Molecular determinants of allergen-induced effector cell degranulation

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Background: Allergen-induced effector cell degranulation is a key event in allergic inflammation and leads to early-phase symptoms, such as allergic rhinitis, conjunctivitis, urticaria, or bronchial asthma.

Objective: We sought to study molecular determinants of effector cell degranulation using a monoclonal IgE antibody specific for a peptide epitope of one of the most important respiratory allergens, the major grass pollen allergen Phl p 1, as a model system.

Methods: A hybridoma cell line producing a monoclonal IgE antibody against a Phl p 1–derived peptide, P1, was established by means of immunization of mice and used to sensitize rat basophil leukemia cells, which were exposed to P1 monomer, P1 dimer, and P1 polymer.

Results: It is demonstrated that the number of IgE epitopes on an allergen molecule and the concentration of allergen-specific IgE antibodies determine the extent of degranulation. The P1 monomer did not cause mediator release and prevented degranulation induced by polymeric P1.

Conclusion: Our results suggest that the number of IgE epitopes on an allergen molecule determines its allergenic activity and explains why increases of allergen-specific IgE levels make patients more sensitive to allergens. Allergen-derived monomeric structures isolated by means of combinatorial chemistry might be used to develop new therapeutic strategies for allergy.

Clinical implications: Our study reveals molecular factors that determine the immediate allergenic activity of allergens and hence influence clinical sensitivity to these allergens. (J Allergy Clin Immunol 2007;119:384-90.)

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Supported by a research grant from the Christian Doppler Association and Biomay, Vienna, and by grants T163, S8811, and F01815 of the Austrian Science Fund (FWF).

Disclosure of potential conflict of interest: R. Valenta has consultant arrangements with Phadia and Biomay. The rest of the authors have declared that they have no conflict of interest.

Received for publication June 27, 2006; revised September 22, 2006; accepted for publication September 22, 2006.

Available online November 10, 2006.

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0091-6749/$32.00

doi:10.1016/j.jaci.2006.09.034

Key words: *Allergy, allergen, epitope, effector cell degranulation*

Cross-linking of FcεRI-bound IgE on mast cells and basophils by allergens is a key event in acute allergic inflammation.[1] It induces the immediate release of inflammatory mediators (eg, histamine and leukotrienes), which are responsible for the most common manifestations of type I allergy (eg, hay fever, asthma, food allergy, and anaphylactic shock).

Considerable advances have been made in understanding how IgE binds to FcεRI and how its signal transduction cascade activates basophil and mast cell degranulation.[2,3] In contrast, relatively little is known about how the IgE-allergen interaction might influence the quality and magnitude of the allergic effector cell reaction.

In the last few years, several *in vitro* and *in vivo* studies have been conducted with defined allergen molecules to analyze determinants of allergic effector cell activation.[4] In the course of these studies, it has been observed that allergenic molecules can strongly differ from each other in terms of their ability to induce mast cell and basophil degranulation and that these differences cannot always be explained by the levels of allergen-specific IgE antibodies in serum or other body fluids.[4] In fact, several studies demonstrate that allergen-specific IgE levels are not always related to the patient's biologic sensitivity (eg, nasal and skin sensitivity and basophil degranulation)[5,6] and have suggested that several other factors, including IgE epitope density and perhaps the geometric arrangement/orientation of IgE epitopes on a given allergen molecule, might influence the immediate allergic reaction. On the other hand, it is known that increases of IgE levels against the same allergen occur after seasonal allergen exposure or controlled respiratory provocation and cause strong increases of clinical sensitivity to these allergens.[7-9]

Here we have established a molecular model system to analyze molecular determinants of allergen-induced effector cell degranulation and mediator release. For this purpose, we have used one of the most important respiratory allergens, the major timothy grass pollen allergen Phl p 1, and Phl p 1–derived peptides[10] as molecular probes.

METHODS

Materials and reagents

Purified rPhl p 1 was purchased from BIOMAY (Vienna, Austria). Phl p 1–derived peptides (P1 monomer, EPVVVHITDDNE EPIAPYHFDLSGHAFGAMA, amino acids 86-116; control peptide P5, CVRYTTEGGTKTEAEDVIPEGWKADTSYESK, amino acids

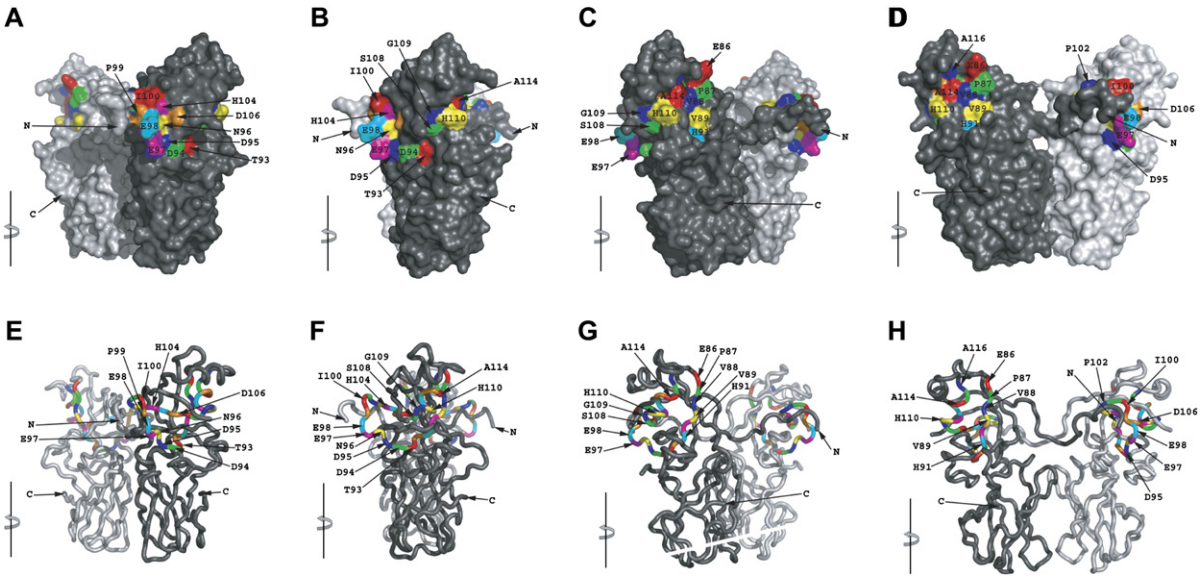

FIG 1. Localization of the P1 peptide in the context of the 3-dimensional structure of Phl p 1 visualized by means of surface **(A-D)** and backbone **(E-H)** representations. The 2 Phl p 1 monomers are shown in *light gray* and *dark gray*, and N- and C-termini are indicated. The P1 peptide comprising amino acids 86-116 of Phl p 1 has been colored, and the amino acids have been numbered.

Abbreviations used
KLH: Keyhole limpet hemocyanin
RBL: Rat basophil leukemia

212-241) were synthesized on the Applied Biosystems peptide synthesizer model 433A (Foster City, Calif).[10] P5 contained an additional, non-Phl p 1–derived cysteine residue at the N-terminus. For the mapping of IgE binding sites on P1, the following peptides were synthesized: P1-A, EPVVVHITDDNEEPI (amino acids 86-100); P1-B, APYHFDLSGHAFGAMA (amino acids 101-116); P1-C, ITDDNEEPIAPYHFDLSG (amino acids 92-109); P1-D, DLSGHAFGAMA (amino acids 106-116); P1-E, HAFGAMA (amino acids 110-116); and P1-F, YHFDLSGHAFGA (amino acids 103-114). A P1 derivate forming stable dimers (EPVVVHITDDNEEPIAPY HFDLSGHAFGAMAC) was obtained by synthesizing the P1 monomer with an additional cysteine residue at the C-terminus. The additional cysteine residue in the P1 derivative also allowed us to produce a P1 polymeric version, KLH-P1, by coupling multiple copies of the peptide to keyhole limpet hemocyanin (KLH; molecular weight, 4.5×10^5 to 1.3×10^7 g/mol; Pierce, Rockford, Ill). The mouse monoclonal IgE antibody against the P1 peptide was obtained by chance when BALB/c mice were immunized with KLH-coupled peptides that had induced IgG antibodies capable of blocking the binding of allergic patients' IgE to Phl p 1 (Charles River, Sulzfeld, Germany).[10]

SDS-PAGE

rPhl p 1 preparations and the peptides were analyzed by means of SDS-PAGE under reducing, as well as nonreducing, conditions.[11] Reducing conditions were obtained by dissolving the peptides or proteins in sample buffer containing 70 mmol/L β-mercaptoethanol. After boiling, samples were separated in 16% SDS-PAGE. Gels were subsequently stained with Coomassie Blue.

Rat basophil leukemia cell degranulation experiments

The rat basophil leukemia (RBL) cell subline RBL-2H3[12] was maintained in RPMI 1640 medium (Biochrom AG, Berlin, Germany) with 10% FCS (PAA, Pasching, Austria). RBL-2H3 cells were plated in 96-well tissue-culture plates (4×10^5 per well; Szabo, Vienna, Austria) and incubated for 24 hours at 37°C in 5% CO_2. Passive sensitization of the cells was performed by incubating the cells with mouse monoclonal P1-specific IgE antibodies for 2 hours. The cell layer was washed 2 times in Tyrode Buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 0.4 mmol/L NaH_2PO_4, 5.6 mmol/L D-glucose, 12 mmol/L $NaHCO_3$, 10 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, and 0.1% w/v BSA, pH 7.2; Sigma-Aldrich, Vienna, Austria) to remove unbound antibodies. Cross-linking of the FcεRI-bound IgE and subsequent degranulation of RBL cells was induced by adding 100 μL of serial dilutions of the allergen in Tyrode Buffer for 30 minutes to the cells in a humidified atmosphere at 37°C.[13]

The effects of preincubation of loaded cells with the P1 monomer on KLH-P1–induced β-hexosaminidase release was studied as follows: sensitized RBL cells were preincubated with various doses of the P1 monomer (for 30 minutes at 37°C and 5% CO_2) before allergen exposure (KLH-P1 for 30 minutes at 37°C and 5% CO_2).

In each of the assays, controls were performed without addition of allergen to measure spontaneous release. The release of β-hexosaminidase supernatants was analyzed by incubating culture supernatants with 80 μmol/L 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) in citrate buffer (0.1 mol/L, pH 4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100 μL of glycine buffer (0.2 mol/L glycine and 0.2 mol/L NaCl, pH 10.7), and fluorescence was measured at an extinction wavelength of 360 nm to the emission wavelength of 465 nm by using a fluorescence microplate reader (CYTO FLUOR 2350; Millipore, Billercia, Mass). Results are reported as the percentage of total β-hexosaminidase released after complete cell lysis achieved by addition of 10% Triton X-100 (Merck, Darmstadt, Germany).

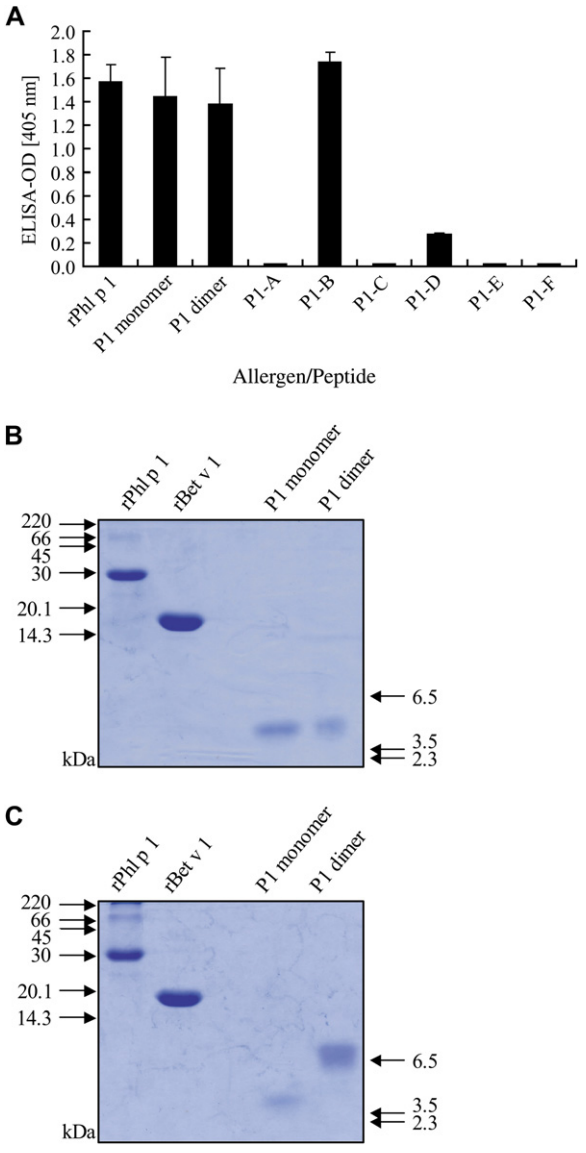

FIG 2. A, Levels of binding (mean OD values, *y-axis*) of the Phl p 1–specific monoclonal IgE to Phl p 1 and Phl p 1–derived peptides *(x-axis)*. **B** and **C**, SDS-PAGE containing allergens (rPhl p 1 and rBet v 1) and peptide preparations (P1 monomer and P1 dimer) under reducing (Fig 2, *B*) and nonreducing (Fig 2, *C*) conditions. Molecular weights are displayed in kilodaltons at the margin.

ELISA and ELISA inhibitions

ELISA plates (Greiner, Kremsmünster, Austria) were coated with rPhl p 1 (1 μg/mL PBS) or each of the peptides (1 μg/mL) at 4°C overnight. The mouse monoclonal IgE against P1 (amino acids 86-116) was either used as such or preincubated either with P1, a control peptide (P5), or buffer alone for 30 minutes at room temperature. Plates were blocked (24 hours) and incubated with mouse IgE for 2 hours at 37°C. Bound mouse monoclonal IgE antibodies were detected with a 1:1000 diluted biotin-conjugated rat anti-mouse IgE mAb (BD PharMingen, San Diego, Calif) at 4°C overnight, followed by the 1:2000 diluted avidin-horseradish peroxidase conjugate (BD PharMingen) for 30 minutes each at 37°C and 4°C. Plates were washed 5 times with PBS-T between incubation steps. Finally, plates were incubated with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) at room temperature, and absorbance was measured at 405 nm. Each assay was performed in triplicate, and results are displayed as mean values ± SD.

RESULTS

A monoclonal mouse IgE antibody recognizing a peptide epitope of the major timothy grass pollen allergen Phl p 1

The 3-dimensional structure of Phl p 1 has been determined recently by using x-ray crystallography.[14] Fig 1 shows a surface and backbone representation of rPhl p 1, which has crystallized as a dimer. Phl p 1 consists of 2 domains formed primarily by β-sheets. Using the Phl p 1–derived peptide P1 comprising amino acids 86-116 of the N-terminal Phl p 1 domain for immunizing BALB/c mice, we generated a monoclonal mouse IgE antibody. The localization of P1 on the 3-dimensional structure of the folded Phl p 1 is illustrated by coloring of the peptide in the surface representation, as well as in the backbone representation, and by indicating the flanking amino acids (Fig 1).

The binding site of the Phl p 1–specific monoclonal IgE was narrowed down by means of ELISA studies with P1 dimer, P1 monomer, and various truncated P1 forms (see Fig 2, *A*, and the Methods section, P1-A to P1-F). Only peptide P1-B (APYHFDLSGHAFGAMA) reacted with the monoclonal IgE with comparable intensity as the full-length peptide, indicating that the major binding epitopes reside within the 16-amino-acid peptide. A smaller peptide (P1-D: DLSGHAFGAMA) comprising amino acids 106-116 showed weak but distinct reactivity (Fig 2, *A*).

rPhl p 1 and the P1 peptide derivatives were analyzed by means of SDS-PAGE under reducing and nonreducing conditions (Fig 2, *B* and *C*, respectively). rPhl p 1 shows a strong tendency to form dimers and oligomers under nonreducing conditions, which could not be completely abrogated even under reducing conditions. When P1 is synthesized with a C-terminal cysteine, it forms dimers, even under denaturing conditions (Fig 2, *C*), which can only be disrupted under reducing conditions (Fig 2, *B*). P1 without the C-terminal cysteine occurs as a monomer, under both reducing and nonreducing conditions (Fig 2, *B* and *C*).

The number of IgE epitopes on a molecule determines the intensity of effector cell degranulation

RBL-2H3 cells were passively sensitized with mouse monoclonal IgE against the Phl p 1–derived peptide P1 (amino acids 86-116) to evaluate the allergenic activity of the Phl p 1 preparations and P1 derivatives. When the RBL cells were incubated with increasing concentrations of nPhl p 1 and rPhl p 1, strong differences regarding the allergenic activity of both protein preparations were noted (Fig 3, *A*). The rPhl p 1 preparation that forms polymers, as observed by means of nonreducing gel electrophoresis (Fig 2, *C*), induced a strong and dose-dependent release of

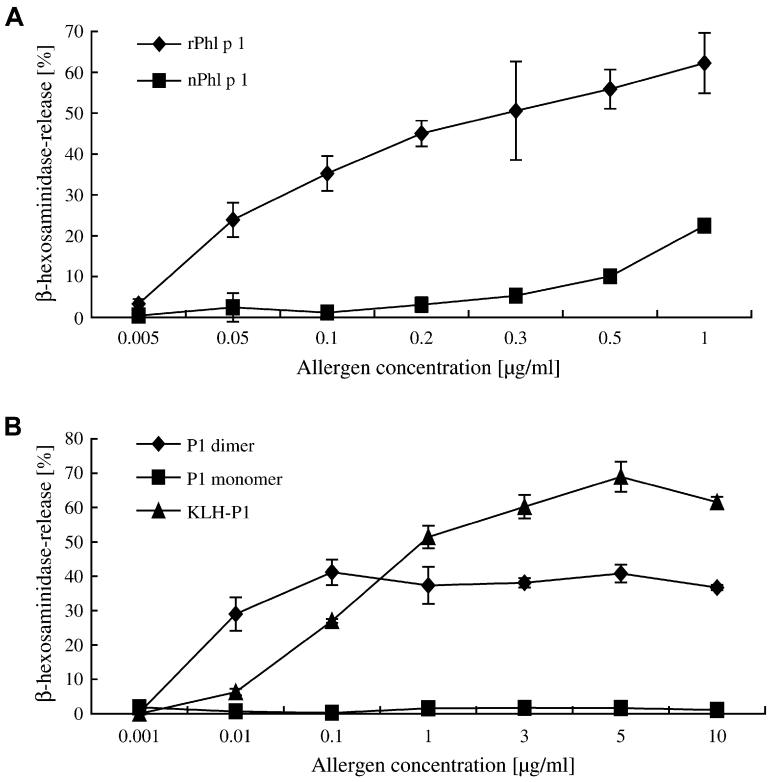

FIG 3. A, Induction of β-hexosaminidase release is displayed on RBL cells with rPhl p 1 and nPhl p 1. RBL cells were sensitized with mouse monoclonal IgE against the Phl p 1–derived peptide P1 (amino acids 86-116) and stimulated with increasing concentrations of allergens *(x-axis)*. β-Hexosaminidase release is displayed on the *y-axis*. **B**, Allergenic activity of P1 monomer, P1 dimer, and P1 polymers. Sensitized RBL cells were stimulated with increasing concentrations of the peptides *(x-axis)*, and the percentage of β-hexosaminidase release is displayed on the *y-axis*.

β-hexosaminidase, starting already at a concentration of 0.05 μg/mL (Fig 3, *A*). In contrast, the monomeric nPhl p 1 preparation[15] did not induce relevant RBL degranulation up to a concentration of 0.5 μg/mL (Fig 3, *A*).

The availability of a defined P1 peptide monomer and dimer and a polymeric P1 form allowed us to investigate the relationship between the number of IgE-reactive epitopes on a molecule and its allergenic activity. When RBL-2H3 cells that had been preloaded with the P1-specific monoclonal IgE were exposed to the P1 monomer, no mediator release could be detected up to the highest concentration tested (ie, 10 μg/mL; Fig 3, *B*). The P1 dimer induced β-hexosaminidase release already at a concentration of 0.01 μg/mL, but the release did not exceed 41%, even at the highest concentration of 10 μg/mL. The polymeric P1, which was generated by coupling P1 to KLH, induced the strongest RBL activation (ie, almost 70% of total β-hexosaminidase release; Fig 3, *B*).

The level of allergen-specific IgE antibodies determines the magnitude of effector cell degranulation

Next we investigated the effects of different IgE concentrations on the intensity of basophil degranulation (Fig 4). RBL cells were loaded with 3 different dilutions of the P1-specific monoclonal IgE (1:50, 1:500, and 1:2000), washed, and then exposed to increasing concentrations of P1 dimer. We found that the percentage of total cellular β-hexosaminidase that was released at a given allergen concentration depended on the concentration of IgE used for loading of the RBL cells (Fig 4, *A*). The higher the IgE concentration, the more β-hexosaminidase was released. Whether the concentrations of IgE used for cell loading affect also the sensitivity to a given allergen concentration was investigated by testing several allergen concentrations (Fig 4, *B*). In these experiments no evidence could be obtained that increasing amounts of cell-bound IgE antibodies cause a shift of the mediator release curve to lower allergen concentrations.

IgE-reactive monomeric structures derived from allergens prevent allergen-induced effector cell degranulation

Next we investigated whether the P1 monomer can block the binding of P1-specific IgE to the complete Phl p 1 allergen. ELISA plate-bound rPhl p 1 was incubated with the P1-specific mouse monoclonal IgE, which had been preincubated with various concentrations of P1 monomer and, for control purposes, with an unrelated peptide, P5, or buffer alone. Preincubation with the P1

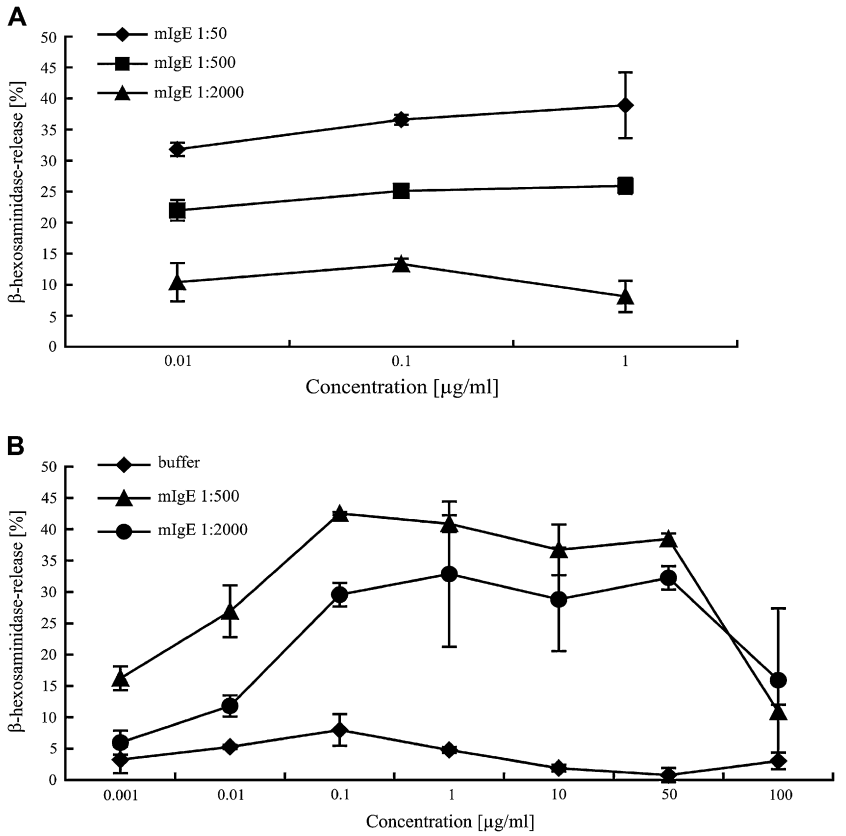

FIG 4. Factors affecting magnitude of mediator release and cellular sensitivity. The magnitude of β-hexosaminidase release *(y-axis)* depends on the titers of IgE used for loading the cells. RBL cells loaded with different concentrations of IgE (1:50, 1:500, and 1:2000) were exposed to different concentrations of P1 dimer (0.01-1 μg/mL in **A** and 0.001-100 μg/mL in **B**, *x-axis*).

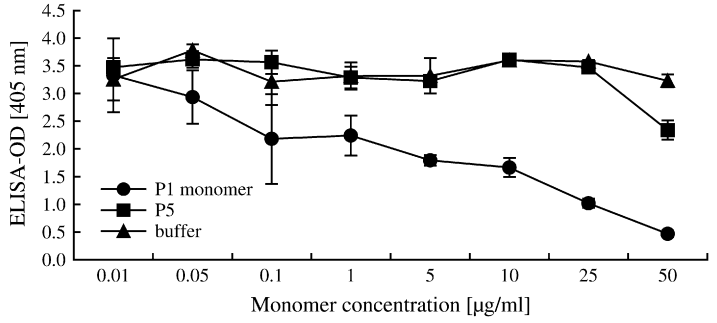

FIG 5. Preincubation of the monoclonal P1-specific IgE with the P1 monomer inhibits binding to rPhl p 1. The P1-specific IgE was preincubated with increasing concentrations *(x-axis)* of the P1 monomer, control peptide P5, or buffer alone. OD values *(y-axis)* correspond to the amount of IgE bound to immobilized rPhl p 1.

monomer yielded a dose-dependent inhibition of IgE reactivity to Phl p 1 (Fig 5). Preincubation with a concentration of 50 μg/mL of the P1 monomer inhibited almost completely IgE reactivity to Phl p 1.

Because the P1 monomer was identified as an IgE-reactive hapten through its IgE reactivity and lack of allergenic activity (Figs 2 and 3, *B*), we investigated whether pre-exposure of sensitized RBL cells with the P1 monomer can inhibit subsequent β-hexosaminidase release induced with the polymeric P1 (Fig 6). Preincubation of the sensitized cells with the P1 monomer induced a dose-dependent inhibition of β-hexosaminidase release

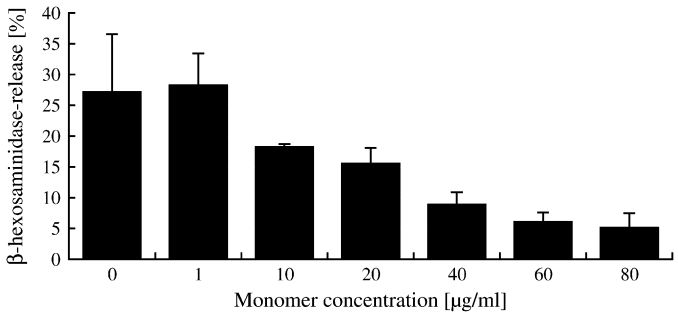

FIG 6. P1 monomer inhibits P1 polymer–induced β-hexosaminidase release. Sensitized RBL cells were incubated with increasing doses of P1 monomer *(x-axis)* and subsequently exposed to P1 polymer, and the percentage of released β-hexosaminidase was recorded *(y-axis)*.

(Fig 6). An approximately 77% inhibition of mediator release was obtained at a concentration of 60 μg/mL of the peptide epitope.

DISCUSSION

Our results demonstrate that the intensity of effector cell degranulation is associated with the number of IgE epitopes on a given allergen molecule. Second, we show that the magnitude of mediator release is associated with the concentration of epitope-specific IgE molecules used for loading of the effector cells. The latter observation might explain why allergic patients with increased levels of allergen-specific IgE after seasonal allergen exposure[7-9] become much more sensitive to allergens. Our data are in agreement with earlier studies having analyzed effector cell activation by using chemically cross-linked IgE preparations,[16-18] monoclonal and/or polyclonal anti-IgE antibodies for cross-linking,[19,20] artificial allergen mimics, or crude allergen extracts.[21-23] In these studies it was noted that human basophils were activated by much lower concentrations of allergen extracts than by anti-IgE antibodies, presumably because of the more efficient cross-linking by oligomeric structures (ie, allergen extracts and polyclonal anti-IgE) compared with bivalent cross-linking by monoclonal anti-IgE antibodies.[19]

The demonstration that IgE epitope monomers can be prepared for protein allergens under controlled conditions might be of relevance for the development of new therapeutic strategies for allergy. These epitopes fail to induce mediator release because they cannot cross-link effector cell–bound IgE. Because many of the most common and relevant allergens have been characterized down to the molecular level,[24,25] several strategies can be envisaged to obtain IgE-reactive epitopes that can prevent allergen-induced effector cell degranulation. First, simply digesting allergen preparations with proteolytic enzymes might be considered, but the end products obtained by means of such a procedure have proved to be difficult to define and standardize.[26] Another possibility might be to produce recombinant or synthetic allergen fragments,[27] but it seems that the majority of IgE epitopes on respiratory allergens belong to the conformational type and hence are difficult to reproduce, even with recombinant DNA technology and synthetic peptide chemistry. The perhaps most straightforward strategy might be the application of random peptide library approaches[28] and of high-throughput screening of compound libraries[29,30] using allergic patients' IgE antibodies as templates to identify IgE-reactive monomeric structures for therapy in the near future. By using such technologies, it should be possible to isolate and select therapeutic cocktails of IgE-reactive epitopes that can be locally applied at mucosal sites of allergic inflammation to block mast cell and basophil activation before allergen exposure.

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