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In contrast to specific B cells, human basophils are unaffected by the toxic activity of an allergen toxin due to lack of internalization of immunoglobulin E-bound allergen

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Clinical and Experimental Allergy

Summary

Background Specific immunotherapy is the only curative therapy for type I allergies and the alarming increase in allergy prevalence emphasizes the need for additional/alternative strategies for curative treatment. Allergen toxins (AT), fusion products of an allergen with an apoptosis inducing cytotoxin, are a new kind of immunotoxin.

Objective AT should allow allergen-specific targeting and elimination of allergy-relevant cells, with B cells being the primary target. An important question is the fate of the effector cells, e.g. mast cells and basophils, which carry allergen-specific IgE: the immunotoxin might even prove to be harmful.

Methods We established a reliable *in vitro* B cell model (using two mouse hybridoma cell lines) for testing specificity and toxicity of P5-ETA', a fusion protein of the major timothy grass pollen allergen Phl p 5b and truncated *Pseudomonas* Exotoxin A. In a second step, we investigated the impact of the AT on human basophils.

Results P5-ETA' reliably eliminated Phl p 5-specific cells in the *in vitro* B cell model, leaving unspecific B cells unharmed. Human basophils of grass pollen allergic donors specifically bound P5-ETA', released IL-4 and up-regulated the activation marker CD203c, but were not subject to the toxic effect because of lack of internalization of IgE-bound allergen.

Conclusion According to our data, basophils are pure effector cells in the context of IgE-bound allergen and not involved in classical antigen presentation.

Keywords allergen, allergen toxin, basophils, hybridoma cells

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Introduction

Today, more than 20% of the population of the industrialized nations suffers from type I allergies with an almost alarming increase in the prevalence rate over the last few decades [1]. The symptoms range from relatively mild hayfever to life-threatening anaphylactic shock. Although our knowledge of the pathomechanism has become more detailed, no curative therapy for allergy exists, apart from specific immunotherapy (SIT). Despite the improvements of SIT already in use or in clinical trials, it can still have adverse side-effects and its use has certain

limitations [2–4]. It is also noteworthy that the mechanisms underlying success or failure of this therapy are still under debate.

A new curative approach could be the use of allergen toxins (AT), allergen-specific immunotoxins. In immunotoxins – most of which are used in strategies for the treatment of cancer – a target cell-specific component is fused to a cytotoxic compound [5, 6]. One of the main difficulties in cancer therapy is that specific target structures on malignant cells are rather hard to find. However, this is not true for type I allergy: allergen-specific B cells have to be eliminated in order for the treatment to be successful and allergen-specific immunoglobulin (especially of the IgE subtype) on the surface of these cells is the target for AT. Binding of AT to immunoglobulin

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(bound to the B cell receptor) via the allergen component should result in internalization and subsequent death of the cell. Probable secondary targets are the effector cells, e.g. mast cells and basophilic granulocytes, that also carry IgE bound via FcεRI, but whether affecting these cells would be only beneficial for the patient is not clear, as they have both protective and homeostatic functions in addition to the role in allergic disease [7]. The function of basophils especially is poorly understood. If production of IgE could be abolished, the natural turnover of the effector cells would result in mast cells and basophils without allergen-specific IgE and thus without reactivity to the corresponding allergens.

Recently, the protection of ovalbumin (OVA)-sensitized mice from allergic shock after treatment of the animals with a recombinant OVA toxin has been reported [8]. A second study showed a decrease of OVA-specific IgE and IgG1, but an increase in OVA-specific IgG2a [9]. Apparently, the AT also had a modulating effect on the immune response toward OVA.

Immunization of mice with OVA is a rather artificial system for development of a new therapy with an AT and therefore an allergen component more relevant for human allergy would be appropriate. An allergen-binding Epstein-Barr virus (EBV)-transfected B cells has previously been used as a model system to investigate the effects of AT in a human *in vitro* model [10]. The AT applied was a newly designed fusion protein of the major timothy grass pollen allergen Phl p 5b and *Pseudomonas aeruginosa* Exotoxin A (PEA). The natural binding domain of PEA, the target structure of which is CD91 (or α-macroglobulin receptor) [11], was exchanged with the allergen, thus leaving only the translocation and the toxic domains in the new protein that was named P5-ETA'. The highly toxic properties of the compound ETA' have been shown in a number of studies [12–16], and fusion of ETA' to a fragment of the Fc region from mouse IgE has been shown to create a molecule that is toxic to mouse mast cells [15].

EBV-transfected B cells have several drawbacks, the most important being their low rate of internalization, which results in a relatively high resistance towards AT. The first aim of this study was to establish a simpler *in vitro* B cell system for testing specificity and toxicity of Phl p 5b based AT and then to investigate the impact of P5-ETA' on human effector cells (e.g. basophilic granulocytes).

Material and methods

Antibodies and antigens

The monoclonal antibodies Bo9 and Bo12 were prepared from supernatants of the corresponding mouse hybridoma cell lines. Bo9 [17], Bo12 and Bo14 are fusions of B cells from murine spleen (mice immunized with timothy grass pollen extract) with the cell line P3X63Ag8U.1 [18] and

are specific for the timothy grass pollen allergens Phl p 5 (Bo9), Phl p 6 (Bo12), and Phl p 1 (Bo14), respectively. Bo9 and Bo12 are producing IgG1 and Bo14 IgM antibodies.

Other antibodies were as follows: anti-Penta His, unconjugated or Alexa Fluor 488 conjugated (Qiagen, Hilden, Germany), anti-human CD203c-PE, anti-human CD14-PE (Immunotech Coulter, Marseille, France), anti-human CD91-FITC (BD Biosciences, Heidelberg, Germany), anti-human HLA-DR-PE (Biozol, Eching, Germany), anti-human IgE (Allergopharma, Reinbek, Germany) labelled with the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions, anti-phospho-Histone H2A.X (Ser 139), FITC conjugated (Biomol (Upstate), Hamburg, Germany), goat anti-mouse IgG⁺M, alkaline phosphatase-conjugated and F(ab')₂ donkey anti-mouse IgG (H+L), PE conjugated (Jackson Immuno Research, West Grove, PA, USA).

Recombinant Phl p 5b (see below) was labelled with the Alexa Fluor 488 Protein Labeling Kit according to the manufacturer's instructions.

Cloning of Phl p 5b into the bacterial expression vector

The major grass pollen allergen Phl p 5b was cloned into the periplasmatic expression vector pBM1.1 [19] – derived from pET27b (Novagen, Madison, WI, USA) – via PCR with pQE9PHLP5b [20] as template (primers: forward, AAGCTTGCCGATGCCCGCTACGCCCC; reverse, GAATCC GACTTTATAGCCACCGGCAG), which introduced 5' *Hind* III and 3' *Eco*RI restriction sites. The PCR product was first cloned into pGEM-T using the pGEM-T Vector System I (Promega, Madison, WI, USA) according to the manufacturer's instructions, and then subcloned into pBM1.1 via the *Hind*III and *Eco*RI restriction sites. The new plasmid was named pBM-PHLP5b.

Protein expression and purification

Periplasmatic expression of Phl p 5b, P5-ETA', and 425(scFv)-ETA'[21] was performed using the plasmids pBM-PHLP5b, pBM-P5-ETA', and pBM-425(scFv)-ETA' in *Escherichia coli* BL21 Star (DE3) (F[−] ompT hsdS_B (rB[−] mB[−]) gal dcm rne131 DE3) under osmotic stress conditions as previously described [21]. Cells were harvested 15 h after isopropyl-β-D-thiogalactopyranoside induction. The pellet was re-suspended in sonication buffer (75 mM Tris/HCl (pH 8), 300 mM NaCl, 1 tablet Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) per 50 mL, 5 mM dithiothreitol, 10% (v/v) glycerol) at 4 °C and sonicated six times for 30 s at 200 W. Alternatively, the bacterial pellet was frozen overnight at −20 °C and slightly modified buffers were used as described before [19, 21]. Recombinant protein was purified by immobilized metal-ion affinity chromatography using nickel-nitriloacetic (Ni-NTA) chelating Sepharose

(Qiagen) followed by size exclusion chromatography using an Äkta Purifier10 with Unicorn Software and a Tricorn Superdex 200 10/300 GL column (GE Healthcare, Uppsala, Sweden) with 0.2 M ammonium hydrogencarbonate as running solvent. Recombinant protein containing fractions were pooled, protein content determined by measuring the optical density at 280 nm, shock-frozen in aliquots in liquid nitrogen and immediately lyophilized. The dried proteins were stored at 4 °C until use.

SDS-PAGE and Western blotting

SDS-PAGE was performed with Tris-tricine gels (13% separation gel, 10% spacer, 4% stacking gel) using a Protean II XI Cell (Bio-Rad, Munich, Germany).

Proteins were transferred onto nitrocellulose membrane, 0.45 µm pore diameter (Schleicher & Schüll, Dassel, Germany) by semi-dry electroblotting using the Kyhse-Andersen buffer system [22]. Membranes were blocked for 30 min in TTBS (0.1 M Tris-buffered saline with 0.05% Tween, pH 7.4) after blotting and then incubated overnight at room temperature (RT) with Moab Bo9 (1 : 20) or anti-Penta His (1 : 5000) in TTBS. Membranes were then washed three times with TTBS and incubated for 2 h with alkaline phosphatase-conjugated goat anti-mouse IgG⁺M. After washing, binding was visualized with substrate solution (0.033% (w/v) nitro blue tetrazolium salt and 0.017 (w/v) 5-bromo-4-chloro-3-indolyl phosphate potassium salt in 0.1 M Tris-buffered saline, pH 9.5).

Cell purification

Human peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood diluted 1 : 2 in HBSS using a PANCOLL, human (PAN Biotech, Aidenbach, Germany) gradient. Blood was taken from five patients with grass pollen allergy and IgE against rPhl p 5b and – as controls – from three healthy donors, who all had agreed to the experiments in informed consent.

Human basophils were isolated from PBMC using the Basophil Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions, but without erythrolysis.

Cell culture

Unless noted otherwise all media and supplements were purchased from PAA Laboratories, Linz, Austria. All cells were incubated at 37 °C and 5% CO₂ in humidified atmosphere.

Hybridoma cell lines were cultivated in 250 mL filter cap Cellstar Flasks (Greiner Bio-One, Frickenhausen, Germany) in RPMI 1640 Medium supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin

(100 U/mL) and streptomycin (100 µg/mL). Medium was changed every 3–4 days and cells passaged once a week.

2 × 10⁶ PBMC per well were cultivated in Nunclon Surface 24-well plates (Nunc, Wiesbaden, Germany) in 2 mL RPMI 1640 Medium supplemented with 10% inactivated FCS (PAA Laboratories), L-glutamine (2 mM), Penicillin (100 U/mL), and streptomycin (100 µg/mL). Isolated basophils were cultivated in 200 µL of the same medium as PBMC (supplemented with recombinant IL-3 (5 ng/mL; R&D Systems, Wiesbaden, Germany), if the cells were cultivated overnight or longer) at 5 × 10⁴ cells per well in 96-well Nunclon Surface flat-bottom plates (Nunc).

IL-4 release from basophils

Testing for IL-4 release from basophils was performed with purified cells from grass pollen allergic donors as previously described [23]. Briefly, 2500 basophils per well of a 96-well plate were incubated overnight with following stimuli: anti-IgE (50 ng/mL), rPhl p 5b (100 ng/mL), P5-ETA' (200 ng/mL) or without stimuli (negative control). IL-4 was measured by ELISA using the IL-4 Eli-pair Kit (Diacclone, Besançon, France) and a Dynatech MR-7000 ELISA reader (Acterna, Germantown, MD, USA).

Toxicity tests with mouse hybridoma cell lines

1 : 2 serial dilutions of P5-ETA', 425(scFv)-ETA' and PEA (List Biologicals, Campbell, CA, USA) were prepared in cold medium and 100 µL/well was filled in 96-well Nunclon Surface flat-bottom plates (Nunc). All dilutions were prepared in triplet. Plates were incubated on ice until addition of cells.

Hybridoma cells were washed twice with excess medium (4 °C), suspended in cold culture medium (without phenol red) to a concentration of 10⁵ cells/mL and incubated on ice for 10 min.

One hundred microlitres of cell suspension was added to all wells (10⁴ cells per well), incubated at 4 °C for 15 min and then for 24 h (37 °C, 5% CO₂) in humidified atmosphere.

For viability assays 50 µL of RPMI 1640 (without phenol red) containing 1 mg/mL XTT sodium salt and 12 µg/mL phenazine metho-sulfate (Sigma-Aldrich, Schnelldorf, Germany) were added to each well. After 18 h incubation (37 °C, 5% CO₂) optical densities at 450 nm (reference wavelength 650 nm) were measured with a Versa max microplate reader (Molecular Devices, Ismaning, Germany) with Softmax Pro 4.7 software. The reduction of protein synthesis relative to untreated control cultures was calculated graphically via Microsoft Excel, ANOVA tests were performed using SPSS (SPSS Inc., Chicago, IL, USA).

Cell stimulation, staining, and flow cytometry

Living cells were discriminated from dead cells by staining with trypan blue (5 : 1; Fluka, Steinheim, Germany)

followed by light microscopy or with propidium iodide (Sigma-Aldrich) and subsequent fluorescence-activated cell sorting (FACS) analysis.

Protein binding was analyzed using 2×10^5 cells (PBMC or hybridoma) first incubated with 2 µg/mL of His-tagged protein in 100 µL staining buffer (1% bovine serum albumin (BSA), 0.1% NaN₃ in phosphate-buffered saline (PBS)) for 30 min on ice and after washing with anti-Penta His-Alexa Fluor 488 (1 : 100 in 100 µL staining buffer) for another 30 min on ice.

Staining for IgE and CD203c or CD14 was performed after 3 days of incubation of PBMC with 5 µg/mL rPhl p 5b, 10 µg/mL P5-ETA' (cultures contained 5 ng/mL IL-3). Isolated basophils were also incubated with 2 µg/mL PEA. After washing, 2×10^5 cells were stained for 15 min at RT with 100 µL staining buffer containing anti-CD203c-PE or anti-CD14-PE (1 : 20) and Alexa Fluor 488 conjugated anti-human IgE (0.5 µg/mL).

Staining for CD91 and CD203c or CD14 was performed with freshly isolated PBMC and anti-CD91-FITC (1 : 40), volume, cells, and anti-CD203c or anti-CD14 as stated above.

Basophil stimulation was performed using 2×10^5 PBMC incubated with 1 µg/mL rPhl p 5b or 2 µg/mL P5-ETA' in RPMI (without IL-3) for 30 min (37 °C, 5% CO₂). After fixation with a solution of 2% formaldehyde, 0.5% BSA in PBS and washing, cells were stained with anti-CD203c-PE (1 : 20 in staining buffer) for 15 min at RT.

Apoptosis was detected using fixed cells incubated with 50 µL anti-Histone H2A.X-FITC (1 : 100) for 10 min in saponin buffer (0.5% BSA, 0.5% saponin (Sigma Aldrich)), at RT.

Internalization and/or release of previously bound allergen by basophils was tested using 5×10^5 PBMC of grass pollen allergic donors incubated for varied periods of time in RPMI (5 min to 18 h, 37 °C, 5% CO₂) with or without 1 µg/mL rPhl p 5b or Alexa Fluor 488 conjugated rPhl p 5b. Afterwards, cells were fixed for 20 min in 2 % formaldehyde solution, washed and stained with anti-CD203c (1 : 20) alone (incubation with conjugated allergen) or anti-CD203c and anti-His (1 : 100; unconjugated allergen) or anti-HLA-DR (1 : 20). Additionally, isolated basophils of allergic donors were incubated with or without (negative control) rPhl p 5 or P5-ETA' for 15 min at 37 °C in medium before or after fixation and with anti-His.

All FACS measurements were performed on an Epics XL Cytometer (Beckman Coulter, Krefeld, Germany).

Listmode files were further analyzed using Win MDI 2.8 software.

Results

Expression and purification of immunotoxins and allergens

P5-ETA', 425(scFv)-ETA' and recombinant Phl p 5b (rPhl p 5b) were expressed in the periplasm of *E. coli* BL21

StarTM (DE3) and purified in a two step chromatographic procedure involving affinity chromatography on Ni-NTA chelating Sepharose followed by SIC. No impurities could be detected in Coomassie-stained SDS-PAGE gels and in immunoblots using anti-Penta His Moab (not shown, see [10]). The AT and rPhl p 5b were bound by IgE from grass pollen allergic patients and the Phl p 5b-specific Moab Bo9, but not by the Phl p 6-specific Moab Bo12 (not shown).

Proof of principle for allergen toxins with two hybridoma cells lines

Two mouse hybridoma cell lines Bo 9 and Bo12 (see above) were chosen to establish a reliable *in vitro* B cell test system for specificity and toxicity of the AT. Staining with anti-mouse IgG revealed that both cell lines carry antibody on their surface, with Bo12 showing a slightly higher amount of surface bound IgG (Fig. 1a); negative control was the cell line Bo14 (specificity for Phl p 1, producing IgM). After incubation with P5-ETA' or 425(scFv)-ETA' followed by staining with anti-His-tag antibody a clear shift in fluorescence activity could be observed for Bo9 incubated with P5-ETA' compared with the negative control. No shift was detectable under all other conditions. Therefore, we conclude that only P5-ETA' but not 425(scFv)-ETA' bound to Bo9, whereas neither of them bound to Bo12 (Fig. 1b).

The possibility that specific binding resulted in a toxic effect was tested using both cell lines incubated with varying concentrations of P5-ETA', PEA (positive control) or 425(scFv)-ETA' (unspecific binding domain; negative control), purified without freezing steps apart from shock-freezing in liquid nitrogen before lyophilisation. Inhibition of protein synthesis achieved with 8 µg/mL PEA was set as 100%. After 48 h of incubation, no living cells could be detected by trypan blue staining (not shown). As all three proteins have a molecular weight of approximately 65 kDa, equal concentrations in µg/mL are also approximately equal in molarity. As differences of up to 15% could be observed between triplets of cells growing in medium without further addition of toxin, only inhibition values greater than 15% were considered positive. The experiment was repeated three times, a typical result is shown in Fig. 2. PEA (the original *Pseudomonas* Exotoxin A) efficiently inhibited protein synthesis of both cell lines, even at low concentrations. For Bo9, maximum inhibition was achieved with 2 µg/mL. P5-ETA' efficiently affected only Bo9 showing 50–80% activity of the corresponding PEA concentrations. There was also a slight inhibition of Bo12 cells at higher concentrations, which could also be observed (even more pronounced) with 425(scFv)-ETA'. The latter showed the same effect on Bo9 at similar concentrations, however, there was a significant difference in inhibition between P5-ETA' and 425(scFv)-ETA' at

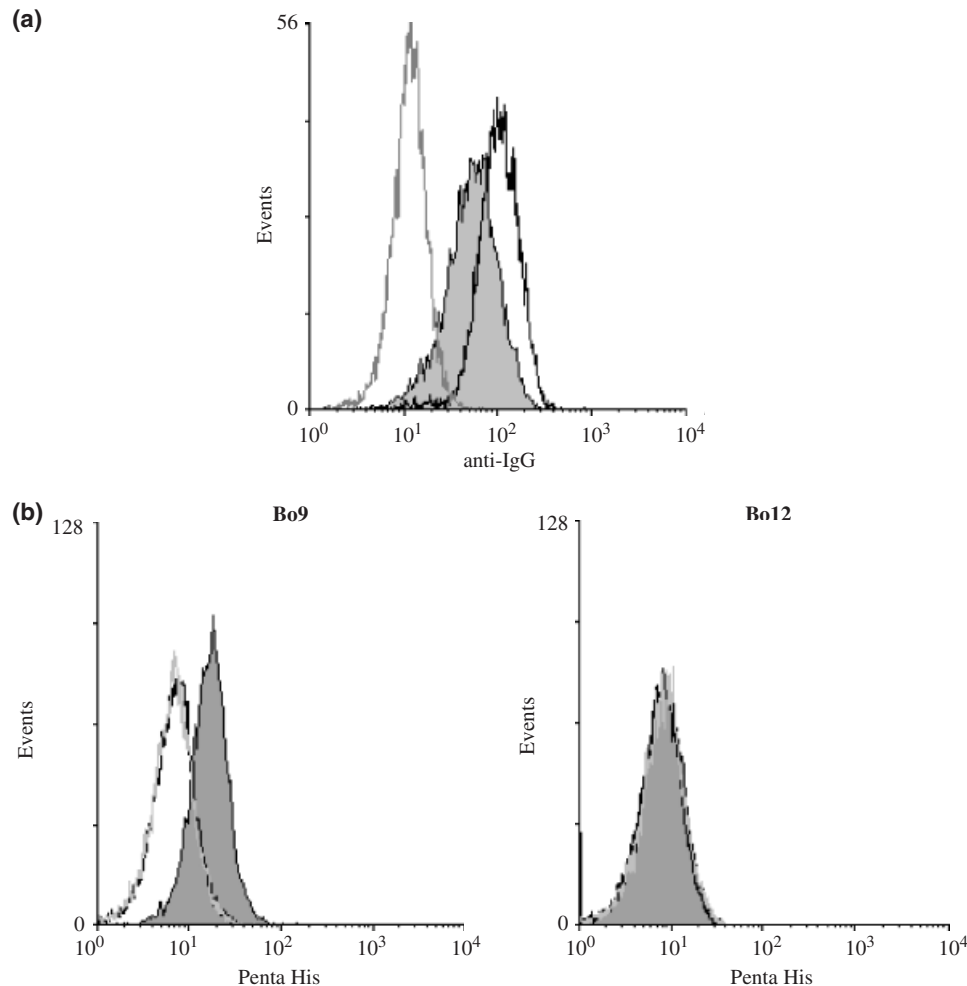


Fig. 1. Specific binding of P5-ETA' to hybridoma cells. (a) Cell lines Bo9, Bo12 (producing IgG1) and Bo14 (producing IgM, negative control) were incubated with donkey anti-mouse IgG (H+L), PE conjugated. Filled curve: Bo9; open curve, black: Bo12; open curve, grey: Bo14. (b) Cell lines Bo9 and Bo12 were incubated with P5-ETA' or 425(scFv)-ETA' and afterwards stained with Alexa Fluor 488 conjugated anti-Penta His. Filled curves: incubated with P5-ETA' open curve, black: incubated with 425(scFv)-ETA' open curve, grey: negative control, stained without previous incubation with ETA' constructs.

all concentrations ($P \leq 0.01$). Of the two immunotoxin constructs, only P5-ETA' caused a pronounced inhibition of protein synthesis in Bo9 cells (and not in Bo12) at concentrations of 1 $\mu\text{g/mL}$ and lower.

AT purified following previously described procedures only had up to 40% of the inhibitory activity of P5-ETA' purified as described above, although binding of AT to Bo9 cells was not influenced by the additional freezing (not shown).

Effect of allergen toxins on human effector cells

Basophilic granulocytes were chosen as a test system to assess the impact of P5-ETA' on effector cells. Isolated basophils of grass pollen allergic patients with IgE against recombinant Phl p 5b (rPhl p 5b) were incubated with P5-ETA' followed by anti-Penta His and anti-CD203c together or anti-Penta His alone, and a corresponding

isotype control. A typical experiment is shown in Fig. 3a. Anti-CD203c reactivity revealed a typical basophil purity of more than 90%, and no significant unspecific binding could be observed with the isotype control. Anti-His-tag reactivity after incubation with P5-ETA' showed that virtually all basophils bound the AT, whereas the anti-His-Tag antibody could not bind without previous treatment with AT.

The possible induction of apoptosis by AT was investigated using basophils of grass pollen allergic patients incubated for 18 h with rPhl p 5b, P5-ETA', or PEA. After incubation, cells were stained for phosphorylation of histone 2A.X (not shown). There was no significant increase of apoptotic cells (compared with the negative control) for both rPhl p 5b and P5-ETA'. Slightly more apoptotic cells could be observed after incubation with PEA. However, more than 60% of the apoptotic cells under this condition were CD203c negative and therefore

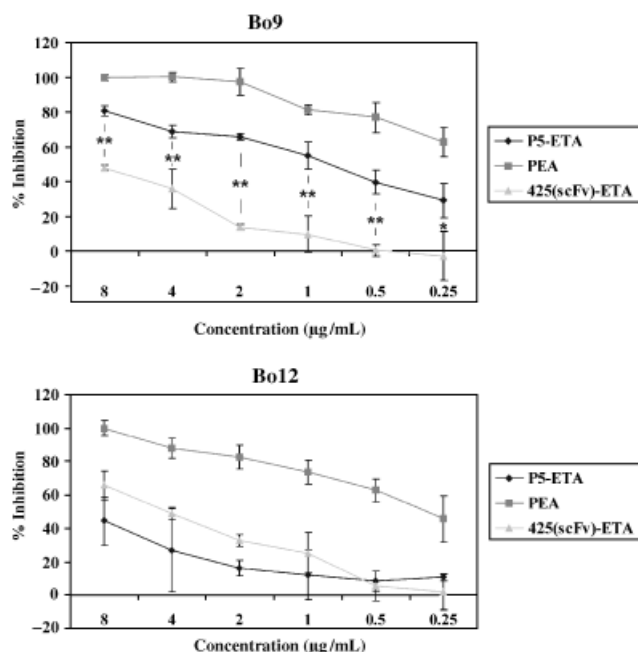


Fig. 2. P5-ETA' has a toxic effect on the hybridoma cell line Bo9, but not on Bo12. Inhibition of protein synthesis of Bo9 and Bo12 cells was measured after incubation with allergen toxin (P5-ETA'), 425(scFv)-ETA' or PEA (natural *Pseudomonas* Exotoxin (a)). Inhibition achieved with 8 µg/mL PEA was set as 100%; all values with standard deviation (2s); * $P \leq 0.01$; ** $P \leq 0.005$ for comparisons between P5-ETA' and 425(scFv)-ETA'.

comprising impurities. When PBMC were stained for CD91 – the receptor for PEA – and CD203c or CD14, the basophils were CD91-negative whereas the monocytes clearly expressed CD91. No increase in dead cells could be observed after staining with trypan blue or propidium iodide in any of the above conditions.

The specific and non-specific effects of AT were tested using PBMC of patients with grass pollen allergy and IgE against rPhl p 5b or of healthy donors. Cells were incubated with rPhl p 5b or P5-ETA' and afterwards stained with anti-CD203c and anti-Penta His. The basophils (CD203c positive) of the allergic patients bound the AT, whereas the basophils of the healthy controls did not (Fig. 3b, typical experiment). Basophilic granulocytes of the allergic patients were activated by the allergen and the AT to the same extent. Both allergen and P5-ETA' cause IL-4 release and up-regulation of CD203c (Figs 4a, b). No P5-ETA' specific binding to non-basophils (e.g. monocytes, T or B cells) could be observed in PBMC of allergic or non-allergic donors (not shown).

In order to test whether the binding of AT resulted in increased death of basophils, PBMC of grass pollen allergic and healthy donors were incubated with P5-ETA' for 3 days. Even for the grass pollen allergic donors (whose basophilic granulocytes clearly bound the AT) no increase in dead cells could be observed. The percentage

of basophils was not influenced by P5-ETA' compared with the allergen control (Fig. 4c). No ETA' specific toxicity could be observed (even with an AT concentration as high as 10 µg/mL), although there was P5 specific binding and activation of the cells. P5-ETA' showed a significant toxic effect on PBMC in these high concentrations (Figs 5a, b). After incubation of PBMC of grass pollen allergic donors with rPhl p 5b for 3 days, the monocytes (CD14⁺) survived and enhanced their size. Even when no additional protein was given, some monocytes remained, although the resulting population was considerably smaller. In contrast, after incubation with P5-ETA' these monocyte-derived cells did not appear and the monocyte population was almost completely depleted. A pronounced effect on lymphocytes could not be observed.

In order to determine whether basophils internalize bound allergen and/or release the allergen into the medium again, PBMC were incubated with rPhl p 5b or Alexa Fluor 488 conjugated rPhl p 5b for varied periods of time and then stained for binding with anti-His (only in case of unconjugated allergen) and anti-CD203c (Fig. 6). As in the experiments described above, the basophils (CD203c positive) could be shown to bind the allergen (conjugated as well as unconjugated) with maximum fluorescence activity being achieved after 5 min of incubation. The fluorescence activity in both cases did not change during the whole experiment (5 min to 18 h of incubation). Bound allergen was accessible on the surface throughout, as shown by the reactivity with anti-Penta His, and was not released after binding (Fig. 6b). There was a change, however, in CD203c expression: After the initial up-regulation, down-regulation occurred, starting at about 20 min and continuing over the rest of the experiment (not shown).

To rule out the possibility of very early internalization during the first 5 min, isolated basophils of grass pollen allergic donors were incubated with rPhl p 5 or P5-ETA' before (no internalization possibly) or after fixation of the cells (Fig. 7). It could be shown that the cells bound the allergen or AT and that the fluorescence activities before or after fixation were the same. When the same experiment was performed with labelled anti-IgE (surface anti-IgE detected with anti-murine IgG) it could be shown that although fixed cells bound/internalized less anti-IgE than untreated cells (Fig. 7b), there was less surface anti-IgE detectable on the cells that were not fixed before the incubation. However, even on the cells that were still able to internalize during the incubation a considerable proportion of anti-IgE was still detectable on the surface (Fig. 7c). These cells also showed a clear up-regulation of CD203c (Fig. 7d). Additionally, we could find no up-regulation of HLA-DR on the basophils even 18 h after incubation with allergen, whereas HLA-DR increased on the cells that were incubated with anti-IgE (not shown). We could detect no loss of surface bound allergen or

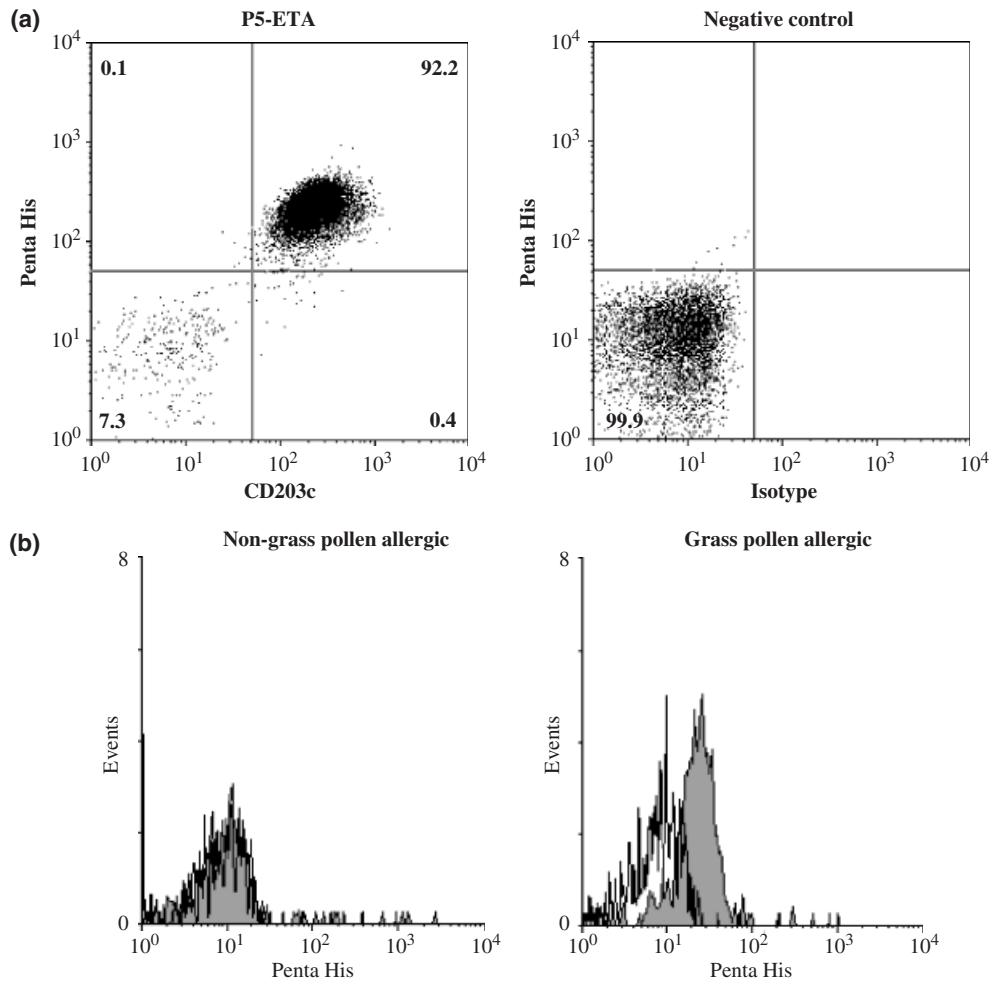


Fig. 3. P5-ETA' binds to basophils of grass pollen allergic donors. (a) Dot plots of isolated human basophils from a grass pollen allergic donor, incubated with 2 µg/mL P5-ETA' (left panel) or without (negative control, right panel). Cells were stained with anti-CD203c-PE (left panel) or isotype control antibody-PE (right panel). Numbers: percentage of cells in corresponding quadrant. (b) Histograms of basophils (CD203c-positive) from peripheral blood mononuclear cells (PBMC) of a non-grass pollen allergic and a grass pollen allergic donor. Filled curve: incubated with 2 µg/mL P5-ETA' and afterwards stained with Alexa Fluor 488 conjugated anti-Penta His; open curve: only anti-Penta His (negative control).

P5-ETA' because of internalization or shedding/release, whereas some of the anti-IgE was internalized by the basophils.

Discussion

Hybridoma cell lines Bo9 and Bo12 constitute a simple and reliable test system for Phl p 5-based allergen toxins

In the hybridoma test system, P5-ETA' shows a specific toxicity only with the Phl p 5-specific cell line Bo9. At concentrations of 1 µg/mL and lower P5-ETA still has a pronounced inhibitory effect on Bo9, which is not seen with all other conditions (425scFv-ETA' and/or Bo12) with the exception of PEA alone. Although there appears to be an element of non-specific toxicity of the ETA' constructs as is apparent from the inhibitory effect of

425(scFv)-ETA' at higher concentrations, the specific effect of P5-ETA' seems to be dominant over the non-specific as inhibition by P5-ETA' is significantly higher than inhibition by 425(scFv)-ETA' at all concentrations. Thus, the *in vitro* test system also gives an idea of the concentration range in which the ETA' domain causes additional unspecific toxic effects, starting at concentrations higher than 1 µg/mL for both immunotoxins tested on both cell lines. As no binding of 425(scFv)-ETA' to both cells and of P5-ETA' to Bo12 could be observed, the non-specific inhibitory effect had to be due to unspecific uptake, e.g. by pinocytosis, which obviously was rather ineffective compared with the specific uptake of AT by Bo9 cells as clear inhibition was seen only at higher concentrations.

Freezing steps during purification of ETA' constructs, other than shock-freezing in liquid nitrogen before

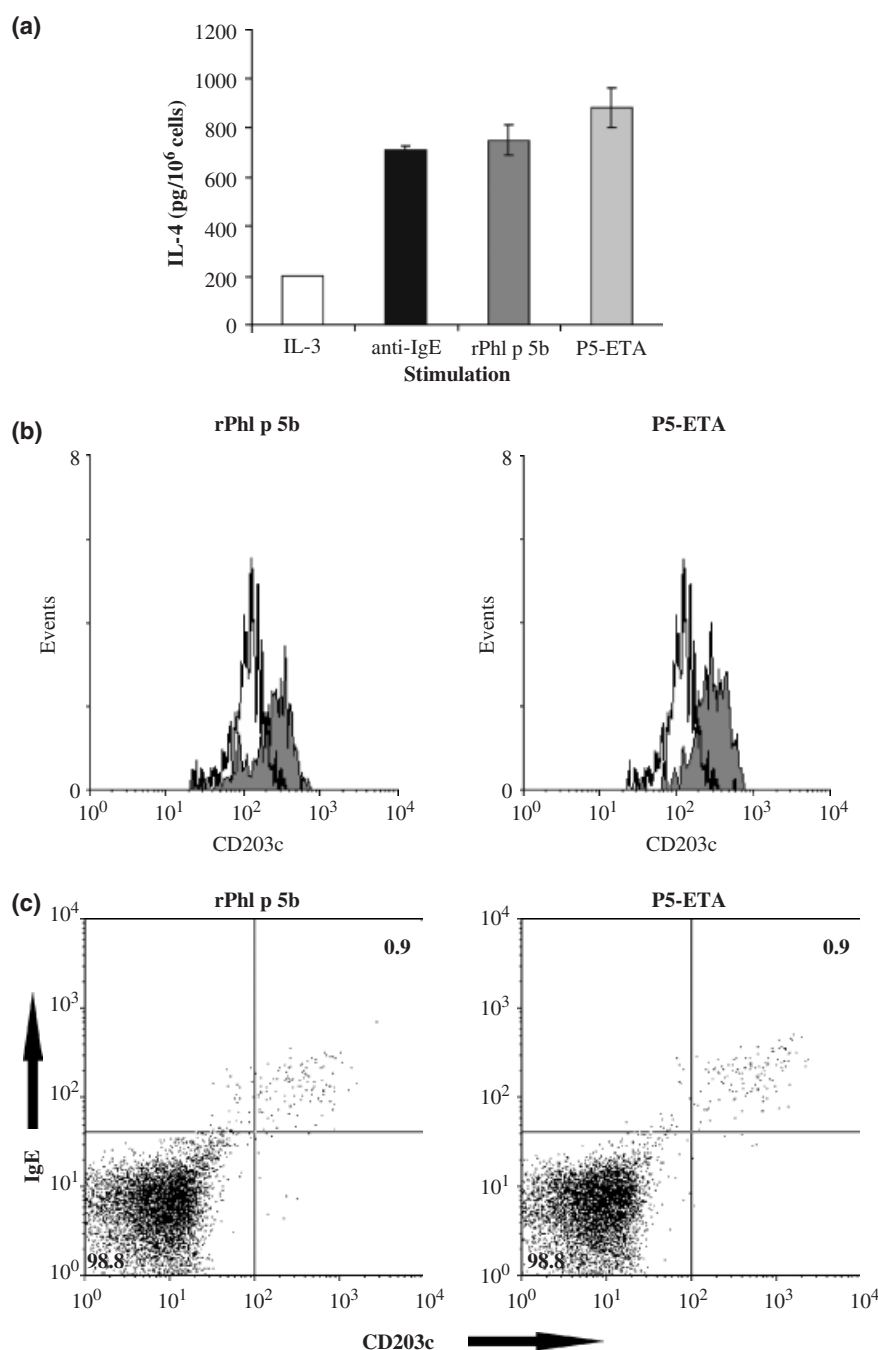


Fig. 4. P5-ETA' activates basophils of grass pollen allergic donors upon binding, but does not cause death even after prolonged incubation. (a) IL-4 release by isolated basophils from a grass pollen allergic donor. IL-4 (pg/10⁶ cells). IL-3: no further stimulation (negative control); stimulation with anti-IgE (50 ng/mL); rPhl p 5b (100 ng/mL); P5-ETA' (200 ng/mL). (b) Histograms of basophils from a grass pollen allergic donor incubated with 1 µg/mL rPhl p 5b (left panel) or 2 µg/mL P5-ETA' (right panel). Filled curves: incubation with AT or allergen; open curves: mock control. Cells were stained with anti-CD203c-PE. (c) Peripheral blood mononuclear cells (PBMC) incubated with rPhl p 5b (5 µg/mL, left panel) or P5-ETA' (10 µg/mL, right panel) for 3 days. Cells were incubated with anti-human IgE-Alexa Fluor 488 and anti-CD203c-PE; gate set on lymphocyte/basophil region. Percentage of cells given in corresponding quadrant.

lyophilization, are to be avoided for they significantly reduced the toxicity of the fusion proteins. Apparently, the additional freezing reduced translocation of ETA' or its ADP-ribosylating activity as binding of P5-ETA' was not influenced.

The Bo9/Bo12 system provides a simple and reliable *in vitro* B cell test for specificity and toxicity of P5-ETA' and it might also be a suitable system for future AT with Phl p 5 as binding element and toxic domains other than ETA'. The results so far are encouraging.

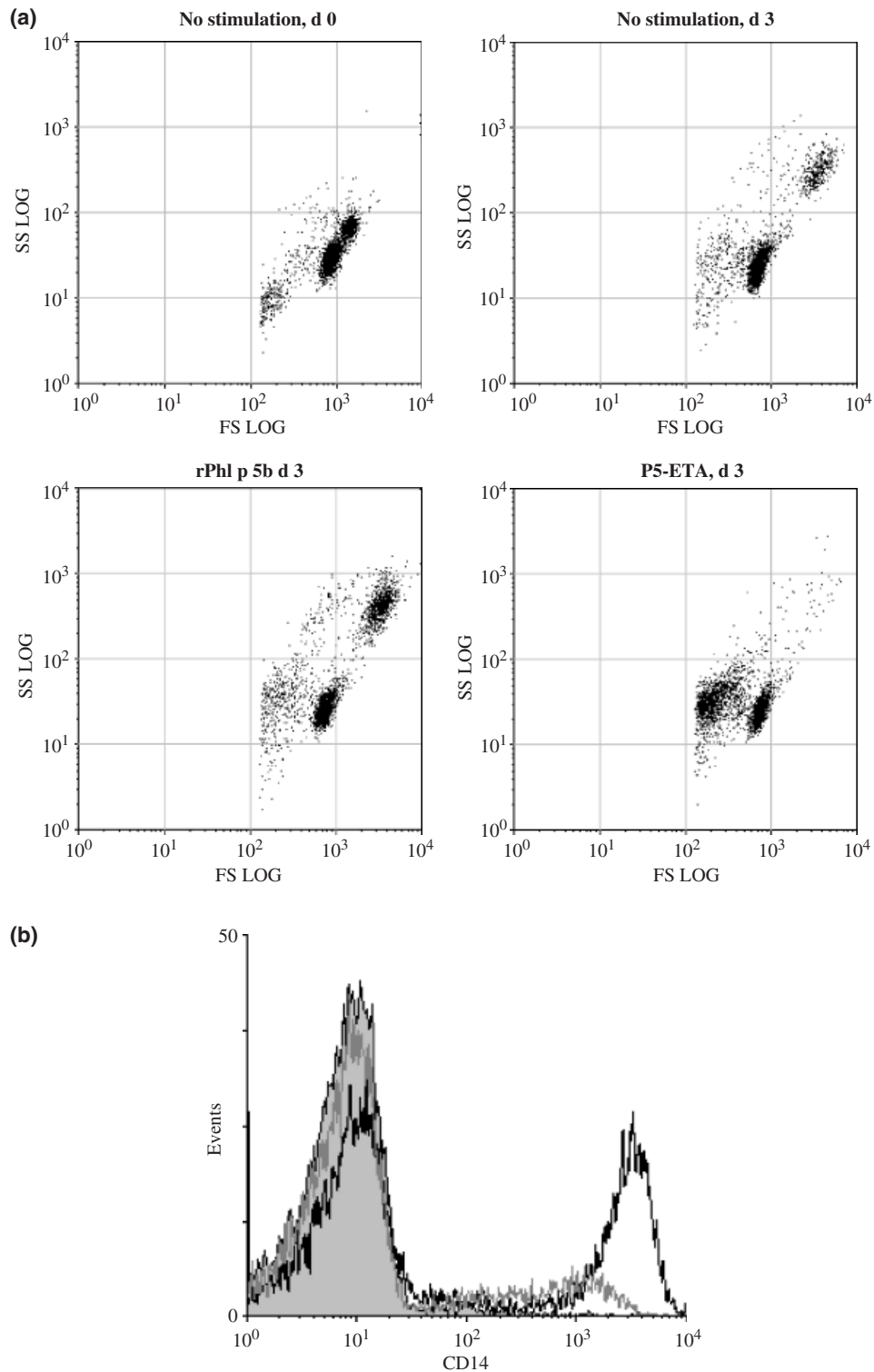


Fig. 5. Effect of P5-ETA' on peripheral blood mononuclear cells (PBMC). (a) Dot plots of PBMC from a grass pollen allergic donor: without stimulation immediately after preparation (upper left panel) and incubated for 3 days (upper right panel); incubated with $5 \mu\text{g}$ rPhl p 5b (lower left) or $10 \mu\text{g}$ P5-ETA' (lower right). SS log: side-scatter, logarithmical scale; FS log: forward scatter, logarithmical scale. (b) Histogram of living cells (10^4 events) after 3 days from 7-A, stained for CD14 for detection of monocytes. Filled curve: Incubation with P5-ETA' ($10 \mu\text{g}/\text{mL}$); open, black: incubation with rPhl p 5b ($5 \mu\text{g}/\text{mL}$); open grey: no stimulation.

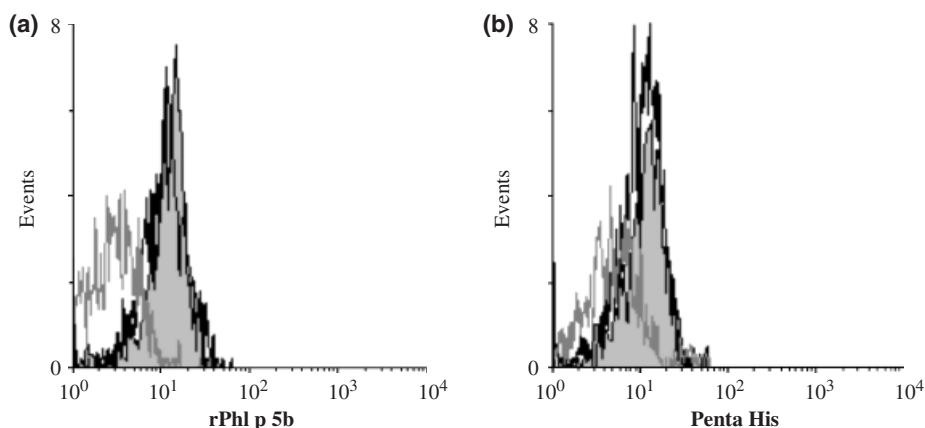


Fig. 6. Human basophils keep allergen bound to their surface. Histograms of CD203c positive cells from a grass pollen allergic donor. (a) Peripheral blood mononuclear cells (PBMC) incubated with 1 µg/mL rPhl p 5, Alexa Fluor 488 conjugated. Open curve, grey: Negative control (no rPhl p 5b added); open curve, black: incubation time 2 h; filled curve: incubation time 10 min. (b) PBMC incubated with rPhl p 5b and anti-Penta His. Open curve, grey: negative control (no rPhl p 5b, only anti-Penta His); open curve, black: incubation for 2 h; filled curve: incubation for 10 min.

Human basophils are not affected by the toxicity of allergen toxins because of lack of internalization of immunoglobulin E bound allergen

Virtually all basophils of every donor with IgE directed against rPhl p 5b bound P5-ETA' (Fig. 3a) and they were also activated by the AT as shown by IL-4 release and up-regulation of surface CD203c [24, 25] (Figs 4a, b). Interestingly, P5-ETA' did not show any toxic effect on the basophils. Neither did it increase the rate of apoptosis nor the rate of cell death of the basophils (Fig. 4c). There are a number of possible explanations as to how basophilic granulocytes might elude toxic damage. First, they might simply be insensitive to the ribosylation by ETA'. The fact that PEA (the original Exotoxin A) has no effect on isolated basophils cannot strengthen this hypothesis, as the basophils simply lack CD91, the receptor for PEA. It does not seem reasonable to us that some molecule exclusively found in basophils could block the ribosylating activity of ETA'. Second, the cells could rapidly cleave the cross-linking AT after uptake, which is rather unlikely given the slow processing rate described for basophilic leukemia cells [26]. Third, the AT might not be internalized. Very rapid internalization of antigen bound to IgE has been described for basophilic leukaemia cells and normal rat mast cells [27, 28]; however, only 50% of the bound antigen was found to be internalized in these experiments. These reports correlate well with our data obtained for human basophils: We also found that a significant proportion of allergen stays on the cell surface. If any at all was internalized the process had to be a rapid one because maximum fluorescence activity was obtained after 5 min of incubation. Fourth, after partial processing of the bound IgE basophilic leukaemia cells release what they have internalized into the supernatant again [26]. We could find no evidence of such a 'cherry-stone like' mechanism in the human basophils. On the contrary, in

our experiments the fluorescence activity remained the same from 5 min to 18 h (Fig. 6). When the basophils were fixed before incubation with allergen or AT, thus preventing internalization, we could show that the same amount of allergen or AT was accessible on the surface whether or not the cells were still able to internalize (Fig. 7a). Obviously, human basophils lack the ability to internalize IgE-bound allergen even after cross-linking and they did not increase surface HLA-DR. According to our data, human basophilic granulocytes are pure effector cells in the context of interaction of allergen and specific IgE, and are not involved in classical antigen presentation. On the other hand, they capture antigen and obviously retain it on their surface for a long-time (Fig. 6) thus presenting it as intact protein to other cells. As the whole protein is presented, B cells are more probable target cells in this context than T cells which are the primary target cells of the professional antigen presenting cells e.g. dendritic cells.

A remarkable difference is seen when the cells are stimulated with anti-IgE (Figs 7b, d): They internalize part of the anti-IgE as has been observed with RBL cells. However, it has to be kept in mind that cross-linking of any two IgE molecules with anti-IgE is far from the natural situation where specific IgE-molecules that can only bind 'their' antigen/allergen are few and far between. Obviously, one has to be careful when drawing conclusions from experiments with anti-IgE. The findings on internalization presented here are the first that have been obtained with human basophils with an *ex vivo* loading of IgE. Considering the unexpected results, other experiments concerning cross-linking and internalization which were almost all performed with RBL-cells and/or a monomeric loading with IgE should probably be repeated with *ex vivo* cells. It seems that there are indeed differences between the stimulation of *ex vivo* mast cells with allergen, anti-IgE or IgE-dimer [29, 30].

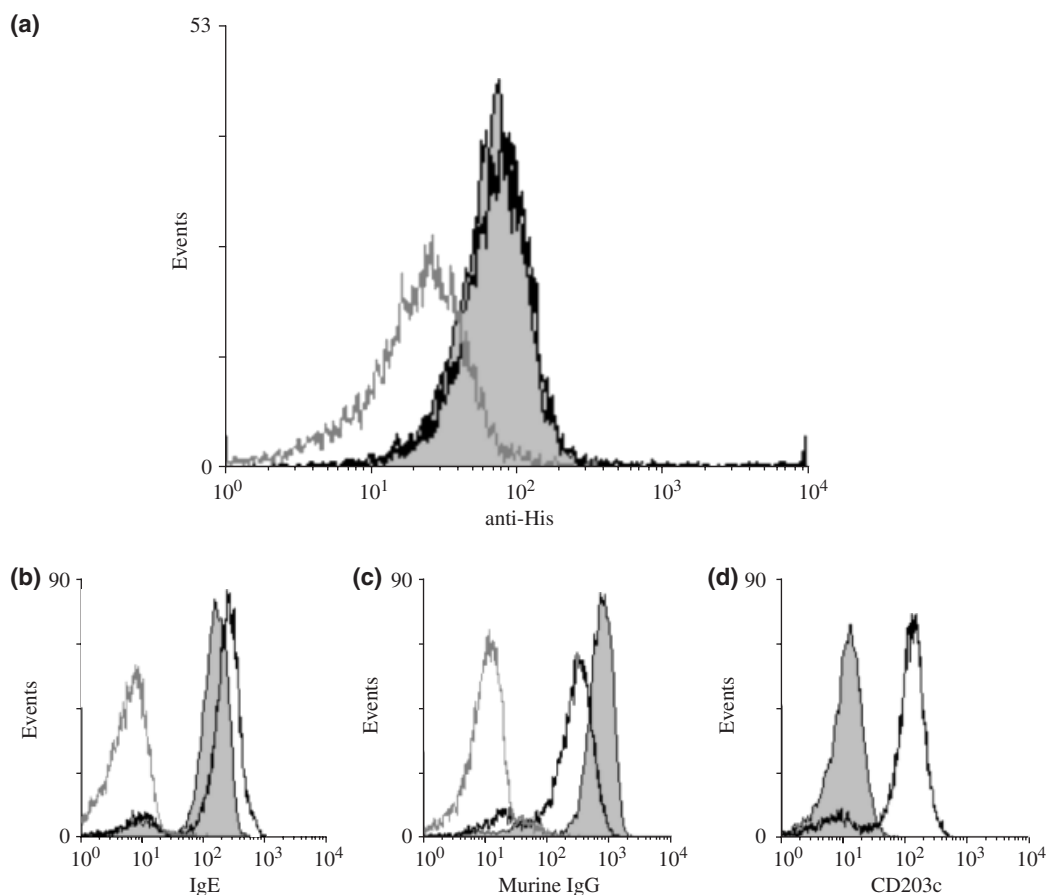


Fig. 7. Human basophils do not internalize bound allergen toxin. Histograms of isolated human basophils of a grass pollen allergic donor. (a) Basophils incubated with 10 $\mu\text{g/mL}$ P5-ETA' and Alexa Fluor 488 conjugated anti-Penta His. Cells fixed before incubation (filled curve) or fixed after incubation (open curve, black) or cells incubated only with anti-Penta His (negative control, open curve, grey). (b) Basophils incubated with Alexa Fluor 488 conjugated anti-IgE. Cells fixed before incubation (filled curve) or fixed after incubation (open curve, black) or cells incubated with anti-Penta His (negative control, open curve, grey). (c) As 7-B, cells incubated with anti-mouse IgG-PE. (d) As 7-B, cells incubated with anti-CD203c-PE.

The resistance of basophils to the AT was quite unexpected after the results for the hybridoma cell lines. Like mast cells, basophils belong to the effector cells of the allergic reaction that accumulate IgE on their surface via Fc ϵ RI. On the one hand the activation of basophils by P5-ETA' has to be kept in mind regarding future therapeutic concepts involving AT in view of the possible risk of anaphylaxis. On the other hand, it is not necessary to eliminate the effector cells to cure allergy, as only B cells and their derivatives produce the allergen-specific IgE, without which no allergen-specific degranulation of mast cells and basophils would occur. Considering that the natural function of basophilic granulocytes is far from being fully understood, their elimination might even bear the risk of severe adverse side-effects.

Effect of allergen toxins on peripheral blood mononuclear cell

When PBMC are incubated with high concentrations of AT, there is an effect on the monocyte population (Fig. 5). While incubation with allergen mediates survival of the

monocytes, P5-ETA' is obviously toxic to the cells. As the monocytes show no significant specific binding of P5-ETA', this toxic effect is likely caused by unspecific uptake of AT. The monocytes seem to clear the AT quite effectively from the medium, at least there was no significant toxic effect of P5-ETA' on the lymphocyte subpopulation. This unspecific toxicity of the ETA' fragment of PEA at very high concentrations is well in line with the results obtained for the hybridoma cell lines with similar concentrations of AT.

The implications of this obviously unspecific uptake of AT by monocytes for therapeutic applications cannot be deduced from the existing data. Allergen specific B cells are the most important target for AT, but they are too scarce in peripheral blood to be detected in experiments as described above. An animal model (mouse) would be a possible solution for testing the impact of P5-ETA' on allergen-specific B cells obtained *ex vivo*.

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References

- Ring J, Kramer U, Schafer T, Behrendt H. Why are allergies increasing? *Curr Opin Immunol* 2001; 13:701–8.
- Nelson HS. Advances in upper airway diseases and allergen immunotherapy. *J Allergy Clin Immunol* 2004; 113:635–42.
- Norman PS. Immunotherapy: 1999–2004. *J Allergy Clin Immunol* 2004; 113:1013–23; quiz 24.
- Valenta R, Vrtala S, Focke-Tejkl M et al. Genetically engineered and synthetic allergen derivatives: candidates for vaccination against type I allergy. *Biol Chem* 1999; 380:815–24.
- FitzGerald DJ, Kreitman R, Wilson W, Squires D, Pastan I. Recombinant immunotoxins for treating cancer. *Int J Med Microbiol* 2004; 293:577–82.
- Schnell R, Borchmann P, Schulz H, Engert A. Current strategies of antibody-based treatment in Hodgkin's disease. *Ann Oncol* 2002; 13 (Suppl. 1):57–66.
- Boyce JA. Mast cells: beyond IgE. *J Allergy Clin Immunol* 2003; 111:24–32; quiz 3.
- Lee BK, Yoo YG, Lee WY, Hong CS, Park JK, Ro JY. Ovalbumin fused with diphtheria toxin protects mice from ovalbumin induced anaphylactic shock. *Yonsei Med J* 2001; 42:91–105.
- Lee BK, Yoo JE, Jang YS, Kim JY, Hong CS, Ro JY. Allergen-specific immunosuppression by ovalbumin fused with diphtheria toxin in mice sensitized with albumins of different origin. *Clin Exp Allergy* 2004; 34:1642–8.
- Stocker M, Klockenbring T, Huhn M et al. Antigen-specific targeting and elimination of EBV-transformed B cells by allergen toxins. *J Allergy Clin Immunol* 2005; 116:910–5.
- Binder RJ, Karimeddini D, Srivastava PK. Adjuvanticity of alpha 2-macroglobulin, an independent ligand for the heat shock protein receptor CD91. *J Immunol* 2001; 166:4968–72.
- Boltansky H, Dyer J, Esworthy S, Kaliner M. IgE-immunotoxins. I. IgE-intact ricin. *Immunopharmacology* 1987; 14:35–45.
- Bruell D, Bruns CJ, Yezhelyev M et al. Recombinant anti-EGFR immunotoxin 425(scFv)-ETA' demonstrates anti-tumor activity against disseminated human pancreatic cancer in nude mice. *Int J Mol Med* 2005; 15:305–13.
- Bruell D, Stöcker M, Huhn M et al. The recombinant anti-EGF receptor immunotoxin 425(scFv)-ETA' suppresses growth of a highly metastatic pancreatic carcinoma cell line. *Int J Oncol* 2003; 23:1179–86.
- Fishman A, Lorberboum-Galski H. Targeted elimination of cells expressing the high-affinity receptor for IgE (Fc epsilon RI) by a Pseudomonas exotoxin-based chimeric protein. *Eur J Immunol* 1997; 27:486–94.
- FitzGerald D, Pastan I. Pseudomonas exotoxin and recombinant immunotoxins derived from it. *Ann NY Acad Sci* 1993; 685:740–5.
- Petersen A, Becker WM, Schlaak M. Epitope analysis of isoforms of the major allergen Phl p V by fingerprinting and microsequencing. *Clin Exp Allergy* 1994; 24:250–6.
- Yelton DE, Diamond BA, Kwan SP, Scharff MD. Fusion of mouse myeloma and spleen cells. *Curr Top Microbiol Immunol* 1978; 81:1–7.
- Matthey B, Engert A, Klimka A, Diehl V, Barth S. A new series of pET-derived vectors for high efficiency expression of Pseudomonas exotoxin-based fusion proteins. *Gene* 1999; 229:145–53.
- Bufe A, Schramm G, Keown MB, Schlaak M, Becker WM. Major allergen Phl p Vb in timothy grass is a novel pollen RNase. *FEBS Lett* 1995; 363:6–12.
- Barth S, Huhn M, Matthey B, Klimka A, Galinski EA, Engert A. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. *Appl Environ Microbiol* 2000; 66:1572–9.
- Kyhse-Andersen J. Electrophoretic blotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984; 10:203–9.
- Haisch K, Schramm G, Falcone FH, Alexander C, Schlaak M, Haas H. A glycoprotein from Schistosoma mansoni eggs binds non-antigen-specific immunoglobulin E and releases interleukin-4 from human basophils. *Parasite Immunol* 2001; 23:427–34.
- Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int Arch Allergy Immunol* 2004; 133:317–29.
- Boumiza R, Monneret G, Forissier MF et al. Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin Exp Allergy* 2003; 33:259–65.
- Iserksy C, Rivera J, Segal DM, Triche T. The fate of IgE bound to rat basophilic leukemia cells. II. Endocytosis of IgE oligomers and effect on receptor turnover. *J Immunol* 1983; 131:388–96.
- Furuichi K, Rivera J, Iserksy C. The fate of IgE bound to rat basophilic leukemia cells. III. Relationship between antigen-induced endocytosis and serotonin release. *J Immunol* 1984; 133:1513–20.
- Pfeiffer JR, Seagrave JC, Davis BH, Deanin GG, Oliver JM. Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. *J Cell Biol* 1985; 101:2145–55.
- Healicon RM, Foreman JC. Characteristics of histamine release from isolated rat mast cells: differences when various ligands are used to induce secretion. *Agents Actions* 1984; 14:365–9.
- Healicon RM, Foreman JC. Rat mast cell activation and inactivation: differences when various ligands are used to induce secretion. *Agents Actions* 1985; 16:155–9.