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## IgE epitope proximity determines immune complex shape and effector cell activation capacity

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### Abstract

**Background**—IgE-allergen complexes induce mast cell and basophil activation and thus immediate allergic inflammation. They are also important for IgE-facilitated allergen presentation to T cells by antigen-presenting cells.

**Objective**—To investigate whether the proximity of IgE binding sites on an allergen affects immune complex shape and subsequent effector cell activation *in vitro* and *in vivo*.

**Methods**—We constructed artificial allergens by grafting IgE epitopes in different numbers and proximity onto a scaffold protein. The shape of immune complexes formed between artificial allergens and the corresponding IgE was studied by negative-stain electron microscopy. Allergenic activity was determined using basophil activation assays. Mice were primed with IgE, followed by injection of artificial allergens to evaluate their *in vivo* allergenic activity. Severity of systemic anaphylaxis was measured by changes in body temperature.

**Results**—We could demonstrate simultaneous binding of 4 IgE antibodies in close vicinity to each other. The proximity of IgE binding sites on allergens influenced the shape of the resulting immune complexes and the magnitude of effector cell activation and *in vivo* inflammation.

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**Conclusions**—Our results demonstrate that the proximity of IgE epitopes on an allergen affects its allergenic activity. We thus identified a novel mechanism by which IgE-allergen complexes regulate allergic inflammation. This mechanism should be important for allergy and other immune complex-mediated diseases.

### Keywords

Allergy; allergen; IgE epitope; immune complex; effector cells

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Antigen-antibody complexes play an important role in the activation of the innate and adaptive immune system and thus in host defense against infectious agents and cancer as well as in immune-mediated diseases such as allergy and autoimmunity.<sup>1</sup> The most common immune complex-mediated hypersensitivity disease is IgE-associated allergy, which affects more than 25% of the population.<sup>2</sup> Allergic patients suffer from various symptoms such as hay fever, food allergy, dermatitis, severe and disabling asthma, and life-threatening anaphylactic shock.<sup>3</sup> Atopic individuals are genetically predisposed to develop IgE antibodies upon contact with *per se* harmless antigens (ie, allergens), whereas healthy persons mount IgG antibody responses.<sup>4</sup> After sensitization, IgE antibodies bind with high affinity to receptors (ie, FcεRI) on immune cells (eg, mast cells, basophils, antigen-presenting cells, and eosinophils).<sup>5,6</sup> Subsequent allergen contact cross-links allergen-specific IgE antibodies on effector cells (eg, mast cells and basophils) leading to the immediate release of biological mediators (eg, histamine and leukotrienes) responsible for acute allergic inflammation.<sup>7,8</sup>

As early as 1924, Karl Landsteiner<sup>9</sup> had demonstrated that polyvalent antigen is required to trigger an allergic reaction, whereas monovalent antigen (ie, hapten) failed to do so and even induced a state of “antianaphylaxis.” An *in vitro* system in which cultured basophils are stimulated with allergen to release histamine was developed by Lichtenstein and Osler<sup>10</sup> to mimic immediate allergic inflammation. Using this system, it is possible to define factors that determine the magnitude of effector cell activation.

Using chemically cross-linked IgE or anti-IgE antibodies, it has been shown that cross-linking of IgE antibodies requires at least 2 IgE epitopes on an allergen molecule for the activation of effector cells.<sup>11–14</sup> However, the in-depth analysis of IgE-allergen complex formation and effector cell activation has been hampered by the lack of defined molecular tools. In the last 2 decades, the molecular structures of most of the allergens with relevance for human allergy have been revealed.<sup>15</sup> Using a peptide epitope of one of the most important respiratory allergens (ie, Phl p 1 from timothy grass pollen) and a corresponding monoclonal IgE antibody, we demonstrated that the extent of effector cell degranulation depends not only on the levels of allergen-specific IgE antibodies but also on the number of IgE epitopes.<sup>16</sup> The molecular analysis of several allergens important for human allergy has indicated that they contain several different IgE binding sites. It has been speculated that they can appear in clusters on allergen surfaces.<sup>17–24</sup> These observations led us to hypothesize that the proximity of IgE binding sites on an allergen may affect its ability to form immune complexes, to induce effector cell degranulation, and thus to determine its allergenic potency.

To investigate whether the proximity of IgE binding sites on an allergen is important for its allergenic activity, we grafted an IgE epitope from the major timothy grass pollen allergen, Phl p 1, in different numbers and proximity onto a *per se* nonallergenic molecule, that is, horse heart myoglobin. Using negative-stain electron microscopy (EM), we studied the shapes of immune complexes formed between the artificial allergens and the corresponding IgE antibodies. When the same epitopes were placed in adjacent proximity on the surface of the artificial allergen, immune complexes with a closed shape (ie, compact ring shape) dominated whereas open complexes in the form of short chains were observed when epitopes were placed on different ends and proximity of the host molecule. Importantly, allergen constructs containing the same epitopes engineered into adjacent positions were more potent in inducing degranulation of basophils loaded with IgE and allergic inflammation in mice, which had been sensitized with IgE, than were constructs containing distantly placed epitopes. Our results thus demonstrate that the proximity of epitopes on a given antigen profoundly affects the shape of the resulting antigen-antibody immune complexes and the potency of the antigen to activate immune cells via these immune complexes.

## Methods

### Materials and reagents

Purified rPhl p 1 was purchased from BIOMAY AG (Vienna, Austria). Myoglobin from equine heart was obtained from Sigma-Aldrich (Vienna, Austria). The mouse monoclonal IgE antibody against the Phl p 1–derived peptide P1 (EPVVVHITDDNNEEPIAPYHFDLSGHAFGAMA, aa 86-116) was obtained by immunizing BALB/c mice with the KLH-coupled peptide as described previously.<sup>16,25</sup>

### Construction, expression, and purification of myoglobin derivatives

cDNAs coding for the His-tagged myoglobin derivatives (MB1N, MB2N, MB1N1aa46-47, MB1N1C, MB2N1C, and MB4N) were obtained as synthetic genes with a codon usage optimized for expression in *Escherichia coli* (ATG-Biosynthetics GmbH, Merzhausen, Germany) and were subcloned into the NdeI/EcoRI sites of the plasmid pET17b.

The recombinant proteins were expressed in *E coli* strain BL21 (DE3) (Stratagene, East Key, Australia), which was grown in lysogeny broth medium supplemented with 100 mg/L ampicillin. Protein expression was induced at an OD<sub>600</sub> of 0.6 by the addition of 0.5 mM isopropyl- $\beta$ -thiogalactopyranoside. After culturing for 2 to 4 hours, cells were harvested by centrifugation (37,500  $\times$  g, 15 minutes, 4°C), resuspended in 6 mol/L guanidium hydrochloride, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris (pH 8), and homogenized for 1 minute at room temperature (Ultra-turrax; IKA, Staufen, Germany). The bacterial cell lysates were centrifuged (45,000  $\times$  g, 20 minutes, 4°C), and the recombinant protein-containing supernatants were purified using Ni-NTA affinity columns (Qiagen, Hilden, Germany). Protein preparations were dialyzed in 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, and checked for purity by Coomassie Brilliant Blue staining of 14% SDS polyacrylamide gels (SDS-PAGE), and the protein concentrations were determined by Micro-BCA Protein Assay Kit (Pierce, Rockford, Ill).

## SDS-Page

Myoglobin derivatives were analyzed by means of SDS-PAGE under reducing as well as nonreducing, conditions.<sup>26</sup> Reducing conditions were obtained by dissolving the proteins in sample buffer containing 70 mmol/L  $\beta$ -mercaptoethanol. After boiling, 5- $\mu$ g aliquots of each protein were separated in 14% SDS-PAGE. Gels were subsequently stained with Coomassie Brilliant Blue.

## Circular dichroism analysis

Circular dichroism (CD) spectroscopy was performed on a JASCO J-810 spectropolarimeter (Japan Spectroscopic Co, Tokyo, Japan). Spectra were recorded from 195 to 260 nm with 0.5 nm resolution at a scan speed of 50 nm/min using a 0.2-cm quartz cuvette and resulted from averaging 3 scans. All measurements were performed in 10 mM phosphate buffer (pH 8). The final spectra were baseline-corrected by subtracting the corresponding buffer spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity  $[\Theta]$  at a given wavelength.

## Gel filtration

Gel filtration of myoglobin derivatives was performed in 20 mM sodium phosphate, pH 7.6, and 150 mM sodium chloride buffer at a flow rate of 0.3 mL/min. Fifty microliter of each sample was loaded onto the Superose6 FPLC column (Pharmacia Biotech, GE Healthcare, Uppsala Sweden) and detected at 280 nm. The column was calibrated with bovine thyroglobulin (670 kDa), bovine  $\gamma$ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B<sub>12</sub> (1.35 kDa) (BIO-RAD, Hercules, Calif).

## Quantitative IgE reactivity as determined by binding to dot-blotted purified myoglobin derivatives

Reactivities of recombinant myoglobin derivatives and the Phl p 1-specific monoclonal IgE were determined by dot blot experiments. For this purpose, different concentrations (2.5, 5, 10 nM/mL) of myoglobin derivatives and horse heart myoglobin, as a control protein, were dotted in 2- $\mu$ L aliquots onto nitrocellulose membrane strips (Schleicher & Schuell, Dassel, Germany). The strips were blocked with buffer A (40 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.6 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 0.5% Tween 20, 0.5% [w/v] BSA) and exposed overnight to 1 mL of the Phl p 1-specific monoclonal IgE diluted 1:10 in buffer A (75 pM/mL). Bound IgE antibodies were detected with a <sup>125</sup>I-labeled antimouse IgE antibody (200,000 cpm/mL), visualized by autoradiography using KODAK X-OMAT films and intensifying screens (Kodak, Heidelberg, Germany),<sup>27</sup> and quantified using a gamma counter (Wizzard Automatic Gamma Counter; Wallac, Turku, Finland). The Mann-Whitney *U* test was used for statistical analysis (GraphPad Prism software; GraphPad, San Diego, Calif).

## Negative-stain EM

Negative-stain EM was performed as previously described.<sup>28</sup> Briefly, IgE and purified soluble myoglobin derivatives at 0.5 to 1.0 mg/mL were diluted in buffered saline borate to a final concentration of 5 to 10  $\mu$ g/mL and affixed to carbon membrane supports for assessment of molecular homogeneity. The membranes were stained with 1% uranyl formate

and mounted on 600-mesh copper grids for analysis. Electron micrographs were recorded at 100,000 × magnification at 100 kV on a JEOL JEM-1200 electron microscope. To generate molecular complexes, the reactants were mixed at an approximately 1:1 molar ratio (0.5 mg/mL IgE and 0.05 mg/mL myoglobin construct, unless otherwise stated) for 30 minutes at room temperature and diluted 20-fold in buffered saline borate just before attachment to carbon membranes. Following EM analysis, the ratios of the reactants were sometimes adjusted to maximize the ratio of the complexes to unreacted molecules or to saturate constructs with IgE (ie, antibody excess). Representative electron micrographs of the antigen-antibody mixtures were digitized and manually scored for the presence and morphology of the resulting cross-linked immune complexes.

### **Rat basophil leukemia cell assay**

Rat basophil leukemia (RBL)-2H3 cells<sup>29</sup> were passively sensitized with different dilutions (1:500, 1:5000) of Phl p 1-specific monoclonal IgE antibody. After washing, cells were stimulated by adding serial dilutions (0.001-102.4 nM/mL) of recombinant myoglobin derivatives. In order to measure spontaneous release, RBL cells were passively sensitized without further allergen challenge (controls). Mediator release was quantified by measuring β-hexosaminidase, and results are reported as the percentage of total β-hexosaminidase, obtained by lysing cells with Triton X-100 (Merck, Darmstadt, Germany). All measurements were performed in duplicate.<sup>16</sup>

### **BIAcore analysis**

BIAcore data acquisition and analysis are detailed in the Methods section in this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org).

### **Passive anaphylaxis model**

BALB/c mice (n = 4/group) were injected intravenously with either 80 μg (4-μg dose experiment) or 96.8 μg (40-μg dose experiment) of peptide-specific monoclonal IgE antibody followed 10 hours later by intravenous challenge with either 4 μg or 40 μg of myoglobin derivatives. The severity of the anaphylactic shock was assessed by decrease in rectal temperature.<sup>30</sup>

## **Results**

### **Construction of artificial allergens containing defined numbers of IgE epitopes in different proximity**

For the construction of artificial allergens, the myoglobin molecule was chosen as a host molecule because it is a strictly monomeric protein with a molecular weight of 17.6 kDa, which is in the range of typical allergens. Myoglobin consists of 153 amino acids and is organized into 8 α-helical regions (A, aa 3-18; B, aa 20-35; C, aa 36-42; D, aa 51-57; E, aa 58-77; F, aa 86-95; G, aa 100-118; H, aa 124-149) (Fig 1).<sup>31</sup>

The IgE-reactive Phl p 1 peptide, comprising 11 amino acids (DLSGHAFGAMA),<sup>16</sup> was grafted on the N-terminus as well as the C-terminus and in the loop region between aa 46-47 of myoglobin (Fig 1, A). We engineered and purified 6 recombinant myoglobin derivatives,

each containing 1 to 4 identical IgE-reactive peptides and a C-terminal His-tag to facilitate purification (Fig 1, *A*).

Fig 1, *A*, shows a schematic overview of the myoglobin derivatives, and Tables E1 and E2 in this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org) display their molecular characteristics and sequences, respectively. MB1N, MB2N, and MB4N contain 1, 2, or 4 copies of the IgE binding site on the N-terminus, respectively. MB1N1C and MB2N1C, respectively, contain 1 or 2 IgE binding sites on the N-terminus plus 1 at the C-terminus, whereas MB1N1aa46-47 has one IgE binding site on the N-terminus and the second in the loop region between aa 46-47. The rationale behind the grafting of the IgE binding sites was 2-fold. To test for the effect of multiple epitopes in close proximity to one another, 2 or 4 IgE epitopes were placed on the N-terminus of myoglobin separated from one another only by small flexible spacers consisting of 6 glycine residues.

To test for the effects of epitopes grafted into fixed positions at defined distances and orientations with respect to one another, IgE-reactive peptides were grafted to the N-terminus and the C-terminus of myoglobin or in the loop region at aa 46-47, which according to the atomic model should place it at a specific distance and position from both the N- and C-termini (Fig 1, *B*).

Fig 2, *A*, shows the purity of the purified recombinant myoglobin derivatives and confirms their predicted molecular masses, which were also determined by mass spectrometry (data not shown). Also, under nonreducing conditions purified myoglobin derivatives migrated as monomeric bands in SDS-PAGE (Fig 2, *B*) and appeared monomeric in the fluid phase as demonstrated by gel filtration (Fig 2, *C*).

CD analysis of the recombinant myoglobin derivatives showed that they contain considerable secondary structure consisting mainly of  $\alpha$ -helices, which is very similar to that of myoglobin. The CD spectra are characterized by minima at 208 nm and 222 nm and a maximum at 195 nm (Fig 2, *D*).

### **IgE reactivity of artificial allergens and formation of immune complexes of different shapes depend on the proximity and the number of epitopes**

To determine the IgE-binding capacity of the recombinant myoglobin derivatives, a quantitative radioallergosorbent test-based IgE binding test was performed with equimolar amounts of purified dot-blotted nondenatured proteins. The results presented in Table I support the assumption that IgE binding increases according to the number of grafted epitopes.

To visualize IgE binding to the derivatives and the resulting immune complexes, negative-stain EM was performed.<sup>28</sup> When monovalent MB1N and IgE antibodies were mixed, mostly monomeric IgE was visible (Fig 3, *A*). Because of the small size of MB1N (18,833 kDa), IgE-associated MB1N molecules could not be reliably detected though some Fab arms did appear to have additional protein mass at the distal tip (Fig 3, *A*, arrows in enlargement). In contrast, mixtures of the di- or polyvalent derivatives with IgE resulted in the formation of various types of immune complexes (Fig 3, *B-F*; see Tables E3 and E4 and Fig E1, *A-E*, in



this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org)). In all cases, a considerable percentage (29.2% to 92.5%) of bound IgEs were in large complexes (ie, containing 4 IgE members). Interestingly, immune complexes with a closed shape (ie, compact ring shape) were most abundant in mixtures containing the constructs with 2 adjacent IgE binding sites located at the N-terminus (ie, 23.6% for MB2N, 17.2% for MB2N1C) (Fig 3, *B* and *E*; see Table E4 and Fig E1, *A* and *D*) as compared to other constructs with 2 epitopes in more distant proximity (ie, 3.1% for MB1N1aa46-7, 3.8% for MB1N1C). These data indicate that despite the close proximity of the MB2N epitopes, the 6xGly linker must allow them to orient in such a way as to minimize steric clashes between the bound IgE Fab arms. In the absence of steric restrictions, rings composed of 2 IgE antibodies and 2 divalent antigens are the energetically most favored form of immune complexes.<sup>32</sup> Short chains and larger rings were also observed in both preparations. Interestingly, the MB2N1C construct, which has an additional epitope appended to the C-terminal end of myoglobin, gave an immune complex distribution with somewhat fewer ring dimers (17.2%) and a greater number of large (average, 11.7 IgE molecules) complexes as compared to MB2N, suggesting that even though the C-terminal epitope played little role in ring formation (Fig 3, *E*; see Fig E1, *D*), it does contribute to cross-linking. This perception was reinforced by results obtained from EM analysis of the MB1N1C + IgE mixture (Fig 3, *D*; see Fig E1, *C*) where only 3.8% of the complexes were dimer rings (see Table E4). For MB1N1C, most of the nonring complexes were composed of just 2 (60.5%) or 3 (23.6%) IgE members and this construct formed the fewest large complexes (see Table E4), suggesting that the C-terminal epitope was less likely than the N-terminal epitopes to participate in the binding necessary for extensive cross-linking. These data were unexpected because the atomic modeling suggests that the distance between the N- and C-terminal epitopes would eliminate steric restrictions and the angular orientation of the epitopes should foster cross-linking and ring formation (Fig 1, *B*). Empirical adjustments to the ratio of antibody to antigen did not lead to enhanced binding or cross-linking (data not shown).

Together with the MB2N1C results described above, we speculate that molecules with epitopes at the N- and C-termini present them to IgE in a configuration that is not conducive to compact ring formation.

Like MB1N1C, MB1N1aa46-47 has 1 N-terminal epitope and 1 additional epitope between aa 46-47 (Fig 1, *A*), a configuration that should foster cross-linking and small ring formation. However, as with MB1N1C, we saw little evidence of ring formation (3.1%), extensive small 2-member (57.9%) and 3-member (25.6%) linear complexes and rather few (13.4%) large complexes (Fig 3, *C*; see Table E4 and Fig E1, *B*). Clearly, the presence of 2 epitopes, *per se*, is not driving the type of complexes formed (compare MB2N with 23.6% of 2 IgE member rings to MB1N1aa46-47 [3.1% rings] and MB1N1C [3.8% rings]; see Table E4). Rather, it is most likely that the relative positions and/or accessibility are driving the differences in formation of immune complexes.

A wide variety of immune complexes containing rings (typically rings of 4 rather than 2), chains, and various combinations of both were observed for MB4N, the derivative containing 4 epitopes at the N-terminus (Fig 3, *F*; see Table E4 and Fig E1, *E*). Many of the complexes were quite large (average, 17.1 IgE molecules) as compared to those for

MB1N1C (average, 7.0 IgE molecules) and MB1N1aa46-47 (average, 8.3 IgE molecules). Such higher order complexes are to be expected if 3 or more epitopes are available on a single molecule. Because of the multiple components and crowded architecture of many of the complexes, the actual functional valency of MB4N was difficult to assess when the reactants were incubated at equivalence. To address this issue, we incubated some samples at a 4-fold IgE molar excess in an attempt to saturate the epitopes and minimize cross-linking potential. Many more examples of 3 and 4 IgE antibodies each with one arm converging on a central focal point (the presumed location of MB4N) were observed, mostly within larger immune complexes. However, clear examples of 3 and 4 isolated IgE antibodies with Fab arms bound to a single focus were observed (Fig 3, *F*, bottom row). Thus, the 3 6xGly linkers appear to provide sufficient flexibility and spacing to allow simultaneous IgE binding to all 4 N-terminal epitopes.

### **Molecules containing IgE epitopes engineered into adjacent positions are more potent at inducing basophil degranulation than are molecules with distantly placed epitopes**

In an attempt to correlate the atomic modeling and EM-derived structural information with functional IgE-mediated basophil triggering, we next compared the allergenic activity of various myoglobin derivatives *in vitro* using the RBL cell model.<sup>29</sup> Cultured RBL-2H3 cells were loaded with monoclonal IgE antibodies, exposed to the myoglobin derivatives, and assayed for  $\beta$ -hexosaminidase release (Fig 4, *A-C*).

When RBL cells were incubated with monovalent MB1N, no relevant mediator release could be detected (Fig 4, *A*). Because MB1N reacted with the monoclonal IgE antibody (Table I), this result can be explained by monovalent, hapten-like IgE binding without induction of cross-linking of Fc $\epsilon$ RI-bound IgE antibodies<sup>16</sup> and is consistent with the paucity of immune complexes observed by EM. Among the other myoglobin derivatives, MB4N, the construct with the largest and most abundant soluble immune complexes, was the most potent inducer of degranulation (Fig 4, *A*). It induced mediator release at an allergen concentration as low as 0.01 nM/mL. Next most potent were MB2N and MB2N1C, and both these constructs yielded similar soluble complexes consisting of rings and chains. Interestingly, the divalent MB1N1C and MB1N1aa46-47 bound approximately the same amount of IgE as MB2N (Table I) but showed much lower allergenic activity than did MB2N (Fig 4, *A*). These results are in general agreement with the EM data, which showed far fewer complexes for the MB1N1aa46-47 and MB1N1C constructs than for MB2N (Fig 3, *B-D*; see Fig E1, *A-C*). When RBL cells were loaded with a 10-fold lower concentration of the monoclonal IgE but challenged with the same concentration of derivatives, the maximal percentage of mediator release was reduced considerably (Fig 4, *B*).

The relative allergenic potencies of the different myoglobin derivatives were not changed when cells were loaded with lower amounts of IgE (Fig 4, *B*). MB4N was again the most allergenic protein followed by MB2N and MB2N1C. Again, MB1N1C and MB1N1aa46-47 were the proteins with the lowest allergenic activity. We then tested the allergenic activity of the 3 divalent constructs, MB2N, MB1N1C, and MB1N1aa46-47, in detail by using 1:2 dilutions of the purified proteins (0.05-102.4 nM/mL) and again found that almost 10-fold less MB2N was needed to induce the same amount of basophil activation as was achieved



with MB1N1aa46-47 (Fig 4, C). The *in vitro* allergenic activity of MB1N1C was reproducibly lower than that of MB2N but higher than that of MB1N1aa46-47 (Fig 4, C). However, MB2N, MB1N1C, and MB1N1aa46-47 contain exactly the same number of IgE epitopes and appear to bind approximately the same number of IgE antibodies (no statistical difference) with comparable association and dissociation profiles (Table I; see Fig E2 in this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org)). The EM and basophil results therefore indicate that the proximity of an allergen's IgE epitope has an effect on its ability to cross-link IgE antibodies on effector cells.

### Allergen molecules with IgE epitopes engineered into adjacent positions show high allergenic activity in sensitized mice

We next studied the *in vivo* allergenic activity of those derivatives giving different results in *in vitro* basophil degranulation experiments in a murine model of anaphylaxis.<sup>30</sup> Mice were primed by injection with the monoclonal IgE and then challenged with 2 different doses (4 µg or 40 µg/mouse) of the individual derivatives (MB1N, MB2N, MB1N1aa46-47, MB1N1C, MB4N) and myoglobin as negative control. No relevant temperature drop indicative of a systemic anaphylactic reaction was observed with myoglobin or the MB1N derivative that contained only 1 IgE binding site (Fig 4, D and E). Mice receiving low-dose myoglobin derivatives showed no decrease in body temperature with MB1N and MB1N1aa46-47, a moderate decrease with MB2N and MB1N1C, and a substantial temperature drop within 20 minutes of injection of MB4N (Fig 4, D). Increasing the allergen dose to 40 mg led to a considerable temperature drop in mice receiving derivatives containing 2 or more IgE binding sites; again, this was most pronounced with MB4N (Fig 4, E). In contrast, no statistically significant differences were observed regarding the induction of peptide- and Phl p 1-specific IgE and IgG<sub>1</sub> antibody levels when mice were sensitized with the myoglobin derivatives (MB1N, MB2N, MB1N1aa46-47, MB1N1C, MB2N1C, MB4N) (see Fig E3, A-F, in this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org)). Airway hyperresponsiveness in murine models is mediated mainly by allergen-specific T-cell responses but not by IgE-mediated mast cell or basophil activation.<sup>33</sup> Because the constructs did not contain grass pollen allergen-specific T-cell epitopes recognized by BALB/c mice, we found no airway hyperresponsiveness toward Phl p 1 in mice sensitized with the constructs (data not shown).

## Discussion

We have shown in a molecular approach with defined reagents that the proximity of epitopes on a given allergen determines the shape of immune complexes consisting of the allergen and the corresponding IgE antibodies, the subsequent ability of these immune complexes to activate immune cells via receptor binding, and the potency of the allergen to induce inflammatory reactions *in vivo*. The specific results would not have been predicted by *a priori* geometric analyses of the anticipated immune complexes resulting from IgE binding to the various molecular constructs investigated. Using negative-stain EM for the visualization of immune complexes, it could be shown that antigens containing epitopes placed in adjacent positions (ie, the 2 constructs with 2N epitopes) resulted in a greater proportion of closed ring immune complexes when reacted with IgE antibodies whereas

antigens containing IgE epitopes in distant positions (ie, toward opposite side of the antigen) mainly formed open chain immune complexes. As early as the 1960s, Valentine and Green<sup>34</sup> used negative-stain EM to show an abundance of dimer rings in an antibody-divalent hapten-carrier model reaction. In this example, the 2 hapten epitopes were located on opposite sides of the carrier. Later, negative-stain EM was used to show that the relative locations of epitope pairs can have a significant effect on the formation of rings versus chains in an idiotype-anti-idiotype immune complex system.<sup>35</sup> However, our study is the first to demonstrate that the formation of different immune complex shapes is related to the proximity of the epitopes on a traditional antigen. The hinge-mediated rotational flexibility of the antibody Fab arms plays an important role in facilitating or inhibiting dimer ring formation, especially in systems with inflexible epitopes. Because IgE lacks a formal hinge region and its Fab arms display slower rates of segmental motion,<sup>36</sup> an interesting potential explanation for the lack of ring forms in some of the divalent constructs is the inability of the Fab arms of IgE to freely rotate around their long axis to accommodate the fixed positions and orientations of some of the epitopes in some constructs. Although IgE does show considerable hinge-like (bending) flexibility, which can result in IgE Fab arm orientations ranging from “T”-like to near parallel orientation,<sup>32</sup> the relative ability of IgE Fab arms to rotate has not been established.

The accessibility and the relative position of the engineered epitopes in the 3-dimensional structure of the constructs is unknown. Nevertheless, results from negative-stain EM support that the epitopes are accessible and in relative positions that differ depending on the design of the construct. Furthermore, we think that negative-stain EM for visualization of antibody-antigen complexes has an advantage over cocrystallization, which requires a homogeneous preparation of each complex, and is performed under nonphysiological antigen/antibody concentrations. In fact, the data were indeed in good agreement with the biological experiments.

Artificial allergen constructs containing epitopes engineered into adjacent positions were more potent in activating effector cells of allergy *in vitro* and inducing allergic reactions *in vivo* using a murine model for anaphylaxis. This effect was most evident at low allergen concentrations, which probably reflect the more clinically relevant situation in patients who normally are exposed to very low quantities of allergens. Our data thus provide a novel explanation for the long sought question: “Why are certain allergens more potent than others.” In fact, our study is the first to demonstrate that not only the number of epitopes but also their proximity determines the allergenic activity of an allergen molecule. We are aware that respiratory allergens contain mainly conformational epitopes<sup>37</sup> but think that the findings made with the grafted sequential epitopes also apply for conformational epitopes because the latter ones also can occur in different proximity. It must also be borne in mind that the IgE response of allergic patients is polyclonal<sup>24</sup> and that the IgE antibodies in such a polyclonal response may have different affinities to the corresponding allergen,<sup>38</sup> which will affect allergenic activity. However, studies using defined reagents such as ours are needed to study the contribution of each of the different factors (ie, IgE levels, number of IgE epitopes, proximity, affinity, polyclonality) to allergenic activity.

Furthermore, we confirmed by *in vivo* experiments that increases in allergen-specific IgE levels lead to stronger allergic reactions, which is in agreement with *in vitro* results obtained with chemically cross-linked epitopes.<sup>16</sup> This finding provides an explanation as to why allergic patients, who have increased allergen-specific IgE levels after allergen exposure, become more sensitive to the allergen and show stronger allergic reactions and increased basophil degranulation.<sup>39,40</sup>

In summary, our results define key parameters that dictate the extent of immediate type inflammatory reactions in allergy induced by allergen-IgE immune complexes. Importantly, they reveal a hitherto unknown effect of the proximity of antibody binding sites on an allergen on the shape of resulting immune complexes and the subsequent activation of Fc receptor-bearing effector cells in *in vitro* and *in vivo* models of allergy. We believe that this result is novel and important for several reasons. First, it sheds light on the as yet not fully resolved question of what factors determine the potency (ie, allergenic activity) of an allergen molecule. Second, it may be possible to use the knowledge, that IgE epitopes located in more distant proximity reduce the potency of allergens, for the engineering of safer allergy vaccines that induce less severe allergic adverse effects upon administration to sensitized patients. It is also quite possible that the results obtained from our artificial allergen-IgE model system may well be generally applicable to immune complexes containing other immunoglobulin classes and other disease-relevant antigens. In this context, one may consider classical immune complex-mediated diseases, such as systemic lupus erythematosus, farmers lung, and classical serum sickness, to name a few. However, our findings may also be important for type II hypersensitivity diseases that involve mechanisms such as antibody-dependent cellular cytotoxicity, which are important in various autoimmune diseases, such as idiopathic thrombocytopenic purpura, Graves disease, and myasthenia gravis and in defense against cancer as well as infectious agents.

In this context, it is tempting to speculate that by positioning of binding sites on receptor binding molecules (ie, ligands), it may be possible to engineer a new class of immunomodulatory molecules for the treatment of various immunologically mediated diseases, such as allergies, autoimmune diseases, cancer, infectious diseases, and immune deficiencies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations used

<b>CD:</b>	Circular dichroism
<b>EM:</b>	Electron microscopy

**RBL:** Rat basophil leukemia

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### **Clinical implications**

The demonstration that the proximity of IgE epitopes affects the allergenic activity of an allergen identifies a hitherto unknown determinant of the allergenic potency of allergens and may be useful for the rational design of allergy vaccines.

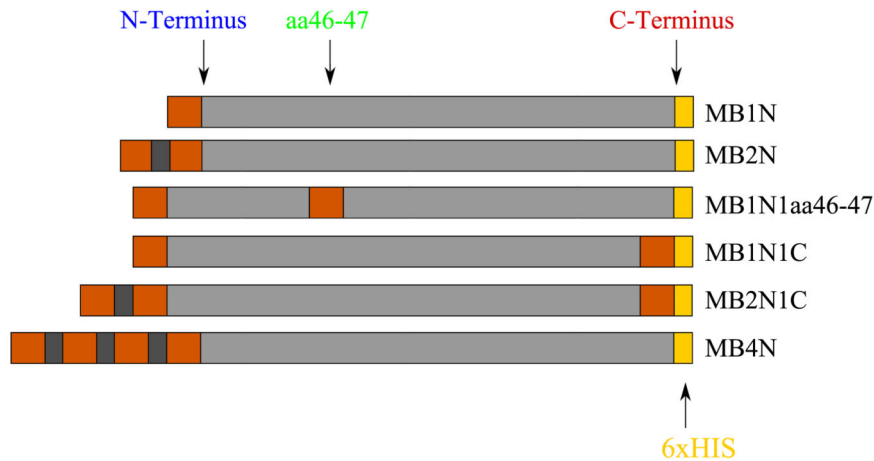
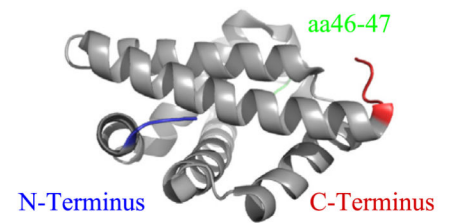
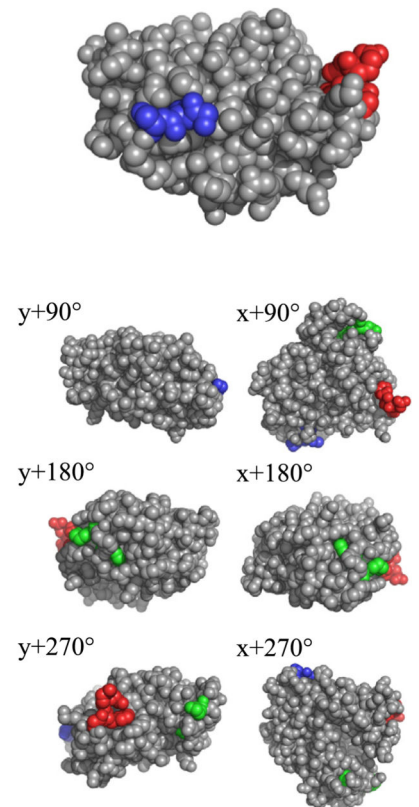


**A****Myoglobin:**

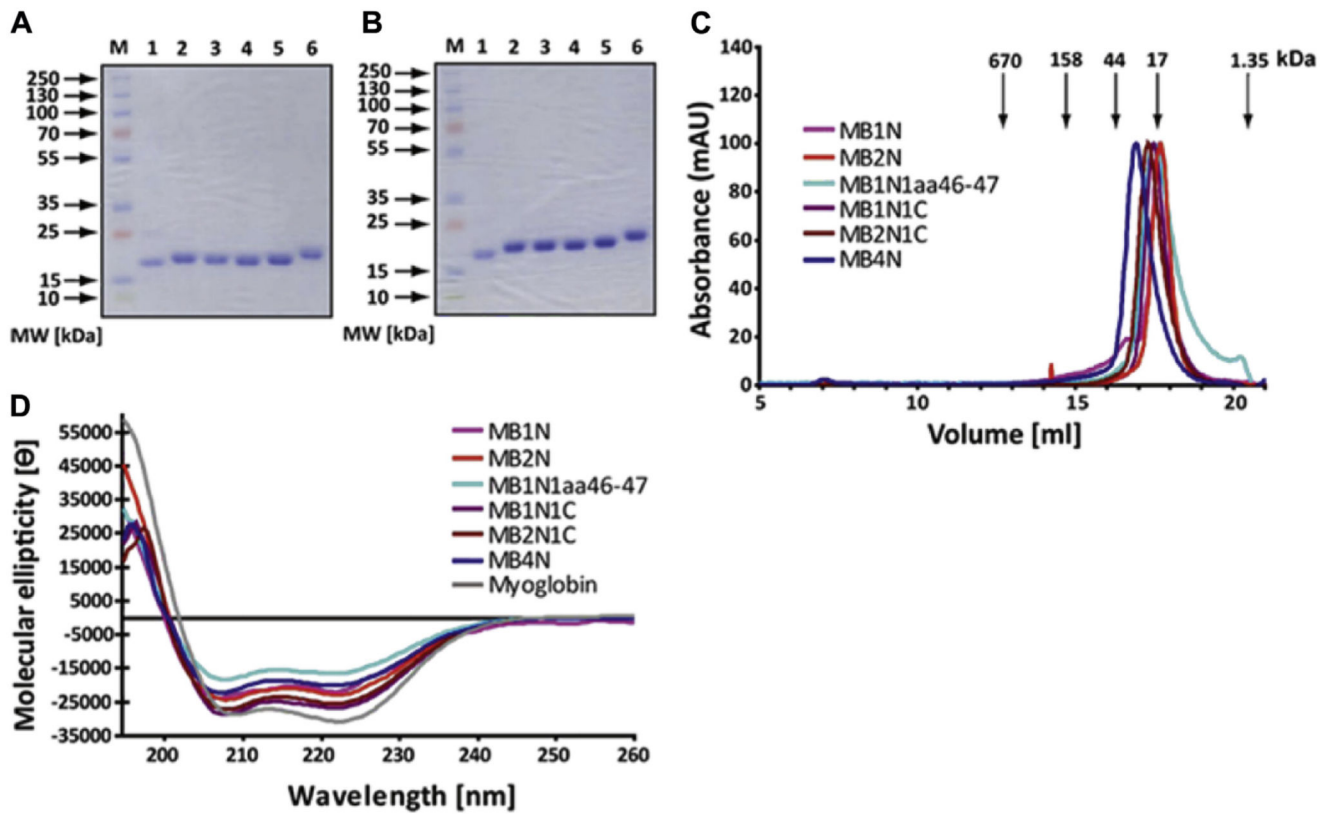
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**IgE-reactive peptide:** DLSGHAFGAMA

**Spacer:** GGGGGG

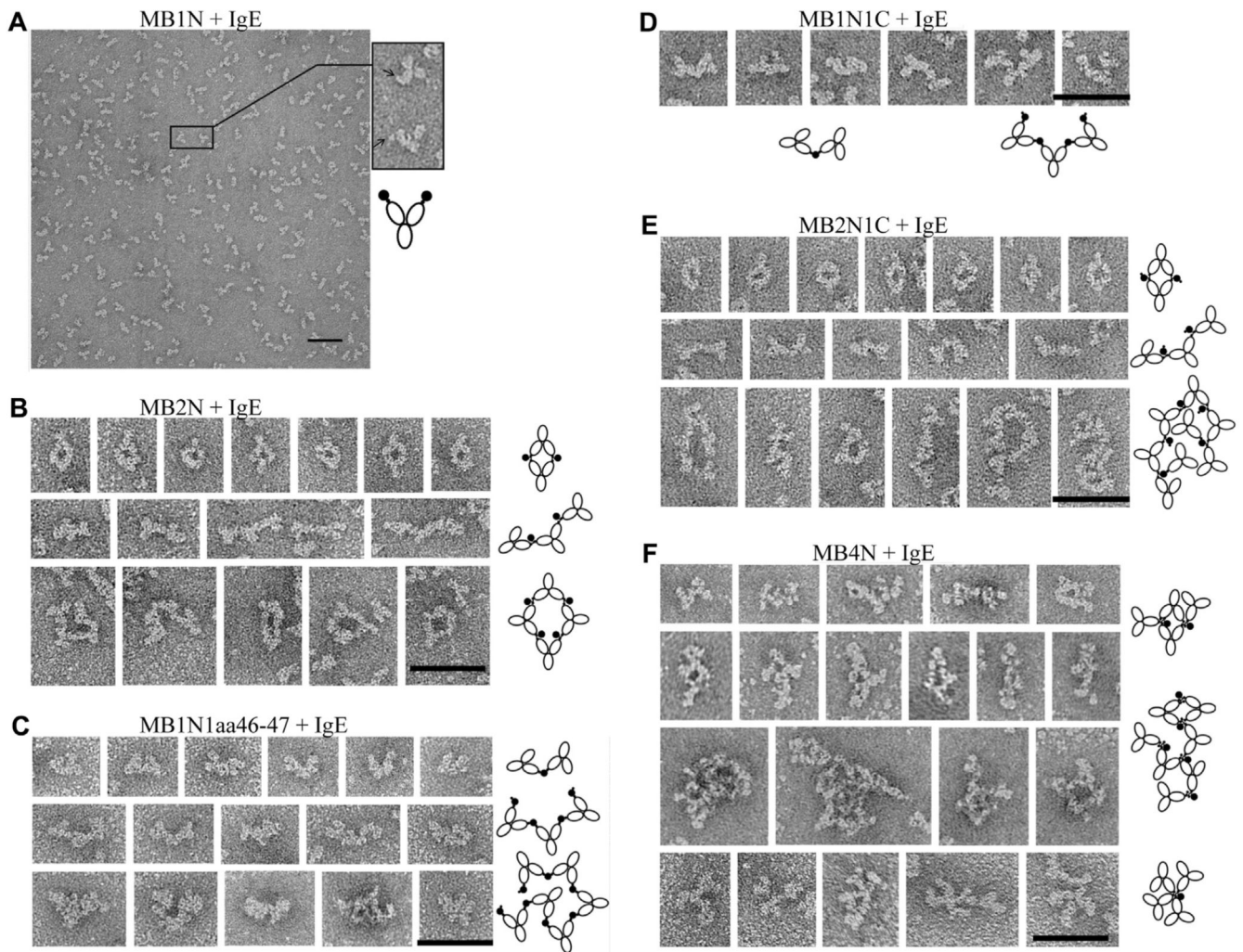
**B****Ribbon representation****Spacefill representation****Fig 1.**

Conversion of horse heart myoglobin into an IgE-reactive protein. **A**, Schematic overview of myoglobin derivatives (MB1N, MB2N, MB1N1aa46-47, MB1N1C, MB2N1C, and MB4N) containing 1 to 4 identical copies of an IgE-reactive peptide (*orange*) in different proximity in the myoglobin sequence (*arrows*). Spacers and hexahistidine tags are indicated with *gray* and *yellow*, respectively. **B**, Ribbon and space-fill representations of the 3-dimensional structure of horse heart myoglobin showing the positions of the 3 N-terminal (*blue*) and 4 C-terminal amino acids (*red*) as well as of amino acids 46 and 47 (*green*). Fig 1, *B*, was made with the MacPyMOL Molecular Graphics System using PDB coordinate file 1wla.

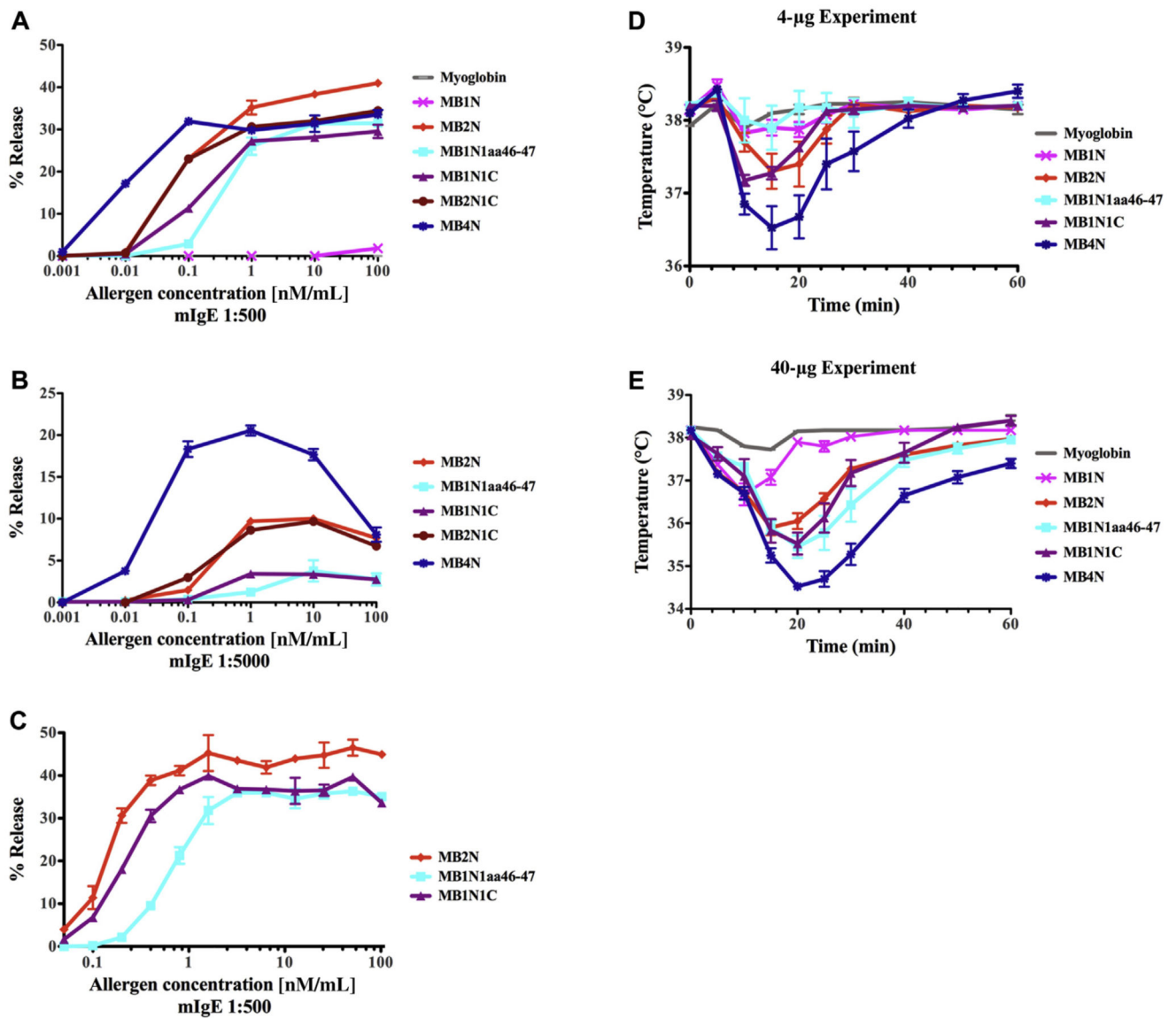


**Fig 2.**

Coomassie-stained SDS-PAGE gels showing the mobility of purified myoglobin derivatives (lanes 1-6: MB1N, MB2N, MB1N1aa46-47, MB1N1C, MB2N1C, MB4N) separated under nonreducing (**A**) and reducing (**B**) conditions. Molecular weights (kDa) are displayed. **C**, Monomeric behavior of the myoglobin derivatives demonstrated by gel filtration (x-axis: elution volumes in mL; y-axis: milli absorbance units at 280 nm). *Arrows* indicate peaks of protein standards (thyroglobulin, 670 kDa; bovine gamma globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; vitamin B<sub>12</sub>, 1.35 kDa). **D**, Far ultraviolet CD analysis showing mean residue ellipticities (Θ, y-axis) of purified myoglobin and myoglobin derivatives recorded at different wavelengths (x-axis).



**Fig 3.** Visualization of complexes consisting of myoglobin derivatives and bound IgE antibodies by negative-stain EM. Representative micrographs and interpretive diagrams showing complexes of IgE with MB1N (A), MB2N (B), MB1N1aa46-47 (C), MB1N1C (D), MB2N1C (E), and MB4N (F). Bars represent 50 nm.

**Fig 4.**

Allergenic activity of myoglobin derivatives analyzed *in vitro* by RBL mediator release assays and in a murine model of passive anaphylaxis. **A** and **B**, RBL cells were loaded with different dilutions (Fig 4, **A**, 1:500; Fig 4, **B**, 1:5000) of the peptide-specific monoclonal IgE antibody and stimulated with increasing concentrations (0.001-100 nM/mL) of myoglobin and the myoglobin derivatives (x-axes). **C**, Detailed analysis of basophil degranulation by myoglobin derivatives containing 2 IgE epitopes in different proximity.  $\beta$ -hexosaminidase release is displayed as percentage of total release on the y-axis (SDs: error bars). **D** and **E**, BALB/c mice ( $n = 4$ /group) were primed with peptide-specific monoclonal IgE antibody and challenged 10 hours later with either (Fig 4, **D**) 4  $\mu$ g or (Fig 4, **E**) 40  $\mu$ g of myoglobin derivatives. Rectal temperatures were determined every 5 minutes for the next 1 hour.

**Table I**

## IgE reactivity of myoglobin derivatives

Antigens	cpm $\pm$ SD		
	2.5 nM/mL	5 nM/mL	10 nM/mL
MB1N	116.5 $\pm$ 18.5	374.0 $\pm$ 37.4	933.6 $\pm$ 134.5
MB2N	266.3 $\pm$ 118.2	651.1 $\pm$ 53.9	2294.6 $\pm$ 961.9
MB1N1aa46-47	199.4 $\pm$ 24	433.3 $\pm$ 146.3	1579.7 $\pm$ 259.0
MB1N1C	232 $\pm$ 42.4	526.0 $\pm$ 69.7	1253.3 $\pm$ 394.6
MB2N1C	776.4 $\pm$ 104.9	1267.9 $\pm$ 50.8	2583.8 $\pm$ 113.5
MB4N	2217.9 $\pm$ 63.6	3112.5 $\pm$ 155.8	5437.1 $\pm$ 47.2
Myoglobin	37.4 $\pm$ 8.31	43.8 $\pm$ 9.6	57.4 $\pm$ 9.7

Nitrocellulose-dotted aliquots (2  $\mu$ L) of myoglobin or myoglobin derivatives (2.5 nM/mL, 5 nM/mL, 10 nM/mL) were exposed to the monoclonal IgE antibody and bound IgE was detected with  $^{125}$ I-labeled anti-IgE and quantified by gamma counting. Results are displayed in counts per minute (cpm).