

## ORIGINAL ARTICLE

# Recombinant monoclonal human immunoglobulin E to investigate the allergenic activity of major grass pollen allergen Phl p 5

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## Clinical & Experimental Allergy

### Abstract

**Background** Allergen recognition by IgE antibodies is a key event in allergic inflammation. **Objective** To construct a plasmid for the expression of human monoclonal IgE antibodies of any desired specificity and to express IgE specific for the major timothy grass pollen allergen Phl p 5.

**Methods** In a first step, the DNA sequence coding for the IgG<sub>1</sub> heavy chain was excised and replaced by the sequence coding for the human  $\epsilon$  constant region gene in plasmid pLNOH2 expressing a human Phl p 5-specific IgG<sub>1</sub> heavy chain. Then, this construct together with a second plasmid expressing the corresponding Phl p 5-specific light chain was co-expressed in COS-7 cells. The Phl p 5-specific IgE (rhuMabEP5) was analysed for allergen-specificity and isotype by ELISA. Cross-reactivity of rhuMabEP5 was investigated by immunoblotting using pollen extracts from various grass species. The allergenic activity of Phl p 5 was studied by exposing rat basophil leukaemia (RBL) cells expressing human-Fc $\epsilon$ RI to rhuMabEP5 and Phl p 5. **Results** We report the construction of vector pLNOH2-P5IgE, for the expression of human IgE and exemplify its usefulness by the production of a complete and functional human monoclonal IgE (rhuMabEP5). rhuMabEP5 is specific for the grass pollen allergen Phl p 5 and cross-reacts with group 5 allergens in natural grass pollen extracts. RBL-release assays with rhuMabEP5 demonstrated that oligomerization does not contribute to the high allergenic activity of Phl p 5.

**Conclusion and Clinical Relevance** Plasmid pLNOH2-P5IgE allowed the production of a fully functional human monoclonal IgE antibody specific for Phl p 5. Recombinant human IgE antibodies of defined specificity represent useful tools to investigate mechanisms underlying IgE-mediated allergies.

**Keywords** allergen, allergy, IgE expression vector, monoclonal human IgE antibodies, Phl p 5  
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### Introduction

The formation of allergen-specific IgE antibodies against *per se* harmless proteins is a hallmark of Type I allergy [1]. Despite low abundance of IgE in blood and other body fluids, immune complexes consisting of allergens and IgE can induce severe inflammatory reactions through interaction with the high-affinity IgE receptor Fc $\epsilon$ RI on mast cells and basophils and the subsequent release of inflammatory mediators, proteases and cytokines [2]. IgE-allergen complexes can be taken up by antigen-presenting

cells (APCs) via Fc $\epsilon$ RI or via the low-affinity receptor for IgE (Fc $\epsilon$ RII, CD23) and subsequently can activate T cells [3, 4]. Minute amounts of allergen are sufficient to boost allergen-specific IgE titres in allergic patients by stimulation and possibly clonal expansion of IgE<sup>+</sup> memory B cells [5]. Reasons for the strong induction of allergic inflammation by IgE-allergen complexes may be the well-established high affinity between IgE and Fc $\epsilon$ RI and the high-affinity recognition of allergens by IgE antibodies [6, 7].

However, there seems to be a strong variability regarding the allergenic activity of individual allergen molecules

[8, 9]. For example, grass pollen contains several allergens of which the major allergen, Phl p 5 and related group 5 allergens not only induce the highest IgE levels but also have high allergenic activity *in vitro* and *in vivo* [10–13]. Phl p 5 represents one of the most frequently recognized and potent respiratory allergens. Several explanations for this high allergenic activity can be found in the literature. Several studies report that allergens form oligomers in solution [14–19]. Furthermore, it has been claimed that such oligomers contain repetitions of the same epitope which can cross-link effector cell-bound IgE and are responsible for degranulation [20]. In addition, it has been shown that certain allergens, including Phl p 5, contain repetitive sequence elements [21–23]. Here we report the construction of an expression vector (pLNOH2-P5IgE) which allows the production of recombinant monoclonal human IgE antibodies of any desired specificity and its use to express a complete human monoclonal IgE antibody specific for Phl p 5, designated rhuMabEP5. rhuMabEP5 was characterized regarding allergen-specificity and IgE-isotype as well as concerning cross-reactivity and biological activity in rat basophil leukaemia cell (RBL) release assays. Using rhuMabEP5 in basophil activation assays, we studied possible factors responsible for the high allergenic activity of Phl p 5.

## Materials and methods

### *Materials, recombinant allergens, sera*

Recombinant allergens Phl p 1, Phl p 2, Phl p 5 and Phl p 6 were obtained from Biomay (Vienna, Austria). Sera were obtained from grass pollen allergic patients and non-allergic individuals after informed consent was obtained. Grass pollen allergy was diagnosed based on case history, skin testing and the detection of allergen-specific IgE antibodies as described [9].

### *Construction of mammalian expression vector pLNOH2-P5IgE*

In order to generate an expression vector system capable of producing complete human monoclonal IgE antibodies with predefined specificity, we used two plasmids (pLNOH2 for heavy-chain expression and pLNOK for light-chain expression) as construction templates [24]. We have earlier engineered a human monoclonal IgG<sub>1</sub> antibody specific for the major grass pollen allergen Phl p 5 by inserting the variable regions of a Phl p 5-specific human IgE Fab into the pLNOH2 and pLNOK plasmids [10]. Here we excised the DNA sequence coding for the IgG<sub>1</sub> heavy chain from pLNOH2 and replaced it by the sequence coding for the human  $\epsilon$  constant region gene which had been obtained by PCR amplification from human blood genomic DNA (Fig. 1) [25].

Genomic DNA was extracted from whole blood as described [26]. The IgE sequence containing introns for enhanced eukaryotic expression was acquired by PCR [27] (GoTaq<sup>®</sup> Polymerase Kit, Promega, Madison, WI, USA). Forward and reverse primers were designed to be located inside intron 1 and 5 of the IgE-encoding DNA, respectively, and comprised restriction sites for *Hind*III and *Bam*HI (underlined) to allow correct insertion in the pLNOH2 expression vector which contains these restriction sites. (Forward Primer: 5' AAGCTTAGGCTGGCACTGACTAGGTTC-3'; Reverse Primer: 5'-GGATCCCACTGCACAGCTGGATGG-3'). The obtained PCR-product was first ligated into the pSTBlue1AccepTor<sup>™</sup> Vector (Novagen, San Diego, CA, USA) to facilitate excision with *Hind*III and *Bam*HI. The sequence of the PCR product was verified by double strand sequencing (Eurofins MWG Operon, Ebersberg, Germany). The IgE-heavy chain-encoding DNA was excised with *Hind*III and *Bam*HI from the acceptor vector and sub-cloned into the expression vector pLNOH2 from which the DNA coding for the constant heavy chain of IgG<sub>1</sub> had been removed with the same restriction enzymes (Fig. 1). Positive clones were selected and examined by restriction analysis using restriction enzymes *Bam*HI and *Hind*III followed by DNA sequencing to verify correct insertion of the constant heavy chain region of IgE into the newly created plasmid pLNOH2-P5IgE (Fig. 1). Following the same principle (i.e., insertion of a genomic IgG<sub>4</sub>-coding DNA), a recombinant human monoclonal IgG<sub>4</sub> antibody comprising the same Phl p 5-specific variable regions, was expressed (rhuMabG<sub>4</sub>P5; C. Madritsch et al., unpublished data).

### *Expression of the monoclonal human Phl p 5-specific immunoglobulin E antibody rhuMabEP5 in COS-7 cells*

DNA from plasmids pLNOH2-P5IgE and pLNOK [10] were produced in *Escherichia coli* XL-1 Blue and extracted with Pure Yield<sup>™</sup> Plasmid Midiprep System (Promega). COS-7 cells were cultured in RPMI 1640 (Biochrome AG, Berlin, Germany) containing 10% FBS (Gibco, Invitrogen, Carlsbad, CA, USA) in T75 cm<sup>2</sup> (Corning, Chelmsford St Lowell, MA, USA) cell culture bottles. For transfection, cells were cultured in 60 mm cell culture dishes (Nunc, Roskilde, Denmark). Transient transfection of cells with both plasmids was performed with DEAE-Dextran [28] (Appl-Chem, Darmstadt, Germany).

### *Enzyme-linked immunosorbent assay experiments*

ELISA plates (Nunc Maxi-Sorp, Roskilde, Denmark) were coated with recombinant allergens (5 µg/mL; in 100 mM NaHCO<sub>3</sub>, pH = 9.6). Plates were incubated for 3 h at 37 °C, washed twice with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBST) and saturated with PBST containing 1% (w/v) bovine serum albumin (BSA)

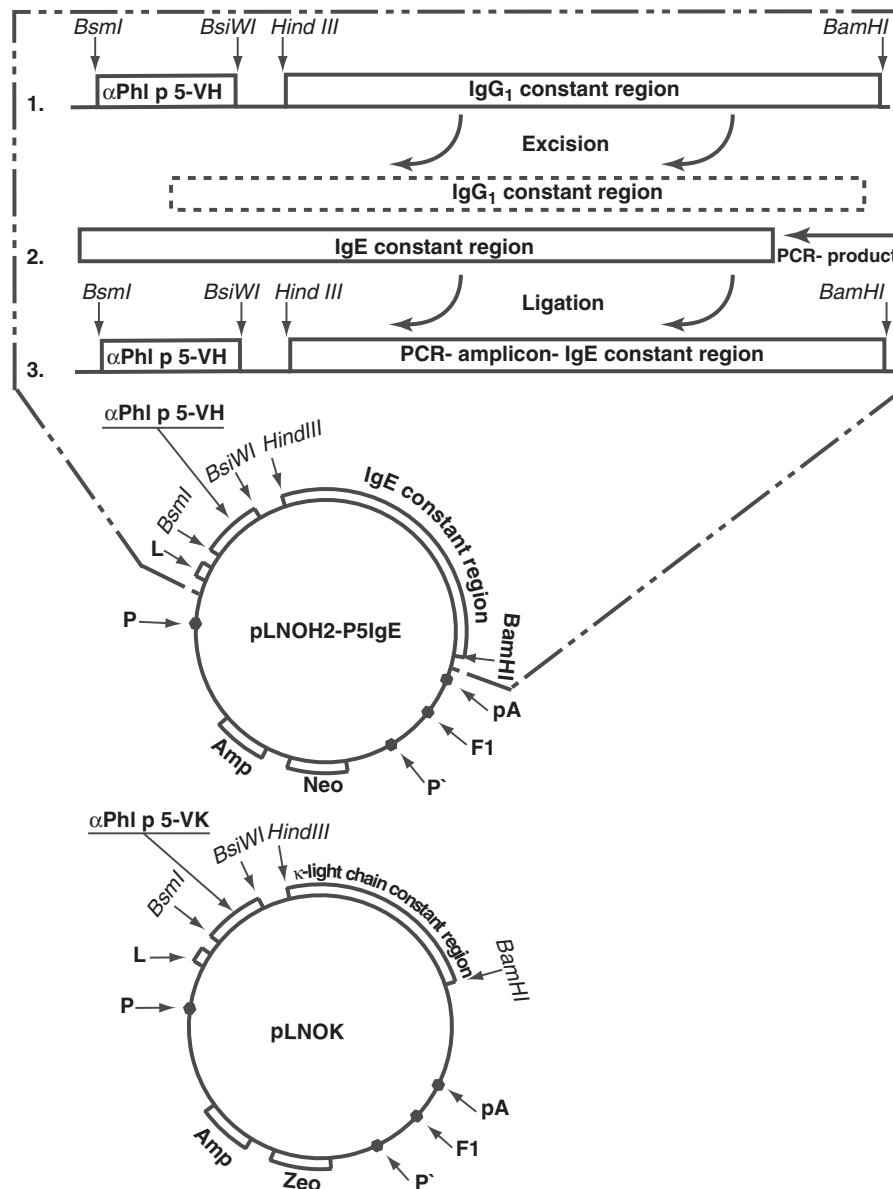


Fig. 1. Expression of a Phl p 5-specific human monoclonal IgE antibody. Plasmid pLNOH2-P5IgE expressing the heavy chain of the Phl p 5-specific IgE was constructed by excision of the IgG<sub>1</sub> constant region-encoding DNA with *HindIII*/*BamHI* (1) which was replaced with the PCR-amplified DNA coding for the IgE constant region (2, 3). Plasmid pLNOK harbours the DNA for the Phl p 5-specific kappa light chain. P, CMV promoter; P', SV 40 Ori; F1, F1 Ori; pA, BGH polyadenylation site; L, leader sequence; Neo, neomycin resistance; Amp, ampicillin resistance; Zeo, zeocin resistance; VH, variable heavy chain region; VK, variable kappa light chain region.

for 3 h at 37 °C. Cell culture supernatants [RPMI 1640 media with 10% fetal bovine serum (FBS)] containing monoclonal Phl p 5-specific IgE, IgG<sub>1</sub> and IgG<sub>4</sub> antibodies were applied in various dilutions overnight. Sera from two grass pollen allergic patients which contained allergen-specific IgE antibodies for each of the four timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6) were identified in pilot experiments and used as positive controls in dilutions of 1 : 5 in PBST/0.5% (w/v) BSA for IgE detection and 1 : 50 for IgG<sub>1</sub>, IgG<sub>4</sub> and  $\kappa$  and  $\lambda$  light chain detection. Cell culture medium (RPMI 1640) supplemented

with 10% FBS was used as negative control. Plates were incubated overnight at 4 °C and washed five times with PBST. Bound antibodies were detected with monoclonal mouse anti-human IgE, IgG<sub>1</sub> and IgG<sub>4</sub> antibodies (BD-PharMingen, San Diego, CA, USA) diluted 1 : 1000 in PBST/0.5% w/v BSA. Human light chains were detected with  $\kappa$  and  $\lambda$  light chain-specific mouse antibodies (Sigma Aldrich, St Louis, MO, USA) diluted 1 : 20 000 and 1 : 25 000, respectively. After washing five times with PBST, horseradish peroxidase-conjugated goat anti-mouse antibodies (GE Healthcare, Little Chalfont, UK)

diluted 1 : 2000 in PBST/0.5% (w/v) BSA were added and incubated for 2 h at 37 °C. Detection followed with 1.7 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS; 1 mg/mL; Sigma Aldrich) in citric acid buffer (60 mM citric acid, 77 mM Na<sub>2</sub>HPO<sub>4</sub> × 2H<sub>2</sub>O, pH 4) supplemented with hydrogen peroxide (3 mM) after washing five times with PBST. Optical density (OD) measurements were carried out at 405 nm on ELISA reader Spectramax Plus (Molecular Devices, Sunnyvale, CA, USA). Results represent means of duplicates with an error of < 10%.

#### Immunoglobulin E immunoblotting

Pollen from nine different grass and corn species were purchased from Allergon (Välinge, Sweden). Hundred milligrams of pollen from *Anthoxanthum odoratum* (sweet vernal grass), *Cynodon dactylon* (bermuda grass), *Lolium perenne* (ryegrass), *Poa pratensis* (kentucky bluegrass), *Secale cereale* (cultivated rye), *Triticum sativum* (cultivated wheat), *Avena sativa* (oat) and *Zea mays* (maize) were mixed with 3 mL 4× SDS-sample buffer and homogenized with an Ultra-Turrax (Ika, Germany) for 2 min [29]. After heating for 5 min at 95 °C, samples were centrifuged for 10 min at 18,400 g in a bench centrifuge to remove insoluble particles. Supernatants were separated by SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue [30]. Comparable amounts of pollen extracts were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) as described [31] and incubated with monoclonal recombinant Phl p 5-specific IgE antibody, two sera of grass pollen allergic patients, with serum from a non-allergic patient and buffer alone (40 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5% v/v Tween 20, 0.5% w/v BSA, 0.05% w/v NaN<sub>3</sub>) overnight at 4 °C. Bound IgE antibodies were detected as described [13].

#### Mediator release experiments with humanized rat basophil leukaemia cells transfected with human FcεRI

RBL-2H3 cells (clone RBL-703/21) transfected with the cDNA coding for the human high-affinity IgE receptor chains α, β and γ, were maintained in RPMI 1640 medium supplemented with 5% FBS, 4 mM L-glutamine and 1 mg/mL geniticine sulphate [32]. Cells were plated in 96-well tissue culture plates (10<sup>5</sup>/well) and incubated overnight at 37 °C, 7% CO<sub>2</sub>. For passive sensitization, half of the culture medium was replaced by cell culture supernatant containing rhuMabEP5 antibodies during overnight incubation. For control purposes, cells were also loaded with grass pollen allergic patient's sera containing Phl p 5-specific IgE which were diluted 1 : 20 in Culture medium and incubated overnight. Cells were washed three times with Tyrode's Buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM

MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM D-glucose, 12 mM NaHCO<sub>3</sub>, 10 mM HEPES and 0.1% w/v BSA; pH 7.2) and incubated with different concentrations of rPhl p 5 (1, 10, 100, 1000 ng/mL) and, for control purposes, with natural timothy grass pollen extract, an unrelated control allergen, Phl p 1, diluted in Tyrode's buffer or buffer alone for 1 h.

Basophil degranulation occurred when cells were loaded with polyclonal serum IgE specific for Phl p 5 and addition of Phl p 5 or grass pollen extract. Additional degranulation experiments were made by adding either (a) anti-human IgE antibodies (b) by super-cross-linking with increasing concentrations of Phl p 5-specific rabbit immune sera (0%, 1%, 10%) or for control purposes, by adding increasing concentrations of the rabbit preimmune serum diluted in Tyrode's buffer to the sensitized RBL cells (Fig. 4a). The release of β-hexosaminidase from activated RBL cells was measured as described [33]. Results are mean values ± SD presented as percentages of total hexosaminidase release.

#### Gelfiltration experiments

Gel filtration of rPhl p 5 was performed in 20 mM sodium phosphate, pH 7.2 and 150 mM NaCl. Twenty microliters of sample was loaded at a concentration of 1 mg/mL. Bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B12 (1.3 kDa) (BIO-RAD, Hercules, CA, USA) were used for calibration. A Superdex<sup>TM</sup> 200 5/150 GL column (GE Healthcare, Uppsala, Sweden) was used with a flow rate of 0.3 mL/min as recommended by the manufacturer.

#### Small angle X-ray scattering experiments

X-ray scattering data for rPhl p 5 were collected on the X33 beam line of the EMBL on the storage ring DORIS III (DESY, Hamburg, Germany). The data were recorded using a MAR345 two-dimensional image plate detector at a sample-detector distance of 2.7 m and a wavelength of λ = 0.15 nm, covering the range of momentum transfer 0.12 < s < 5.55 nm<sup>-1</sup> (s = 4πsinθ/λ, where 2θ is the scattering angle). The data were averaged after normalization to the intensity of the incident beam and the scattering of the buffer was subtracted. All data manipulations were performed using the program package PRIMUS [34].

rPhl p 5a protein solutions were prepared in 50 mM HEPES, pH 7.5, and data were collected at protein concentrations of 8.4, 3.08 and 1.0 mg/mL. Blank measurements using the identical buffer were taken before and after each protein measurement. BSA (4.5 mg/mL) was used as a standard. The low and high concentration data were merged for the subsequent data analysis. The forward scattering I(0) and the radius of gyration (R<sub>g</sub>) were evaluated using the Guinier-approximation [35]. These parameters and the maximum diameter D<sub>max</sub> of the

particle were also computed from the entire scattering patterns using the program GNOM [36]. The excluded volume  $V_p$  of the particle was computed from the Porod invariant [37]. The molecular masses of the solutes were evaluated by comparison of the forward scattering with that from a reference solution of BSA [molecular mass (MM) = 66 kDa] according to the formula.

$$\frac{I(0)^{\text{BSA}}}{\text{MM}^{\text{BSA}}} = \frac{I(0)^{\text{Phlp5A}}}{\text{MM}^{\text{Phlp5A}}}$$

## Results

### *Construction of the expression vector pLNOH2-P5IgE for human Immunoglobulin E. Expression of a recombinant human monoclonal Immunoglobulin E antibody, rhuMabEP5, specific for the major grass pollen allergen Phl p 5*

An expression plasmid for the expression of the complete IgE heavy chain designated pLNOH2-P5IgE was constructed (Fig. 1). A complete human IgE antibody specific for the major grass pollen allergen Phl p 5 was expressed in COS-7 cells. Supernatants collected from transiently transfected cells typically yielded 9.51 kUA/L of Phl p 5-specific IgE antibodies as determined by ImmunoCAP<sup>®</sup> measurements. The recombinant human monoclonal IgE antibody was designated rhuMabEP5 and reacted specifically with Phl p 5a but not with Phl p 5b or other grass pollen allergens in the CAP assay (data not shown).

### *Demonstration of allergen-specificity and immunoglobulin E isotype of rhuMabEP5*

In ELISA experiments, rhuMabEP5 bound specifically to Phl p 5 and showed no reactivity with three other major timothy grass pollen allergens Phl p 1, Phl p 2 and Phl p 6 (Fig. 2a).

The reactivity of rhuMabEP5, of the IgE isotype, was compared with monoclonal IgG<sub>1</sub> (rhuMabG<sub>1</sub>P5) and IgG<sub>4</sub> (rhuMabG<sub>4</sub>P5) containing the very same variable regions. Figures 2a–c demonstrate the isotype-specific reactivity of the three human monoclonal antibodies. None of the monoclonal IgG antibodies reacted with the other grass pollen allergens Phl p 1, Phl p 2 or Phl p 6 confirming also their allergen-specificity (Figs 2b and c). For control purposes, we used sera containing IgE and IgG antibodies, which reacted with Phl p 1, Phl p 2 and Phl p 6, confirming that these allergens were immobilized to the ELISA plates.

Next we studied the correct pairing of heavy and light chains in rhuMabEP5. Using a monoclonal anti-human  $\kappa$  light chain antibody, we could detect binding of rhuMabEP5 to Phl p 5 indicating the correct assembly of the heavy and light chain in rhuMabEP5 (Fig. 2d). For control purposes, we used a detection system specific for  $\lambda$  light chains which did not show reactivity with rhuMabEP5

demonstrating the specific utilization of a  $\kappa$  light chain in rhuMabEP5 (Fig. 2e). Allergic patients' sera displayed mixed  $\kappa$  and  $\lambda$  responses to each of the tested allergens. Cell culture media alone did not show reactivity to the allergens in any of the assays (Fig. 2a–e).

### *rhuMabEP5 specifically detects group 5 allergens in various grass and corn species*

Pollen extracts from nine different grass and corn species were blotted onto nitrocellulose and incubated with rhuMabEP5 (Fig. 3a). rhuMabEP5 reacted with group five allergens in Timothy grass (Phl p), Kentucky Blue grass (Poa p), ryegrass (Lol p) and weakly with rye (Sec c), wheat (Tri s) and oat (Ave S) between 28 and 35 kDa (Fig. 3a). No reactivity to Sweet Vernal grass (Ant o), Bermuda grass (Cyn d) and Maize (Zea m) was observed. Figures 3b and c show the IgE reactivity profile of two grass pollen allergic patients to the nitrocellulose-blotted pollen extracts. Both patients contained IgE against group 5 allergens but also reacted with several other grass pollen allergens.

### *rhuMabEP5 for probing the allergenic activity of Phl p 5*

In order to investigate whether oligomerization or repetition of epitopes on Phl p 5 may contribute to its allergenic activity we used rhuMabEP5 to analyse its allergenic activity in RBL mediator release assays. RBL cells expressing the human high-affinity IgE receptor (Fc $\epsilon$ RI) were loaded with rhuMabEP5 and exposed to Phl p 5. No degranulation was found when Phl p 5 was added in increasing concentrations (1 ng/mL–1  $\mu$ g/mL) to RBL cells passively sensitized with rhuMabEP5 (Fig. 4a). However, mediator release was induced when the Fc $\epsilon$ RI–IgE–allergen complexes were super-cross-linked with polyclonal Phl p 5-specific rabbit IgG. The addition of increasing concentrations of rabbit-anti-Phl p 5 IgG increased the magnitude of degranulation (Fig. 4a). Mediator release occurred only with rabbit immune serum but not with the rabbit's pre-immune serum.

In order to confirm, that RBL cells had bound rhuMabEP5 and were functional, we demonstrated that degranulation could be induced when anti-IgE antibodies were added to RBL cells which had been incubated with rhuMabEP5 or with patient's sera (Fig. 4b). No degranulation was observed with anti-IgE antibodies when RBL cells had been incubated with buffer alone (Fig. 4b). As additional control, we demonstrated that RBL cells that had been loaded with grass pollen allergic patients polyclonal IgE-released mediators when Phl p 5 or grass pollen extract, but not when buffer alone was added (Fig. 4c).

The specificity of the degranulation experiments performed by super-cross-linking with rabbit anti-Phl p 5 antibodies was confirmed by the following control experiments: First, we showed that degranulation only occurred

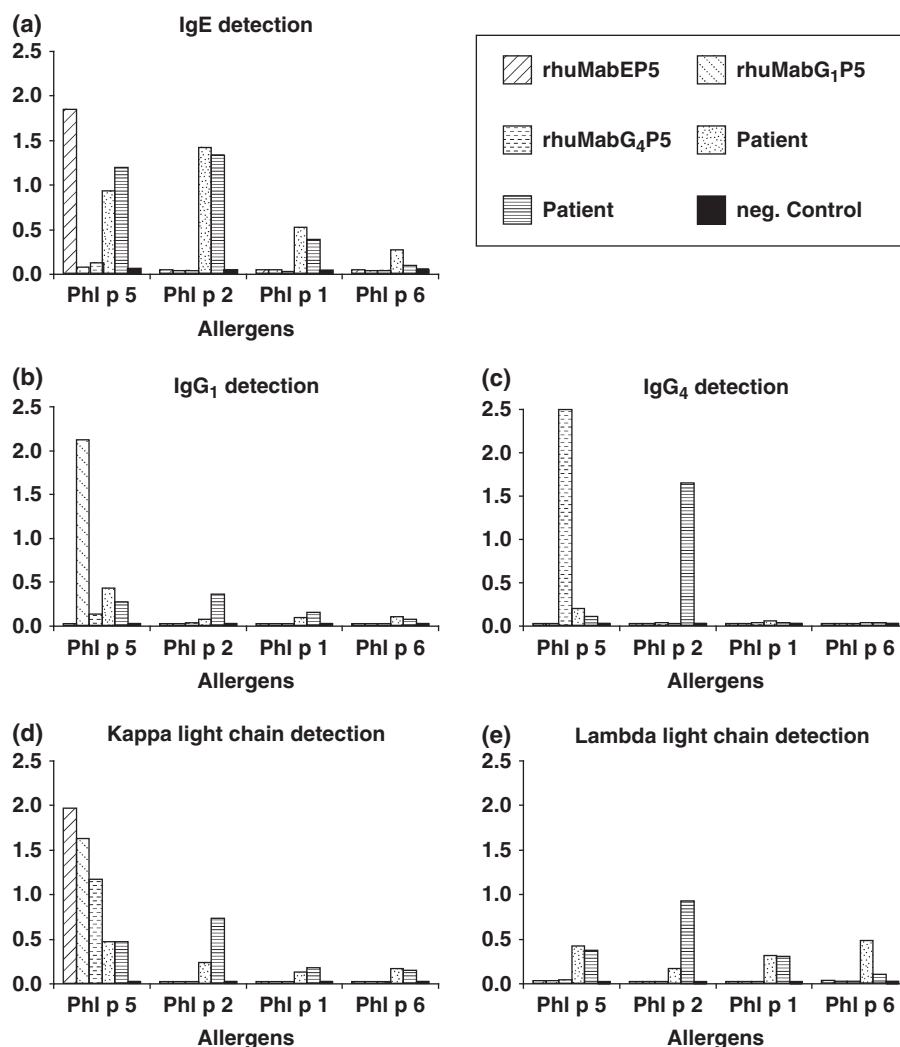


Fig. 2. Allergen-specificity and IgE-isotype determination of rhuMabEP5. ELISA plate-bound grass pollen allergens Phl p 5, Phl p 2, Phl p 1 and Phl p 6, were probed with rhuMabEP5, rhuMabG<sub>1</sub>P5, rhuMabG<sub>4</sub>P5, with sera from grass pollen allergic patients and with cell culture medium (negative control). Bound antibodies were detected with antibodies specific for human IgE (a), IgG<sub>1</sub> (b), IgG<sub>4</sub> (c), kappa (d) and lambda light chains (e). OD values (y-axis) correspond to the amount of bound antibodies.

in the presence of Phl p 5 and Phl p 5-containing grass pollen extract but not when the unrelated allergen Phl p 1 was added (Fig. 4d). Furthermore, it is demonstrated that super-cross-linking with subsequent degranulation occurred only when cells were loaded with rhuMabEP5 together with Phl p 5, but not when either rhuMabEP5 or Phl p 5 were omitted (Fig. 4e).

#### *rPhl p 5 is a monomeric protein in solution*

The elution profile of Phl p 5a in the gel filtration experiment shows that the protein represents a single species with an exclusion volume that would correspond to a globular protein of 60 kDa (Fig. 5a). According to the molecular weight calculated from the Phl p 5 sequence (i.e., 28.6 kDa), the protein appeared to be a dimer in the

gel filtration experiments. However, when the molecular mass and aggregation state of Phl p 5a was analysed by small angle X-ray scattering experiments (SAXS) it could be shown that the protein occurs as strictly monomeric species. The experimental SAXS curve from Phl p 5a is displayed in Fig. 5b. The derived MM of the protein,  $25 \pm 3$  kDa, is compatible with the monomeric form of Phl p 5a ( $MM^{\text{calc}} = 28.6$  kDa). This finding is further corroborated by the excluded volume of the sample,  $V_p = 55 \pm 5$  nm<sup>3</sup>, noting that for sufficiently large proteins the hydrated volume in nm<sup>3</sup> should numerically be about twice the MM in kDa. Figure 5c shows the Guinier-approximation from which  $I(0)$  and  $R_g$  can be deduced. Interestingly, the values of  $R_g$  ( $3.5 \pm 0.15$ ) nm and  $D_{\text{max}}$  ( $12.2 \pm 0.5$  nm) (data not shown) for Phl p 5a are significantly larger than it would be expected from a globular

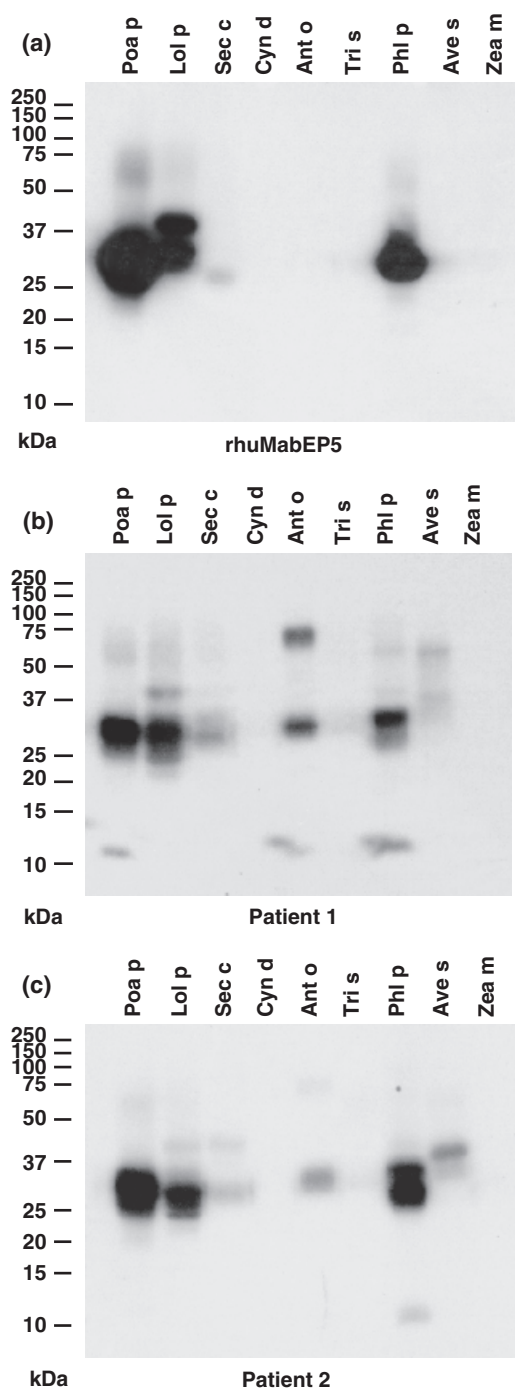


Fig. 3. Detection of group 5 allergens with rhuMabEP5 in different grass and corn species. Nitrocellulose-blotted pollen extracts (Poa p, Kentucky bluegrass; Lol p, ryegrass; Sec c, cultivated rye; Cyn d, Bermuda grass; Ant o, sweet vernal grass; Tri s, cultivated wheat; Phl p, Timothy Grass; Ave s, oat and Zea m: maize) were exposed to huMabEP5 (a) and to sera from two grass pollen allergic patients (b, c). Bound IgE was detected with  $^{125}\text{I}$ -labelled anti-human IgE antibodies. Molecular masses are indicated on the left margin in kDa.

protein of this molecular mass. For comparison, the globular BSA protein, which was used as the MM standard (MM = 66 kDa) yielded an  $R_g$  of  $2.9 \pm 0.1$  nm. These find-

ings indicate that the Phl p 5a molecule adopts a rather elongated conformation in solution. The elongated conformation vastly increases its viscosity radius in comparison to spherical proteins and is responsible for the elution behaviour in the gel filtration experiment.

## Discussion

IgE antibodies are key molecules in Type I allergy but they occur in serum and body fluids of allergic patients only in extremely small amounts and due to their polyclonal nature recognize various epitopes on allergen molecules. To obtain human monoclonal IgE antibodies of predefined specificity and to study mechanisms of allergic inflammation, we constructed an IgE expression vector. This vector termed pLNOH2-P5IgE should allow the expression of complete monoclonal human IgE antibodies of any desired specificity provided that the cDNAs coding for the heavy and light chain variable regions with antigen-specificity are available. Using pLNOH2-P5IgE, we expressed a human monoclonal antibody, designated rhuMabEP5, which is specific for one of the most frequently recognized and most potent respiratory allergens, the major grass pollen allergen Phl p 5, in COS-7 cells [10–13]. There are examples of allergen-specific recombinant chimeric human IgE antibodies [38, 39] but to our knowledge only three entirely human recombinant allergen-specific IgE antibodies have been reported [40–42]. Here we report in detail, the construction of an expression plasmid for human IgE and the expression of a functional recombinant human allergen-specific IgE antibody.

The IgE variable regions have been isolated from a combinatorial IgE library from peripheral blood RNA of an allergic patient [43]. It is therefore possible that the pairing of heavy and light chains is not identical with the natural pairing process. rhuMabEP5 showed identical epitope specificity as the Fab isolated from the combinatorial library and cross-reacted specifically with natural group 5 allergens. rhuMabEP5 could discriminate the Phl p 5a isoform from Phl p 5b in timothy grass [10]. In a series of experiments we demonstrated that rhuMabEP5 resembles the features of a complete biologically active human IgE antibody, which correctly recombines a Phl p 5-specific IgE heavy chain with a  $\kappa$  light chain.

Next we used rhuMabEP5 to study the allergenic activity of Phl p 5. Phl p 5 is known as one of the most potent allergenic molecules in grass pollen [9, 44]. Several explanations have been discussed how allergens can efficiently cross-link effector cell-bound antibodies. They include the idea that allergens can form oligomers which can present the same epitopes in a repetitive manner [14–20], the possibility that allergens occur as monomers containing repetitive epitope sequences [21–23] and finally, the model that allergens contain several different IgE epitopes which cluster on certain surface-exposed

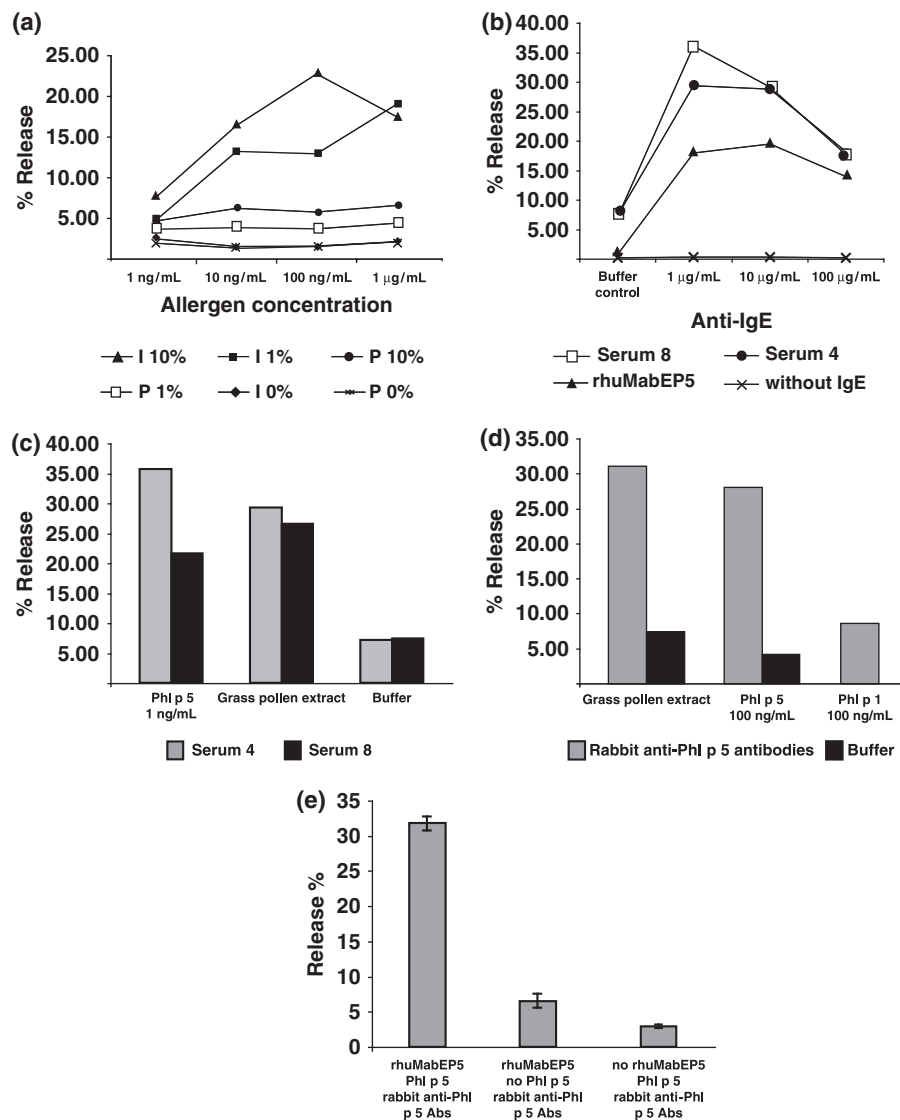


Fig. 4. Basophil degranulation experiments with rhuMabEP5. (a) RBL-2H3 cells transfected with human FcεRI were passively sensitized with rhuMabEP5, exposed to increasing concentrations of rPhl p 5 (x-axis) and then incubated with increasing concentrations of Phl p 5-specific rabbit antibody serum (I) or the rabbits pre-immune serum (P) (0%; 1%; 10%v/v). (b) RBL cells were passively sensitized with rhuMabEP5, with sera from grass pollen allergic patients (serum 4 and 8) or buffer without IgE and then incubated with increasing concentrations of an anti-human IgE antibody. (c) RBL cells were passively sensitized with sera from grass pollen allergic patients (serum 4 and 8) and then incubated with Phl p 5, grass pollen extract or buffer alone. (d) RBL cells were passively sensitized with rhuMabEP5, incubated with grass pollen extract, Phl p 5 or Phl p 1 and then exposed to rabbit anti-Phl p 5 antibodies or buffer alone. (e) Control experiments in which RBL cells were incubated with combinations of agonists indicated on the x-axis (rhuMabEP5, Phl p 5, rabbit anti-Phl p 5 antibodies). Mediator release is displayed as percentage of total β-hexosaminidase release (y-axis).

areas [10, 15, 45]. In an earlier study we have suggested that the majority of IgE epitopes and of the allergenic activity of Phl p 5 resides in an allergen fragment which is defined by rhuMabEP5 [10, 46]. In order to study whether oligomerization may contribute to the allergenic activity of Phl p 5, we loaded RBL cells with rhuMabEP5 and exposed the cells to Phl p 5. Our finding that no basophil degranulation occurred suggested that oligomerization is not a relevant factor for the allergenic activity of Phl p 5. Furthermore, the epitope defined by rhuMabEP5 does not

occur in a repetitive manner on the Phl p 5 allergen. SAXS experiments confirmed that Phl p 5 occurs in solution as monomeric molecule with an elongated shape. The higher viscosity radius caused by the elongated shape of the Phl p 5 molecule seems to be the reason why it shows a migration behaviour in gel filtration which is different from globular proteins of similar size. The correct binding of the Phl p 5-rhuMabEP5 immune complexes to the effector cells was demonstrated by two independent experiments. First we could show that RBL cells can bind rhuMabEP5 because

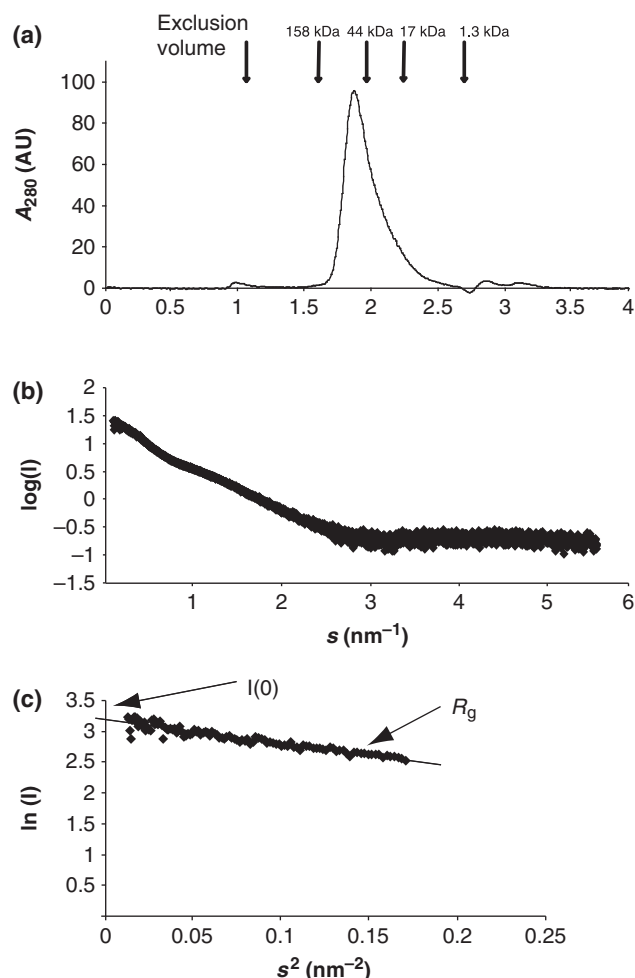


Fig. 5. (a) Elution profile of rPhl p 5 after gel filtration. Exclusion volumes (x-axis: mL) are indicated for the marker molecules with defined molecular masses (arrows). The y-axis shows the adsorption at 280 nm for the eluted Phl p 5. (b) Small angle X-ray scattering of Phl p 5. Experimental X-ray scattering pattern of Phl p 5 (merged curve from three concentrations). The intensity  $\log(I)$  is shown on the y-axis vs. the scattering vector ( $s = 4\pi\sin\theta/\lambda$ ) on the x-axis. (c) The Guinier-approximation was used to determine the forward scattering intensity  $I(0)$ . The inner part of the scattering curve (Fig. 5b) can be approximated by a Gaussian function. In this diagram [ $\ln(I)$  vs.  $s^2$ ], the scattering curve can be fitted by a straight line yielding  $I(0)$  as the y-axis intercept and  $R_g$  as the slope.

cross-linking and degranulation can be induced with anti-IgE antibodies (Fig. 4b). Second, RBL cells loaded with rhuMabEP5 can be super-cross-linked with rabbit anti-Phl p 5 antibodies in the presence of Phl p 5 but not when an unrelated control allergen, i.e., Phl p 1, is used (Fig. 4d).

Using the experimental approach of studying the allergenic activity of a major allergen, with a defined chimeric monoclonal IgE antibody for loading effector cells has already been earlier used to demonstrate that the major birch pollen allergen, Bet v 1, does not form allergenic oligomers [47].

We believe that the described expression vector system and the resulting IgE antibodies will be useful also to study mechanisms of allergic inflammation such as IgE-facilitated allergen presentation, effector cell activation or down regulation by co-cross-linking of FcεRI and Fcγ-receptors. Furthermore, rhuMabEP5 can be used for the standardization of natural allergen extracts that are in use for diagnostic and therapeutic purposes and specifically for the calibration of IgE detection methods such as quantitative IgE measurements and allergen chips. Another important aspect is the environmental monitoring of Phl p 5 which reportedly causes severe asthma attacks particularly after heavy rain falls and thunderstorms because group 5 allergens are liberated in the form of respirable submicronic particles from pollen after rainfall [48, 49].

In conclusion, the IgE expression vector and rhuMabEP5 will represent useful tools for studying mechanisms of allergic inflammation.

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