BSA conjugates have been shown to be potent stimulators of allergic responses^{15–17}. Therefore, we first used hapten-conjugated BSA to optimize our system and assess any cross-reactivity between the antibody-hapten pairs by using the well-established rat basophilic leukemia (RBL) mast cell degranulation model. RBL cells were primed with IgE^{DNP}, IgE^{dansyl} or an equimolar solution of both antibodies. The relative amounts of each IgE on the surface of the mast cells reflected the relative IgE ratios in solution, as determined using a fluorescein-DNP ligand (Supplementary Fig. 4). Our results with BSA allergens dansyl₁₄-BSA and NF₁₇-BSA (14 and 17 average haptens per BSA respectively) demonstrated that both allergens triggered degranulation when the RBL cells were primed with the hapten-specific IgE or the equimolar solution of both antibodies (Fig. 4a). The lack of a response by cells primed with IgE^{DNP} using dansyl₁₄-BSA as the allergen or cells primed with IgE^{dansyl} using NF₁₇-BSA as the allergen demonstrated the specificity of each hapten for the corresponding IgE. These results were consistent with the monovalent affinity assays and further demonstrated that there was no cross-reactivity between the hapten-IgE pairs. This result indicates that (i) both antibodies were able to bind the FcεRI receptors on the cell surface, (ii) the presence of either antibody did not inhibit the other from binding to its hapten and (iii) maximum degranulation does not require complete cross-linking of mast cell-bound IgE. These control experiments validated that RBL cells primed with both IgE^{DNP} and IgE^{dansyl} antibodies provide a suitable experimental system to test the tetravalent synthetic allergens.

Next, we evaluated HmTA [dansyl₄], HmTA [NF₄] and HtTA [dansyl₂NF₂]. HmTA [dansyl₄] proved to be the most potent allergen, stimulating a bell-shaped dose response from 5 nM to 5 μM with a maximum response at 500 nM (Fig. 4b). Conversely, HmTA [NF₄] did not initiate a degranulation response. Markedly, HtTA [dansyl₂NF₂], which is composed of both dansyl and NF haptens, each with a valency of 2, stimulated a bell-shaped degranulation response from 50 nM to 5 μM, similar to HmTA [dansyl₄], with a maximum response occurring at 500 nM. These results were in agreement with our previous work performed with homotetravalent allergens, where we determined that a hapten must have a $K_d < 235$ nM to stimulate a response²⁹. These results suggested a mechanism of activation where a single hapten binds a single IgE on the surface of a mast cell, and then the allergen-IgE complex diffuses laterally across the surface of the mast cell until a second IgE is encountered, and the hapten binds the second IgE, thus cross-linking the receptors. Accordingly, a HmTA with low-affinity haptens will not stay bound to an IgE long enough for complexes to form^{29,33}. Control experiments were performed with HmBA [dansyl₂], which did not stimulate a response under any condition. These results were consistent with previous literature reports that bivalent allergens are incapable of stimulating degranulation^{34,35}. Combined, these results indicate that the response observed with HtTA [dansyl₂NF₂] was not due to the dansyl moieties alone (Fig. 4b). Additional control experiments were performed with increased allergen exposure time and increased allergen concentrations. HmTA [dansyl₄] and HtTA [dansyl₂NF₂] reached their maximum degranulation potential at ~60 min, whereas HmTA [NF₄] and HmBA [dansyl₂] did not induce degranulation even when the allergen exposure time was increased to 120 min (Fig. 4c). These observations did not change even when the HmTA [NF₄] and HmBA [dansyl₂] concentration was increased to 10 μM (Supplementary Fig. 5). Given that the individual components of HtTA (HmTA [NF₄] and HmBA [dansyl₂]) failed to elicit a degranulation response, these results illustrate the importance of low-affinity epitopes in mast cell

degranulation, particularly when presented simultaneously with high-affinity epitopes on the same allergen.

HBI inhibits mast cell degranulation *in vitro*

Previously, we described HBIs that selectively inhibited allergen binding to IgE antibody, thereby inhibiting mast cell degranulation¹⁷. HBI (5) was composed of the NF hapten (K_d of $41 \pm 4 \mu\text{M}$ for IgE^{DNP}) conjugated to a nucleotide-binding site ligand, indole-3-butyric acid (IBA; K_d of $4.5 \pm 0.6 \mu\text{M}$ for IgE), with an ethylene glycol linker (Fig. 5a). This design enabled simultaneous targeting of the antigen-binding site as well as of the adjacent nucleotide-binding site located in the Fab of antibodies (Fig. 1d). Simultaneous bivalent binding to both sites provided HBI with greater than 120-fold enhancement in avidity for IgE^{DNP} compared to monovalent NF¹⁷. In this study, we investigated the potential of HBI to inhibit mast cell degranulation stimulated by HtTA [dansyl₂NF₂] by selectively and exclusively inhibiting the weak-affinity epitope interactions, specifically the NF-IgE^{DNP} interactions. We predicted that HBI would partially inhibit the binding of HtTA [dansyl₂NF₂] to mast cell-bound IgE by blocking the NF-IgE^{DNP} interaction and that this partial inhibition of allergen binding would effectively lower the valency of the allergen, decreasing its potential to stimulate a response. To test our hypothesis, RBL cells were primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl} and then were exposed to HtTA [dansyl₂NF₂] with increasing concentrations of the HBI (Fig. 5b). HBI inhibited the degranulation response with a half-maximum effective concentration of $2 \mu\text{M}$. The individual components of HBI, ethylene glycol-conjugated NF as a monovalent inhibitor (MI, 6; Fig. 5a) or IBA did not inhibit degranulation (Fig. 5c,d). These results demonstrated that both moieties, IBA and NF, were required for the enhanced avidity of HBI for IgE^{DNP} that enabled the competitive inhibition of HtTA [dansyl₂NF₂] binding to IgE^{DNP}. An additional experiment was performed using the weak-affinity allergen HmTA [NF₄] as the inhibitor, and, as expected, HmTA [NF₄] did not inhibit HtTA [dansyl₂NF₂] degranulation as the tetravalent allergen design only allows monovalent binding to a single IgE and therefore does not provide avidity enhancement for IgE (Supplementary Fig. 6). Next, to demonstrate the specificity of the HBI for the NF-IgE^{DNP} interaction, HmTA [dansyl₄] was used in place of HtTA [dansyl₂NF₂]. As NF does not bind IgE^{dansyl}, HBI did not inhibit HmTA [dansyl₄]-induced degranulation (Fig. 5e). Combined, these studies demonstrated that partial inhibition of HtTA [dansyl₂NF₂]-IgE binding by HBIs, which exclusively inhibit only the weak affinity NF-IgE^{DNP} interactions, was sufficient to prevent mast cell degranulation.

HtTA induced degranulation in PCA mouse allergy model

The next step in validating the HtTA experimental system was to demonstrate the ability of HtTA to induce an allergic response *in vivo*. We selected the passive cutaneous anaphylaxis (PCA) mouse allergy model, a well-established model for studying anaphylaxis *in vivo*^{19,37}. The HtTA design requires the presence of two IgE antibodies each specific for a different hapten to stimulate degranulation. Consequently, a mixture of 50 ng of IgE^{DNP} and IgE^{dansyl} (right ear) or PBS (left ear) was injected intradermally into C57BL/6 mice. Twenty-four hours later, 1.5 nmol of HtTA [dansyl₂NF₂] was injected intravenously, causing swelling in the ear that received the IgE injection. Then, by comparing the tissue thickness of the ear that received the IgE injection to the ear that received the PBS injection using both micrometer measurements and histology, the severity of the allergic reaction was quantified. The 1.5-nmol dose of HtTA [dansyl₂NF₂] corresponded to an initial blood concentration of $\sim 1,000 \text{ nM}$, slightly above the concentration that elicited the maximum degranulation response *in vitro* (assuming mouse blood volume of 1.5 mL). HtTA [dansyl₂NF₂] significantly increased tissue swelling 2 h after challenge when compared with the naive group ($P < 0.05$, Fig. 6). Mice treated with DNP-BSA were used as a control and showed a

similar increase in ear thickness (Supplementary Fig. 7). These results validated the utility of the HtTA design as an experimental model system that enables both *in vitro* and *in vivo* evaluation of mast cell activation.

HBI inhibits degranulation in PCA mouse allergy model

To evaluate the therapeutic potential of HBI, we next evaluated its inhibitory potential in the PCA mouse allergy model. C57BL/6 mice were injected intradermally with an equimolar mixture of IgE^{dansyl} and IgE^{DNP} (right ear) and PBS (left ear). Twenty-four hours later, mice were challenged intravenously with 1.5 nmol HtTA [dansyl₂NF₂] alone or in combination with HBI as indicated in Figure 6. The amounts of HBI, 0.5 nmol, 5 nmol and 10 nmol per mouse, were chosen on the basis of the *in vitro* inhibition data and correspond to initial blood concentrations of ~0.3–7 μM (1.5-ml blood volume), approximating the range where inhibition was observed *in vitro*. HBI significantly inhibited ear swelling when compared to nontreated mice in a dose-dependent manner, reaching near-complete inhibition at 10 nmol ($P < 0.05$, Fig. 6). This result indicated that HBI blocks the effects of HtTA [dansyl₂NF₂] on tissue inflammation and edema induced by mast cell degranulation. Additionally, these results provided further evidence that inhibition of all of the IgE epitopes on an allergen is not required to effectively inhibit the degranulation response. In fact, inhibition of only a few low-binding epitopes may be sufficient to prevent mast cell degranulation, thus highlighting the therapeutic potential of the HBI design.

Discussion

Previous studies have addressed the role of high-affinity epitopes in mast cell degranulation by using simplified allergen models; however, the importance of low-affinity epitopes have not been sufficiently or properly evaluated. In this study, we established a well-defined synthetic tetravalent allergen system that models the epitope heterogeneity and polyclonal response of natural allergy systems through the multivalent presentation of high- and low-affinity hapten molecules on the same scaffold. The bivalent presentation of each hapten on the HtTA required the presence of two distinct IgE antibodies to elicit a mast cell degranulation response. As such, the HtTA system better reflected the complexity of IgE-epitope interactions observed in natural allergy systems and allowed for the elucidation of the role weak-affinity epitopes have in mast cell degranulation. The tetravalency of the synthetic allergens provided a realistic approximation of the number of immunodominant epitopes present in several common allergens, and having precise control over the valency and structure of the scaffold enabled us to study the effect of affinity on mast cell degranulation without other confounding factors.

Using the HtTA allergen system, we demonstrated that low-affinity epitopes have an important role in mast cell degranulation when presented in combination with high-affinity epitopes on a multivalent allergen. The importance of low-affinity epitopes was demonstrated with the synthetic allergen HtTA [dansyl₂NF₂], which was a potent stimulator of mast cell degranulation, whereas a tetravalent version of NF, HmTA [NF₄], and a bivalent version of dansyl, HmBA [dansyl₂], did not elicit a response. These results challenge the previous theory that due to the extremely low IgE serum concentration, IgE must have a moderate to high affinity for the specific allergen to stimulate mast cell degranulation³⁸. Combined, these experiments illustrate the importance of valency, affinity and cooperativity in allergen-IgE binding interactions in mast cell degranulation, thus highlighting the importance of weak-affinity epitopes in the induction of an allergic response.

To evaluate the therapeutic potential of selective inhibition of weak-affinity epitopes for the inhibition of an allergic response, we used a molecular engineering approach to design the HBI. The HBI was engineered to selectively inhibit only the weak-affinity hapten-IgE

interactions by simultaneously targeting the antigen- and nucleotide-binding sites present on the Fab arm of antibodies. The simultaneous bivalent binding provided the HBI with 120-fold enhancement in affinity for IgE^{DNP} compared to monovalent NF, making it a potent inhibitor of mast cell degranulation. Markedly, HBI was a potent inhibitor of HtTA-induced mast cell degranulation in both the *in vitro* RBL mast cell model and the *in vivo* PCA mouse model of allergy. Using the HBI design in conjunction with the HtTA allergy model, we demonstrated that inhibition of every epitope-IgE interaction on an allergen was not required for complete inhibition of mast cell degranulation. Rather, partial inhibition of allergen binding to the mast cell-bound IgEs to reduce the allergen valency was sufficient to inhibit the overall mast cell degranulation response. Combined, the results presented here demonstrate the utility of the conserved nucleotide-binding site for the inhibition of allergic reactions and established the HBI design as an effective inhibitor of mast cell degranulation. A major challenge in the current therapies used to treat allergic responses involves the nonspecific suppression of the immune system that puts patients at risk for infections and generates other side effects. Therefore more selective treatment options are needed. Given that most allergens have one to five immunodominant epitopes and that selective inhibition of only a few epitopes are sufficient to inhibit mast cell degranulation, short peptide sequences that mimic an IgE epitope can be used in the HBI design as potential therapeutics for the selective inhibition of food allergies, environmental allergies and asthmas.

Online Methods

Synthesis of the tetravalent and bivalent synthetic allergens

The synthetic allergens were synthesized using Fmoc solid phase synthesis as previously described³³. Fmoc-protected residues were activated with HBTU or HATU in DMF with DIEA for 3 min, and coupling was monitored with Kaiser tests. Fmoc protecting groups were removed by three exposures to 20% piperidine in DMF for 3 min. The synthetic allergens were synthesized using multiple lysine derivatives to achieve branching while *N*-Fmoc-amido-dPEG₈-acid was used to provide the EG₈ linkers. The syntheses of the synthetic allergens were identical except for the conjugation of the different haptens. The synthetic strategy was as follows: Boc-Lys(Fmoc)-OH was conjugated to NovaPEG Rink Amide resin. After removal of the Fmoc group, a second Boc-Lys(Fmoc)-OH group was added to the molecule, and Fmoc was removed. Next, Fmoc-Lys(ivDde)-OH was conjugated to the molecule. After removal of the Fmoc group, Fmoc-Lys(Fmoc)-OH was conjugated to the ligand to facilitate branching. Both Fmoc groups were removed simultaneously, providing two primary amines for the conjugation of the ethylene glycol linkers. After removing the Fmoc groups on the ethylene glycol linkers, Boc-Lys(Fmoc)-OH was conjugated to each chain followed by the removal of the Fmoc groups. This provided two primary amines for the conjugation of the first set of haptens, NF, dansyl or acetyl. The next step was the removal of the orthogonal protecting group, ivDde, from the previously conjugated lysine residue using 2% hydrazine in DMF. The same branching process was repeated by sequentially conjugating Fmoc-Lys(Fmoc)-OH, *N*-Fmoc-amido-dPEG₈-acid and Boc-Lys(Fmoc)-OH to the molecule, thus providing two additional primary amines for the conjugation of the second set of haptens, dansyl or NF. Conjugation of the haptens was achieved using: dansyl chloride (5-(dimethylamino)naphthalene-1-sulfonyl chloride) for dansyl, 5-nitro-2-furoic acid for NF and acetic anhydride to create the acetyl group. Following synthesis, all of the molecules were cleaved from the resin by two exposures to 92:4:4 TFA/H₂O/TIS for 30 min. The TFA solution was removed under vacuum, and the molecule was purified using RP-HPLC with a semipreparative Zorbax C18 column (9.4 mm × 250 mm) with a linear solvent gradient of 2.5% min⁻¹ increments in acetonitrile at a 4.0 mL/min flow rate. The purified molecules were characterized with a Bruker microTOF II mass spectrometer. Purity was determined with the described HPLC system using a Zorbax

C18 analytical column (4.6 mm × 150 mm). The purity of all of the synthesized ligands was estimated to be >97% (Supplementary Fig. 9). The calculated exact mass of HtTA [dansyl₂NF₂] (C₁₆H₂₈N₂O₅S₂) was 3,606.9518 Da; found 3,607.9477 Da. The calculated exact mass of HmTA [dansyl₄] (C₁₇₈H₃₀₃N₂₇O₅₃S₄) was 3,795.0727 Da; found 3,796.0856 Da. The calculated exact mass of HmTA [NF₄] (C₁₅₀H₂₆₃N₂₇O₆₁) was 3,418.8308 Da; found 3,419.84091 Da. The calculated exact mass of HmBA [dansyl₂] (C₁₅₈H₂₈₅N₂₅O₅₁S₂) was 3,412.9918 Da; found 3,414.0026 Da. Characterization matched literature values^{29,33}.

Synthesis of HBI

The HBI was synthesized using Fmoc solid phase synthesis as previously described in detail¹⁷. The purity was estimated to be >97% (Supplementary Fig. 9) by an analytical injection using the previously described HPLC system and was characterized with a Bruker micrOTOF II mass spectrometer. The calculated exact mass of HBI (C₃₈H₅₆N₆O₁₃) was 804.3905 Da; found 805.3928 Da. Characterization matched literature values¹⁷.

Synthesis of BSA-conjugated allergens

NF was conjugated to BSA using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and dansyl was conjugated to BSA using dansyl chloride as previously described¹⁷. BSA conjugates were purified using 0.5 mL 10-kDa molecular weight cut-off spin concentrators (Millipore), and purity was determined using RP-HPLC and found to be >95% (Supplementary Fig. 8)

Fluorescence quenching assay for determination of IgE-hapten binding affinities

The monovalent binding constants of NF and dansyl for IgE^{DNP} and IgE^{dansyl} were determined using the previously described fluorescence quenching assay¹⁷. Briefly, NF and dansyl quench the fluorescence from the IgE tryptophan residues, occurring at 335 nm, only when the two molecules are in proximity to each other (<10 nm). The monovalent haptens were titrated into a 96-well plate containing a 200-μL solution of 10 nM IgE in PBS. All of the experiments were repeated in at least triplicate.

IgE-HtTA complex formation

Dynamic light scattering experiments were performed with a Malvern Zetasizer Nano S. Monomeric IgE was evaluated at a concentration of 0.8 μM (0.4 μM IgE^{DNP} and 0.4 μM IgE^{dansyl}) in PBS. IgE complex formation was evaluated with an IgE concentration of 0.8 μM (0.4 μM IgE^{DNP} and 0.4 μM IgE^{dansyl}) with a stoichiometric amount of HtTA (0.4 μM) in PBS with an estimated refractive index of 1.45.

RBL degranulation assay

RBL cells and IgE^{DNP} were kindly provided by Dr. B Wilson (University of New Mexico), and IgE^{dansyl} (clone 27-74) was purchased from BD Biosciences. RBL cells were maintained as described previously¹⁶. For the degranulation assays, 100 μL of cells were plated at 0.5×10^6 cells/mL in a 96-well plate and were incubated for 24 h followed by a 2-h incubation with the IgE antibodies as indicated in Figures 4 and 5 at a total IgE concentration of 1 μg/mL. Cells were washed immediately before experiments and were stimulated with the concentration of allergen as indicated in Figures 4 and 5 for 60 min unless otherwise stated. When testing the allergy inhibitors, the inhibitor was added to cells 30 min before the allergen. Degranulation was detected spectroscopically by measuring the activity of the granule-stored enzyme β-hexosaminidase secreted into the supernatant on the substrate *p*-nitrophenyl-*N*-acetyl-β-O-glucosamine. All of the degranulation assays were

repeated in at least triplicate. In all of the experiments, the total IgE concentration was kept constant at 1 $\mu\text{g/mL}$.

Animals

C57BL/6 female mice (7–8 weeks) were obtained from Harlan Biosciences (Indianapolis, IN). Mice were maintained in pathogen-free conditions, and studies were approved by the Indiana University Institutional Animal Care and Use Committee.

PCA

PCA was performed as previously described¹⁹. Briefly, mice were injected intradermally with PBS (left ear) and an equimolar solution of IgE^{DNP} and IgE^{dansyl} (100 ng total IgE) (right ear). After 24 h, mice were injected intravenously with HtTA [dansyl₂NF₂] (1.5 nmol) and treated with the amount of HBI indicated in Figure 6. The thickness of ears was measured before HtTA [dansyl₂NF₂] and at 1 h and 2 h after challenge. The data were expressed as change in thickness compared with values before HtTA [dansyl₂NF₂] inoculation.

Statistical analysis

A one-way analysis of variance (ANOVA) for multiple comparisons was performed, and $P < 0.05$ was considered statistically significant as indicated in Figure 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Metzger H. Transmembrane signaling—the joy of aggregation. *J Immunol.* 1992; 149:1477–1487. [PubMed: 1324276]
2. Blank U, Rivera J. Assays for regulated exocytosis of mast cell granules. *Curr Protoc Cell Biol.* 2006;15.11–15.11.18. [PubMed: 18228483]
3. Wang J, et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J Allergy Clin Immunol.* 2010; 125:695–702. [PubMed: 20226304]
4. López-Torrejón G, et al. An experimental and modeling-based approach to locate IgE epitopes of plant profilin allergens. *J Allergy Clin Immunol.* 2007; 119:1481–1488. [PubMed: 17397911]
5. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *J Allergy Clin Immunol.* 2008; 122:298–304. [PubMed: 18572230]
6. Rougé P, et al. Mapping and conformational analysis of IgE-binding epitopic regions on the molecular surface of the major Ara h 3 legumin allergen of peanut (*Arachis hypogaea*). *Mol Immunol.* 2009; 46:1067–1075. [PubMed: 18995911]
7. Pacios LF, et al. Mimotope mapping as a complementary strategy to define allergen IgE-epitopes: peach Pru p 3 allergen as a model. *Mol Immunol.* 2008; 45:2269–2276. [PubMed: 18242709]

8. Stanley JS, et al. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophys*. 1997; 342:244–253. [PubMed: 9186485]
9. Tanabe S. Epitope peptides and immunotherapy. *Curr Protein Pept Sci*. 2007; 8:109–118. [PubMed: 17305564]
10. Cerecedo I, et al. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J Allergy Clin Immunol*. 2008; 122:589–594. [PubMed: 18774394]
11. Cong Y, Lou F, Xue W, Li L, Chen M. Characterisation of the IgE-binding immunodominant epitopes on Ara h1. *Food Agric Immunol*. 2008; 19:175–185.
12. Denery-Papini S, et al. Immunoglobulin-E-binding epitopes of wheat allergens in patients with food allergy to wheat and in mice experimentally sensitized to wheat proteins. *Clin Exp Allergy*. 2011; 41:1478–1492. [PubMed: 21771117]
13. Tordesillas L, et al. Characterization of IgE epitopes of Cuc m 2, the major melon allergen, and their role in cross-reactivity with pollen profilins. *Clin Exp Allergy*. 2010; 40:174–181. [PubMed: 20205701]
14. Guex N, Peitsch M. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 1997; 18:2714–2723. [PubMed: 9504803]
15. Passante E, Frankish N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflamm Res*. 2009; 58:737–745. [PubMed: 19669619]
16. Andrews NL, et al. Small, mobile FcεR1 receptor aggregates are signaling competent. *Immunity*. 2009; 31:469–479. [PubMed: 19747859]
17. Handlogten MW, Kiziltepe T, Moustakas DT, Bilgic B. Design of a heterobivalent ligand to inhibit IgE clustering on mast cells. *Chem Biol*. 2011; 18:1179–1188. [PubMed: 21944756]
18. Collins AM, Basil M, Nguyen K, Thelian D. Rat basophil leukaemia (RBL) cells sensitized with low affinity IgE respond to high valency antigen. *Clin Exp Allergy*. 1996; 26:964–970. [PubMed: 8877164]
19. Hsu CL, Neilsen CV, Bryce PJ. IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *PLoS ONE*. 2010; 5:e11944. [PubMed: 20689814]
20. James LC, Tawfik DS. The specificity of cross-reactivity: promiscuous antibody binding involves specific hydrogen bonds rather than nonspecific hydrophobic stickiness. *Protein Sci*. 2003; 12:2183–2193. [PubMed: 14500876]
21. Hlavacek WS, Posner RG, Perelson AS. Steric effects on multivalent ligand-receptor binding: exclusion of ligand sites by bound cell surface receptors. *Biophys J*. 1999; 76:3031–3043. [PubMed: 10354429]
22. Xu K, Goldstein B, Holowka D, Baird B. Kinetics of multivalent antigen DNP-BSA binding to IgE-FcεRI in relationship to the stimulated tyrosine phosphorylation of FcεRI. *J Immunol*. 1998; 160:3225–3235. [PubMed: 9531278]
23. Rajagopalan K, et al. Novel unconventional binding site in the variable region of immunoglobulins. *Proc Natl Acad Sci USA*. 1996; 93:6019–6024. [PubMed: 8650212]
24. Alves NJ, et al. Selective photocrosslinking of functional ligands to antibodies via the conserved nucleotide binding site. *Biomaterials*. 2013; 34:5700–5710. [PubMed: 23601661]
25. Alves NJ, et al. A small molecule based affinity chromatography method for antibody purification via nucleotide binding site targeting. *Anal Chem*. 2012; 84:7721–7728. [PubMed: 22928545]
26. Schweitzer-Stenner R, Licht A, Luscher I, Pecht I. Oligomerization and ring-closure of immunoglobulin-E class antibodies by divalent haptens. *Biochemistry*. 1987; 26:3602–3612. [PubMed: 3651400]
27. Hunt J, et al. A fluorescent biosensor reveals conformational changes in human immunoglobulin E Fc implications for mechanisms of receptor binding, inhibition, and allergen recognition. *J Biol Chem*. 2012; 287:17459–17470. [PubMed: 22442150]
28. Baird EJ, Holowka D, Coates GW, Baird B. Highly effective polyethylene glycol architectures for specific inhibition of immune receptor activation. *Biochemistry*. 2003; 42:12739–12748. [PubMed: 14596588]

29. Handlogten MW, Kiziltepe T, Alves NJ, Bilgicer B. Synthetic allergen design reveals the significance of moderate affinity epitopes in mast cell degranulation. *ACS Chem Biol.* 2012; 7:1796–1801. [PubMed: 22877585]
30. Stefanick JF, Kiziltepe T, Handlogten MW, Alves NJ, Bilgicer B. Enhancement of antibody selectivity via bicyclic complex formation. *J Phys Chem Lett.* 2012; 3:598–602.
31. Bilgiçer B, Moustakas DT, Whitesides GM. A synthetic trivalent hapten that aggregates *anti*-2,4-DNP IgG into bicyclic trimers. *J Am Chem Soc.* 2007; 129:3722–3728. [PubMed: 17326636]
32. Bilgiçer B, et al. A non-chromatographic method for the purification of a bivalently active monoclonal IgG antibody from biological fluids. *J Am Chem Soc.* 2009; 131:9361–9367. [PubMed: 19534466]
33. Handlogten MW, Kiziltepe T, Bilgicer B. Design of a heterotetravalent synthetic allergen that reflects epitope heterogeneity and IgE antibody variability to study mast cell degranulation. *Biochem J.* 2013; 449:91–99. [PubMed: 23050868]
34. Sil D, Lee JB, Luo D, Holowka D, Baird B. Trivalent ligands with rigid DNA spacers reveal structural requirements for IgE receptor signaling in RBL mast cells. *ACS Chem Biol.* 2007; 2:674–684. [PubMed: 18041817]
35. Posner RG, et al. Trivalent antigens for degranulation of mast cells. *Org Lett.* 2007; 9:3551–3554. [PubMed: 17691795]
36. Baird B, Zheng Y, Holowka D. Structural mapping of IgE-Fc- ϵ -RI, an immunoreceptor complex. *Acc Chem Res.* 1993; 26:428–434.
37. Dinneswara Reddy G, Park S, Cho HM, Kim T, Lee ME. Antiallergic activity profile *in vitro* RBL-2H3 and *in vivo* passive cutaneous anaphylaxis mouse model of new sila-substituted 1,3,4-oxadiazoles. *J Med Chem.* 2012; 55:6438–6444. [PubMed: 22770426]
38. Lund G, et al. Antibody repertoire complexity and effector cell biology determined by assays for IgE-mediated basophil and T-cell activation. *J Immunol Methods.* 2012; 383:4–20. [PubMed: 22683539]

Figure 1. Design of the HtTA and the HBI for the selective inhibition of mast cell degranulation
(a) Structure of the melon allergen Cuc m 2 with the four identified epitopes displaying both high and low affinity for IgE antibodies. A HtTA consisting of two distinct haptens each with a valency of 2 was synthesized to model the multiple different epitopes present on a natural allergen such as Cuc m 2. To mimic the high- and low-binding IgE epitopes, the first hapten was chosen to have a high affinity and the second hapten was chosen to have a low affinity for their respective IgEs. **(b)** In an allergic reaction, the allergen binds the polyclonal IgE antibodies present on the surface of mast cells. Multivalent allergen binding to surface-bound IgE antibodies cause aggregation of the IgE receptor, FcεRI, initiating a signaling cascade that results in mast cell degranulation. **(c)** The HtTA design mimics multiple distinct epitopes as well as the polyclonal IgE response present in natural allergic responses by using two IgE antibodies, IgE-1 and IgE-2, each with different hapten specificity. **(d)** Crystal structure of an IgG antibody with enlarged Fab region to demonstrate the location of the nucleotide- and antigen-binding sites. The HBI was designed to simultaneously bind both the antigen- and the nucleotide-binding sites on an IgE. **(e)** HBI selectively inhibits the low-affinity hapten/IgE interactions, effectively lowering the valency of the allergen rendering it incapable of causing sufficient IgE cross-linking to stimulate a degranulation response.

Figure 2. Chemical structures of the haptens and tetraivalent synthetic allergens

(a) Structures of the haptens NF and dansyl used to synthesize the allergens. (b) The structure of the tetraivalent scaffold and the compositions of the HtTA, HmTAs and HmBA are shown.

Figure 3. Characterization of hapten/IgE binding interactions

(a) Binding curves for monovalent dansyl (●) binding to IgE^{dansyl} and monovalent NF (□) binding to IgE^{DNP}. Binding was observed by monitoring the fluorescence quenching of the tryptophan residues in the IgE antibodies. Values for the binding constants are $K_{d,dansyl} = 54 \pm 4$ nM and $K_{d,NF} = 41 \pm 4$ μ M. Control experiments with NF-IgE^{dansyl} and dansyl-IgE^{DNP} did not show any cross-reactivity in the hapten IgE pairs (Supplementary Fig. 1). Data represent the mean $\pm$ s.d. of triplicate experiments. (b) Dynamic light scattering data demonstrate the aggregation of IgE^{DNP} (left) and IgE^{dansyl} (right) in response to the addition of a stoichiometric concentration of HtTA. The size of the equimolar solution of IgE^{DNP} and IgE^{dansyl} was 10 nm and was increased to 20 nm upon the addition of HtTA [dansyl₂NF₂].

Figure 4. Mast cell degranulation in response to tetravalent synthetic allergens

(a) RBL cells were primed with IgE^{DNP}, IgE^{dansyl} or an equimolar solution of both antibodies. Dansyl₁₄-BSA and NF₁₇-BSA both stimulated a response when the hapten-specific IgE was present on the surface of the mast cells, but did not stimulate a response if it was absent. (b) RBL cells were primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl} and then exposed to increasing concentrations of the indicated synthetic allergen. (c) RBL cells were primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl} followed by exposure to a constant allergen concentration for the indicated period of time. HtTA [dansyl₂NF₂] and HmTA [dansyl₄] were used at 500 nM, whereas HmTA [NF₄] and HmBA [dansyl₂] were used at 10 μ M. Triton X (1%) was used to determine the percent degranulation. Data represent the mean $\pm$ s.d. of triplicate experiments.

Figure 5. Inhibition of mast cell degranulation via the HBI design

(a) Structures of the HBI and the control molecule MI are shown. HBI is composed of the NF hapten molecule conjugated to IBA with an ethylene glycol linker, whereas MI is composed of the ethylene glycol–conjugated NF hapten only. (b) The inhibitory potential of HBI was evaluated using mast cells primed with an equimolar solution of IgE^{DNP} and IgE^{dansyl} and stimulated with 500 nM of HtTA [dansyl₂NF₂]. HBI effectively inhibited mast cell degranulation with a half-maximum inhibitory concentration of 2 μM. (c,d) In control experiments performed with MI (c) or IBA (d), no inhibition of mast cell degranulation was observed. (e) In another control experiment, HmTA [dansyl₄], was used in place of HtTA [dansyl₂NF₂] to stimulate degranulation, and HBI was unable to inhibit the response. Triton X (1%) was used to determine the percent degranulation. Data represent the mean ± s.d. of triplicate experiments.

Figure 6. Inhibition of PCA via the HBI in an *in vivo* mouse allergy model

(a) Ear swelling during PCA was determined 1 h and 2 h after systemic HtTA [dansyl₂NF₂] challenge in mice receiving no HBI treatment or 0.5 nmol, 5 nmol or 10 nmol of HBI. The data was expressed as change in thickness compared with values before HtTA [dansyl₂NF₂] inoculation, measured with a micrometer caliper. Naïve mice did not receive injection of IgE, HtTA [dansyl₂NF₂] or HBI. (b) Ear tissue was stained with hematoxylin and eosin stain at 2 h after systemic challenge. Representation of dermal thickness in naïve (left), HtTA [dansyl₂NF₂]–challenged (middle) and HtTA [dansyl₂NF₂]–challenged (plus 10 nmol HBI) (right) mice with HBI are shown. Scale bar, 200 μ m. Data represent the mean $\pm$ s.e.m. of five mice from two independent experiments. * $P < 0.05$.