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Allergological implication of the quaternary hexameric structure of the cockroach allergen Per a 3

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Summary

Background Cockroach allergens play a very important role in allergic diseases, especially asthma. The major allergen of the American cockroach (*Periplaneta americana*), Per a 3, naturally occurs as isoforms of hexamers.

Objective The aim of this study was to investigate whether the hexameric structures of Per a 3 influence their allergenicity and immunogenicity.

Methods Therefore, we compared the different effects of native hexamers and dissociated monomers of cockroach haemolymph (HL), containing almost only Per a 3 proteins (HL-Per a 3), on proliferation and T-helper type 1 (Th1)/Th2 cytokine production of human CD4⁺ T cells in co-culture with allergen-pulsed monocyte-derived autologous dendritic cells (DC) as well as the leukotriene release of basophils.

Results In *P. americana*-sensitized and non-sensitized donors the HL-Per a 3 monomers were internalized faster by immature DC and induced higher proliferation and IFN- γ production than the hexamers. While in non-sensitized donors IL-4 and IL-5 as well as IL-10 production were also increased after stimulation with monomeric HL-Per a 3-pulsed DC, Th2 cytokine and IL-10 production were only enhanced in *P. americana*-sensitized donors using hexameric HL-Per a 3-pulsed DC. Furthermore, in the leukotriene release assay the monomers were less effective than the hexamers.

Conclusion Our data indicate that the quaternary structure can influence both allergenicity and immunogenicity, also depending on the sensitization status. The monomeric variant of Per a 3 allergens could be a possible candidate for a specific immunotherapy because the IgE-mediated allergic reaction and the Th2-inducing capacity are diminished while the Th1-inducing capacity is retained.

Keywords allergy, cockroach, dendritic cells, leukotriene release, Th1/Th2

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Introduction

Cockroaches have been described to trigger allergy and especially asthma in many parts of the world including Taiwan, Japan, United States, Thailand, Singapore, India, South Africa and parts of Europe. The prevalence of cockroach allergy ranges from 6% to 41% in several studies and is associated with low socio-economic status [1–3]. The incidence of asthmatic patients who are sensitized to cockroach allergens ranges from 40% to 81% depending on the geographical location [4, 5]. The most common cockroach species, which are frequently found in

homes, are *Blattella germanica* and *Periplaneta americana* [4, 6].

Over the last 10 years, seven allergens of *B. germanica* (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 6, Bla g 7 and Bla g 8) and seven allergens of *P. americana* (Per a 1, Per a 3, Per a 6 and Per a 7, and later also Per a 2, Per a 4 and Per a 5) have been cloned [1, 7–14]. While IgE cross-reactivity between Bla g 1 and Per a 1 and between the muscle protein tropomyosin from cockroaches (Bla g 7, Per a 7), mites (Der p 10, Der f 10) and crustaceans (Pen a 1, Cha f 1 and Pan s 1) has been identified [15], and further cross-reactivities of Bla g 5, Bla g 6 and Bla g 8 with members of these protein families have to be assumed, no cross-reactivity of Per a 3 to other allergens is known. The major

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allergen Per a 3, which causes IgE antibody responses in 48–94% of patients allergic to cockroaches, was first described by Wu and Lan [16] and includes two isoforms: Per a 3.01 (78 kDa) and Per a 3.0201 (72 kDa). The group from Wu and colleagues has also shown that purified Per a 3 allergens induce lymphocyte proliferation and IL-4 production in cockroach skin-sensitive patients [17]. Per a 3 isoallergens share 20.1–36.4% sequence homology with insect haemolymph (HL) proteins (hexamerins, arylphorins) and arthropod haemocyanins, which are members of the haemocyanin superfamily [18–23]. Like haemocyanins, the native forms of Per a 3 allergens exist as hexamers. In a recent study, it turned out that the subunit types Per a 3.01 and Per a 3.0201 form a hexameric isoform with a molecular mass of 465 kDa. In addition, another isoform with a molecular mass of 512 kDa was found which is composed by a new Per a 3.03 subunit type [23]. Analysis of linear IgE-binding epitopes revealed that four peptides of Per a 3 are recognized by human IgE that are necessary for IgE cross-linking [24]. Besides the presence of at least two identical, conformational epitopes, IgE cross-linking and subsequent mediator release can occur by di- or multimerization, a mechanism that has been described for several allergens, e.g. Bet v 1, Phl p 1, Phl p 5b, Phl p 7, Fel d 1, Api m 4, Ara h 2 and Ves v 5 [25]. Further immunologic consequences of oligomerization have not been analysed so far. The role that the quaternary structure of Per a 3 plays in its allergenicity and immunogenicity is analysed in the following investigation.

Material and methods

Preparation of haemolymph from American cockroach and purification of the two isoforms of Per a 3 allergen

Nymphs and adults of both sexes of the American cockroach (*P. americana*) were obtained from Bayer CropScience (Monheim, Germany). Before HL withdrawal, the cockroaches were anaesthetized with carbon dioxide and then decapitated. Approximately 100 µL HL was collected from each animal with a pipette and immediately diluted 1 : 10 with ice-cold extraction buffer [100 mM TRIS/HCl (pH 7.8), 5 mM CaCl₂, 5 mM MgCl₂ and 0.01% (w/v) phenylthiourea] to prevent coagulation and HL browning. The HL was centrifuged at 4 °C for 10 min at 18.740 g (Universal R16; Hettich Zentrifugen, Tuttlingen, Germany) and the yellowish-turbid supernatant was stored at –32 °C until purification.

A two-step protocol using ion exchange chromatography and size exclusion chromatography was developed to purify the two isoforms of Per a 3 allergen (Per a 3.01/Per a 3.0201 and Per a 3.03) from the HL [23]. First, the HL was diluted with a loading buffer containing 20 mM TRIS/HCl (pH 7.0) with a ratio of 1 : 3 to lower its salt concentration

and allow binding to the column. Then, it was centrifuged for 10 min at 18.740 g and filtered with an Acrodisc syringe filter (pore diameter 0.2 µm; PALL Gelman, Dreieich, Germany) to remove particulate matter. Then, 1 mL of diluted HL was separated by ion exchange chromatography on a fast protein liquid chromatography (FPLC) system at 4 °C using a Bio-Scale Q2 (2 mL; BioRad, Munich, Germany). The bound proteins were eluted with a flow rate of 1 mL/min in a gradient with a high salt elution buffer containing 20 mM TRIS/HCl and 1 M NaCl (pH 7.0) at 4 °C, and elution was monitored by their absorption at 280 nm. To improve purification, fractions of the two isoforms of Per a 3 were concentrated in centrifugal filters (Vivaspin 20; 30 000 Mwco; Omnilab, Bremen Germany) and chromatographed by size exclusion chromatography using an S-300 16/60 column (Pharmacia, Erlangen, Germany) at a flow rate of 0.5 mL/min. For this purification step, stabilization buffer containing 0.1 M TRIS/HCl, 5 mM CaCl₂ and 5 mM MgCl₂ (pH 7.3) was used.

Dissociation of haemolymph extract

One millilitre of HL was diluted with a ratio of 1 : 201 with dissociation buffer containing 50 mM glycine, 20 mM EDTA and 2.5 M urea (pH 10.5) and incubated at room temperature for 1 week. Then, it was concentrated in centrifugal filters (Vivaspin 20, 30000 Mwco; Omnilab) and eluted in phosphate-buffered saline (PBS). The success and stability of dissociation was verified with sedimentation runs in an analytical ultracentrifuge (Beckman Optima XL-I, Palo Alto, CA, USA), and by separation on a native PAGE with following Western blot experiments.

Blood samples

Heparinized or EDTA-blood was obtained from atopic donors who were sensitized to *P. americana* or from healthy controls. Specific sensitization was documented by allergen-specific IgE in the sera (CAP class ≥ 2 measured with the ImmunoCAP[®]-specific IgE blood test, Phadia AB, Uppsala, Sweden). This study was approved by the local ethics committee. Informed consent was obtained from all subjects before the study.

Generation of monocyte-derived dendritic cells

PBMC were isolated from heparinized blood by Ficoll-Paque 1.077 g/mL (PAA Laboratories GmbH, Cölbe, Germany) density centrifugation. To enrich CD14⁺ monocytes, 1×10^7 PBMC per well were incubated for 45 min in a six-well-plate (Greiner, Frickenhausen, Germany) in Iscove's modified Dulbecco's medium containing L-glutamine and 25 mM Hepes (IMDM, PAA Laboratories GmbH) supplemented with an antibiotic-antimycotic solution containing 100 µg/mL streptomycin, 100 U/mL penicillin

and 250 ng/mL amphotericin B (PAA) and 3% autologous plasma at 37 °C. After washing the non-adherent cells with pre-warmed PBS, the remaining monocytes (purity >90%) were incubated in 3 mL/well IMDM supplemented with 1% heat-inactivated autologous plasma, 1000 U/mL IL-4 (Strathmann Biotech GmbH, Hannover, Germany) and 200 U/mL GM-CSF (Leukine[®], Immunex Corp., Seattle, WA, USA). Cells were fed with fresh medium every other day. On day 6, the resulting immature dendritic cells (DC) were pulsed with different amounts of monomeric or hexameric HL-Per a 3 as indicated, or 1 µg/mL tetanus toxoid (Behring-Werke, Marburg, Germany), and further stimulated with 1000 U/mL TNF- α , 2000 U/mL IL-1 β (Strathmann Biotech GmbH) and 1 µg/mL PGE₂ (Minprostin[®] E₂; Pharmacia & Upjohn GmbH, Erlangen, Germany) to induce their full maturation. Mature DC were harvested 48 h after stimulation, washed twice and used for T cell stimulation assays. Mature DC expressed high levels (>90%) of CD80, CD83, CD86 and MHC-class II molecules as controlled by flow cytometry.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMC using antibody-coated paramagnetic MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. Separation was controlled by flow cytometry (purity >98%).

Coculture of T cells and autologous allergen-pulsed dendritic cells

For proliferation assays, 1×10^5 CD4⁺ T cells were co-cultured in 96-well plates (Greiner) in triplicate with 1×10^4 autologous allergen-pulsed DC in 200 µL IMDM supplemented with 5% heat-inactivated autologous plasma. After 5 days, the cells were pulsed with 37 kBq/well of [³H]TdR ([methyl-³H]thymidine, ICN, Irvine, CA) for 6 h, and [³H]TdR incorporation was evaluated in a beta counter (1205 Betaplate, LKB Wallac, Turku, Finland).

For cytokine production assays, 5×10^5 CD4⁺ T cells were cultured in 48-well plates in the presence of 5×10^4 autologous allergen-pulsed DC in 1 mL IMDM supplemented with 5% heat-inactivated autologous plasma. After 1 week of culture, T cells were re-stimulated with newly generated allergen-pulsed DC (5×10^4), and supernatants were collected 24 h later.

Quantification of cytokine production by an enzyme-linked immunosorbent assay

Human IL-4, IL-5, IL-10 and IFN- γ were measured by ELISA according to the instructions of the distributors of the pairs of antibodies used (BD Biosciences, Heidelberg,

Germany). The detection limit was 8 pg/mL for IL-4 and 32 pg/mL for all other cytokines.

Determination of leukotriene release

Dextran (250 µL) was added to 1 mL EDTA-blood and incubated for 90 min at room temperature to allow sedimentation of erythrocytes. Then, peripheral blood leucocytes (PBL) were centrifuged at 130 g for 15 min and re-suspended in 1 mL stimulation buffer containing IL-3. Two-hundred microlitres of PBL were stimulated with 50 µL allergen (monomeric or hexameric HL-Per a 3) or anti-IgE receptor antibody for 40 min at 37 °C. Finally, the cells were centrifuged and supernatants were analysed for leukotriene concentration by CAST[®]-2000 ELISA according to the instructions of the manufacturer (Bühlmann Laboratories AG, Schönenbuch, Switzerland).

Flow cytometry analysis and confocal laser microscopy

Monomeric and hexameric HL-Per a 3 was labelled with the Alexa Fluor[®] 488 Protein Labelling Kit according to the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). The degree of labelling (moles dye per mole protein) was assessed by measurement of the absorbance at 280 and 494 nm and was identical for the monomeric and hexameric HL-Per a 3. Labelled allergen (10 µg/mL) was added to immature DC on day 6 of culture and internalization was analysed after 5, 30, 60 and 120 min in a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) equipped with CELLQuest Software. For confocal laser microscopy, DC were labelled with 60 nM LysoTracker Red DND-99 (Molecular Probes) 10 min before allergen addition. Then, internalization of labelled allergen was filmed for 1 h using the Zeiss Laser Scanning System LSM 510 (Zeiss, Jena Germany) equipped with a CO₂ and temperature-controlled chamber (H. Saur, Reutlingen, Germany).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot

HL, Per a 3.01/3.0201 or 3.03 protein (5 µg/lane) were mixed with loading buffer (Roti Load, 4 × concentrated, Roth, Karlsruhe, Germany), heated for 5 min at 96 °C and subjected to SDS-PAGE on a 7.5% polyacrylamide gel containing 1% SDS at 200 V. A pre-stained protein standard (Bio-Rad Laboratories, Hercules, CA, USA) was run in parallel for quantification of the molecular weights. Proteins were stained overnight with Roti[®]-Blue colloidal coomassie solution (Roth). For native PAGE, 20 µg of native or dissociated HL were separated on a 7.5% polyacrylamide gel and stained overnight with coomassie blue (Roth).

Proteins were blotted onto a 0.2 µm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using

a semidry blotting unit (Trans-Blot SD; Bio-Rad Laboratories) in a Roti[®]-Blot anode and a cathode buffer (Roth) for 1.5 h at 0.8 mA/cm². After transfer, the membranes were blocked in blocking buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk powder) for a minimum of 1 h. For detection, the membranes were incubated with anti-Per a 3.01/anti-Per a 3.0201 or anti-Per a 3.03 polyclonal rabbit antisera diluted 1 : 2000 in TBS/1% milk powder for 2 h and with AP-conjugated secondary antibodies (Sigma-Aldrich, Taufkirchen, Germany) at a dilution of 1 : 5000 in TBS/1% milk powder for 1 h. Blots were developed using BCIP (5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt) and NBT (nitro blue tetrazolium) substrate buffer and scanned with CanoScan 8000F (Canon Deutschland GmbH, Krefeld, Germany).

Statistics

Student's *t*-test was used to test the statistical significance of the results; $P \leq 0.05$ was considered to be significant.

Results

The haemolymph of the American cockroach mainly consists of the major allergen Per a 3

The major allergen Per a 3 of the American cockroach, which is known to appear as a hexamer in nature [18, 23], can be isolated from HL. However, during purification of the hexameric isoforms of Per a 3, consisting of the subunit types Per a 3.01/Per a.30201 and of the subunit type Per a 3.03, and subsequent dissociation into their monomers, considerable protein is lost. SDS-PAGE and Western blot data revealed that the HL mainly consists of Per a 3.01/Per a 3.0201 and Per a 3.03 (Figs 1a and b). It should be noted that the subunit types Per a 3.01 and Per a 3.0201 overlap in an SDS-PAGE yielding one band [23]. Therefore, we performed dissociation of the native hexamer into monomers and all further immunological experiments with the HL calling it HL-Per a 3. Successful and stable dissociation was confirmed by sedimentation runs and by blue native PAGE, which revealed that the strong hexameric band detected in the HL was no longer present (Fig. 1c).

*The monomeric haemolymph-Per a 3 induces higher proliferation than the hexameric native form in *Periplaneta americana*-sensitized and non-sensitized donors*

To analyse the immunogenic capacity of monomeric and hexameric Per a 3 allergens, monocyte-derived DC from *P. americana*-sensitized donors or healthy controls were pulsed with different concentrations of the monomeric and hexameric HL-Per a 3 or with tetanus toxoid as the control at an optimal dose of 1 µg/mL during their maturation phase. Two days later, autologous CD4⁺ T cells were

stimulated with these allergen-pulsed DC and proliferation was measured after 5 days of culture. In Fig. 2, it is shown that both allergens, monomeric as well as hexameric HL-Per a 3, induced strong proliferative responses in CD4⁺ T cells compared with the antigen tetanus toxoid. Proliferation was stronger after stimulation with monomeric HL-Per a 3-pulsed DC compared with DC pulsed with the hexameric native form. This higher stimulatory capacity of monomeric HL-Per a 3-pulsed DC could be observed at almost all concentrations used for DC pulse. There were no significant differences in DC-induced T cell proliferation between *P. americana*-sensitized and non-sensitized donors.

*In healthy non-sensitized donors, the monomeric haemolymph-Per a 3 enhances the production of all cytokines, but in *Periplaneta americana*-sensitized donors it only enhances the production of the T-helper type 1 cytokine interferon-γ, while the T-helper type 2 cytokines interleukin-4 and interleukin-5 and interleukin-10 were enhanced by the hexameric haemolymph-Per a 3*

For measurement of IL-10 as well as Th1 (IFN-γ) and Th2 (IL-4, IL-5) cytokine production, co-cultures of allergen-pulsed DC and CD4⁺ T cells were re-stimulated after 1 week with newly generated allergen-pulsed DC, and supernatants were collected 24 h later. DC pulsed with the monomeric HL-Per a 3 induced a stronger production of IFN-γ, IL-4, IL-5 and IL-10 in CD4⁺ T cells from healthy controls, which was not significant for IL-5, and a stronger IFN-γ production in T cells from *P. americana*-sensitized donors than DC pulsed with the hexameric native HL-Per a 3 (Fig. 3). Overall, IL-4 production induced by monomeric or hexameric HL-Per a 3 was stronger in *P. americana*-sensitized compared with non-sensitized donors. In contrast to non-sensitized donors, IL-4, IL-5 and IL-10 production of CD4⁺ T cells from the sensitized group were slightly enhanced after stimulation with hexameric HL-Per a 3-pulsed DC compared with monomeric HL-Per a 3-pulsed DC (Fig. 3).

Monomeric haemolymph-Per a 3 is internalized faster by immature dendritic cells than the hexameric form

To analyse whether there are differences concerning internalization of the native hexameric and the dissociated monomeric form of HL-Per a 3, the two proteins were labelled with Alexa Fluor[®] 488 and added to immature DC. In Fig. 4a, it is shown that the mean fluorescence intensity of internalized monomeric HL-Per a 3 was significantly increased compared with hexameric HL-Per a 3 already one hour after addition (Fig. 4a). After 24 h, the mean fluorescence intensity of the monomeric allergen still remained significantly elevated (data not shown). The faster and stronger uptake of monomeric HL-Per a 3 labelled green first close to the surface

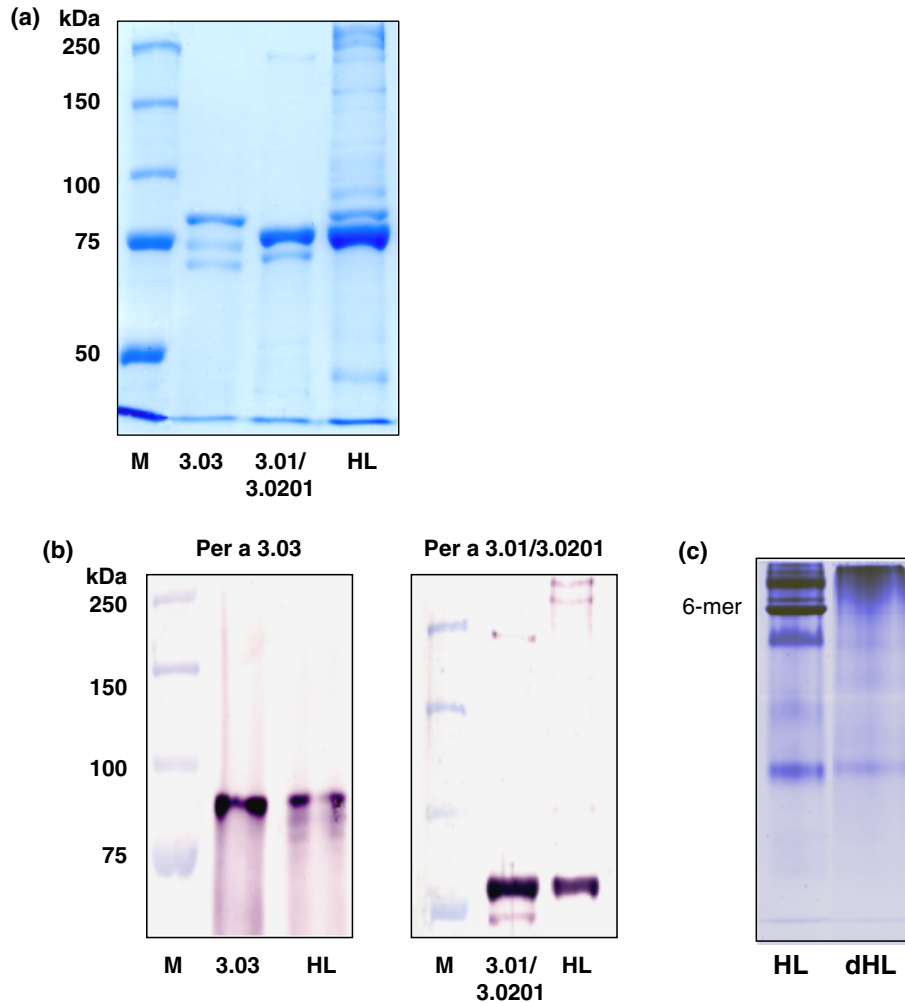


Fig. 1. The haemolymph of the American cockroach is mainly composed of the major allergen Per a 3. (a) Five microgram of haemolymph (HL) or Per a 3 allergens consisting of the hexameric isoforms Per a 3.01/Per a 3.0201 and Per a 3.03 were subjected to SDS-PAGE on a 7.5% polyacrylamide gel and stained overnight with coomassie blue or (b) analysed for Per a 3.01/Per a 3.0201 or 3.03 expression by Western blot as described in 'Materials and methods'. (c) 20 µg of native HL or dissociated HL (dHL) were separated on a 7.5% polyacrylamide gel by native PAGE and stained overnight with coomassie blue.

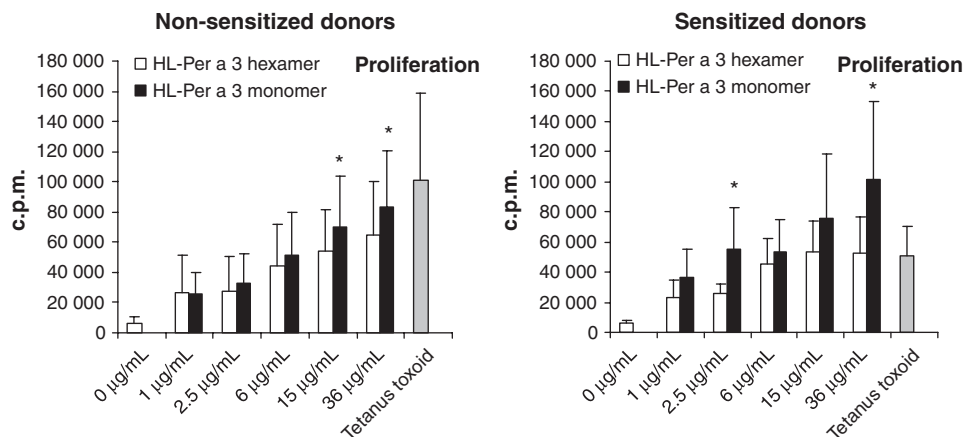


Fig. 2. Enhanced proliferation of CD4⁺ T cells after stimulation with monomeric compared with hexameric HL-Per a 3-pulsed DC. 1×10^5 CD4⁺ T cells were co-cultured with 1×10^4 autologous mature DC, which were pulsed with different concentrations of monomeric or hexameric HL-Per a 3. After 5 days of culture, proliferation was assessed by [³H]-thymidine incorporation. The mean $\pm$ SD from 7 *Periplaneta americana*-sensitized and 11 healthy donors are shown. *Statistically significant differences between monomeric and hexameric HL-Per a 3-pulsed DC, $P \leq 0.05$. HL, haemolymph; DC, dendritic cells.

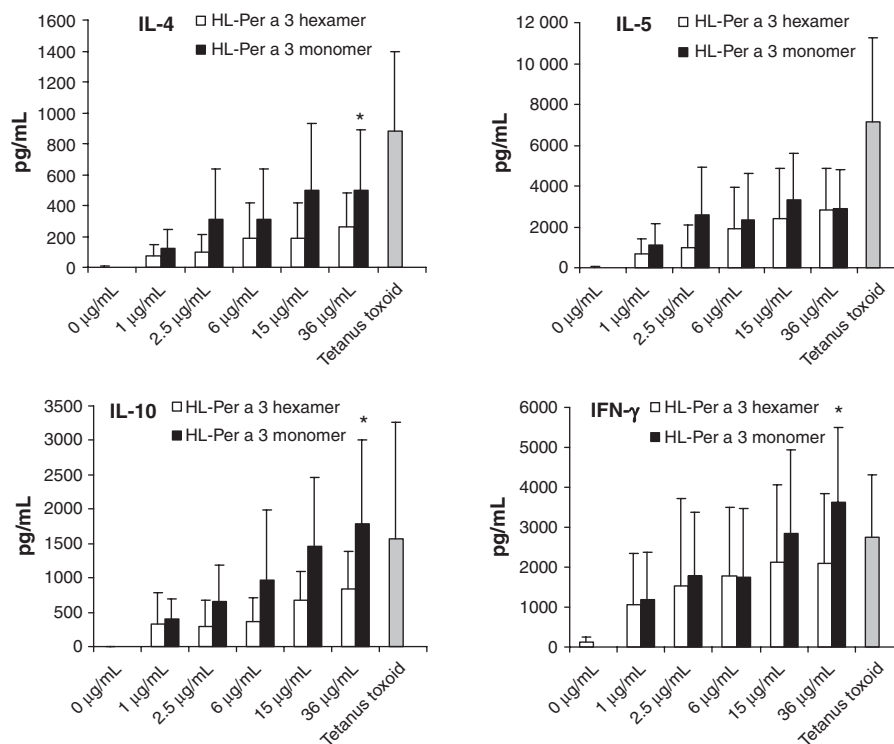
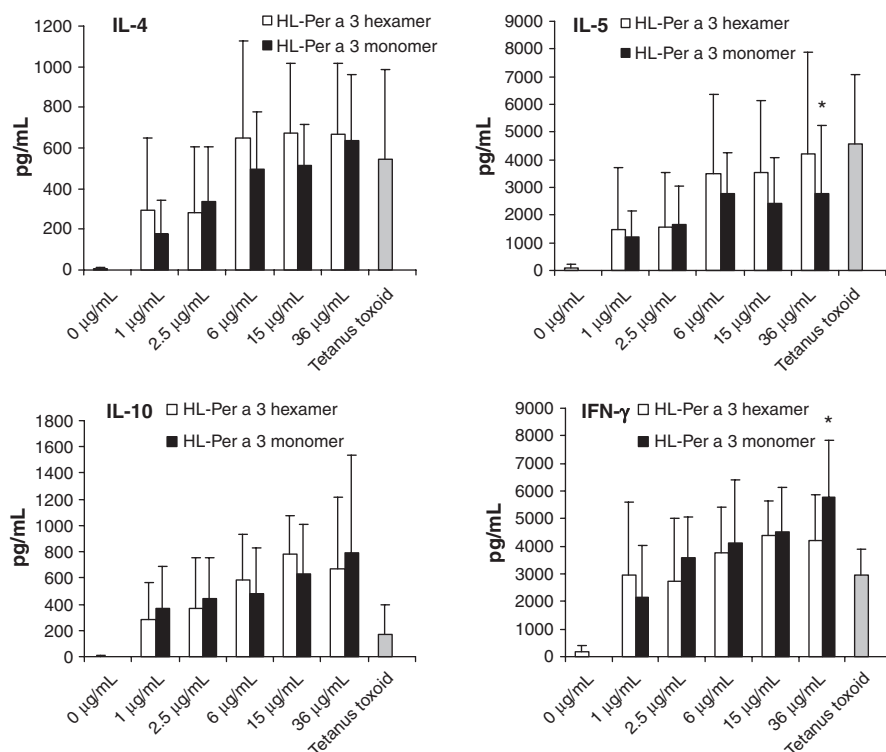
(a) Non-sensitized donors**(b) Sensitized donors**

Fig. 3. Enhanced Th1 but not Th2 cytokine production of CD4⁺ T cells after stimulation with monomeric compared with hexameric HL-Per a 3-pulsed DC in sensitized donors. 5×10^5 CD4⁺ T cells were co-cultured with 5×10^4 autologous mature DC that were pulsed with different concentrations of monomeric or hexameric HL-Per a 3. After 1 week of culture, T cells were re-stimulated with newly generated 5×10^4 allergen-pulsed DC and cytokine content was assessed by ELISA 24 h later. The mean $\pm$ SD from 8 *Periplaneta americana*-sensitized and nine healthy donors are shown. *Statistically significant differences between monomeric and hexameric HL-Per a 3-pulsed DC, $P \leq 0.05$. HL, haemolymph; DC, dendritic cells.

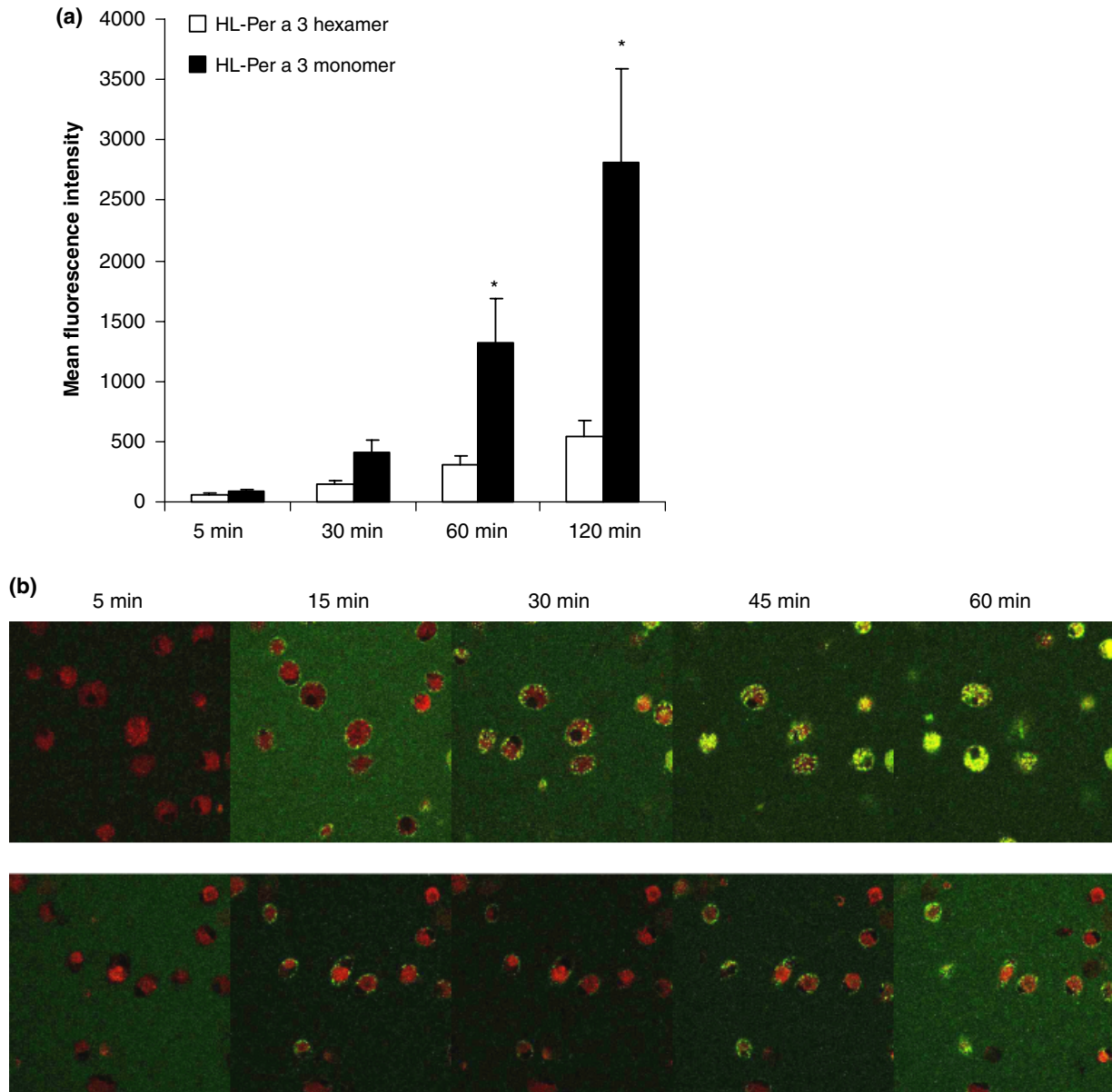


Fig. 4. Faster internalization of monomeric compared with hexameric HL-Per a 3 by immature DC. (a) Alexa Fluor[®] 488-labelled monomeric or hexameric HL-Per a 3 (10 µg/mL) was added to immature DC on day 6 of culture, and internalization of labelled allergen was analysed by flow cytometry after 5, 30, 60 and 120 min. The mean $\pm$ SD from four healthy donors are shown. *Statistically significant differences between monomeric and hexameric HL-Per a 3-pulsed DC, $P \leq 0.05$. (b) Immature DC were labelled with 60 nM LysoTracker Red DND-99 on day 6 of culture, and Alexa Fluor[®] 488-labelled monomeric (upper panel) or hexameric HL-Per a 3 (lower panel) was added 10 min later. Internalization of labelled allergens was filmed over 1 h. A sequential view after 5, 15, 30, 45 and 60 min is shown. HL, haemolymph; DC, dendritic cells.

membrane of the DC and later into their lysosomes labelled red (resulting in a yellow colour) was confirmed by confocal laser microscopy (Fig. 4b).

Hexameric haemolymph-Per a 3 induces a stronger leukotriene release from basophils than the monomeric form

To investigate the allergenicity of the monomeric and hexameric HL-Per a 3, PBL from *P. americana*-sensitized

donors were stimulated with the respective allergens and leukotriene release from the basophils was measured. In Fig. 5, it is shown that both allergen forms induced a leukotriene release almost as high as the release observed after stimulation with the positive control anti-IgER. Owing to the high variability of the leukotriene releases among sensitized donors, representative results are shown (and not means). Overall, hexameric HL-Per a 3 induced a stronger leukotriene release than monomeric HL-Per a 3. In non-sensitized

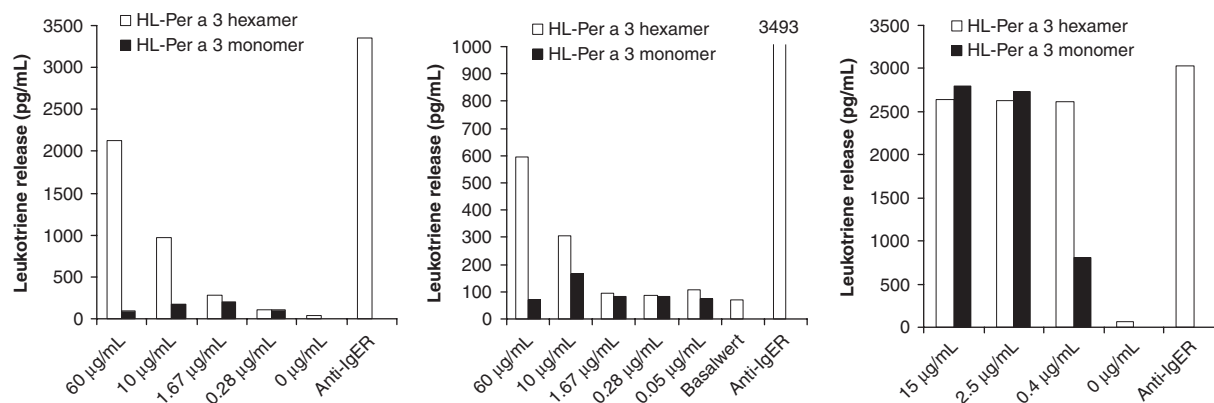


Fig. 5. Enhanced leukotriene release of effector cells after stimulation with hexameric compared with monomeric HL-Per a 3. PBL from *Periplaneta americana*-sensitized donors were stimulated with different concentrations of monomeric or hexameric HL-Per a 3 as described in 'Materials and methods', and the supernatants were analysed for leukotriene release by ELISA after 40 min. Three representative experiments out of 12 independent experiments are shown. HL, haemolymph; PBL, peripheral blood leucocytes.

donors, no induction of leukotrienes after stimulation with HL-Per a 3 was observed.

Discussion

The data presented indicate that the quaternary structure of HL-Per a 3 allergen plays an important role in its allergenicity and immunogenicity. In T cells from non-sensitized individuals, the proliferation and production of all cytokines were enhanced after stimulation with monomeric compared with hexameric HL-Per a 3-pulsed DC. However, in CD4⁺ T cells from *P. americana*-sensitized donors, DC pulsed with the monomeric HL-Per a 3 induced significantly higher proliferation and Th1 cytokine production but less Th2 cytokine and IL-10 production compared with DC pulsed with the hexameric native HL-Per a 3. Additionally, the hexameric HL-Per a 3 was more potent for stimulation of effector cells than the monomeric form.

The fact that the proliferative responses of CD4⁺ T cells were enhanced after stimulation with monomeric compared with hexameric HL-Per a 3-pulsed DC can be explained by the observed faster internalization and presentation of the smaller monomeric form of HL extract by DC. Like haemocyanins, the hexameric HL-Per a 3 molecules might aggregate further, additionally complicating internalization [18, 26, 27]. Another reason could be that, due to its high stability (temperature, pH, and enzymatic activity), the hexameric HL-Per a 3 may be not sufficiently processed in the lysosomes of DC.

Our results concerning better stimulation of basophils by the hexameric HL-Per a 3 are in line with a recent report from Schöll et al., who have investigated whether dimerization or oligomerization of the major birch pollen allergen Bet v 1 influences IgE cross-linking and subsequent mediator release from mast cells and B lymphocytes. In skin tests with Bet v 1-sensitized mice, the

authors demonstrated that only dimeric Bet v 1 elicited positive reactions at the application sites while skin tests with monomeric Bet v 1 remained negative [25]. In another study, Gieras et al. [28] have also shown that only polymeric Phl p 1-derived peptide, P1, caused effector cell degranulation while monomeric P1 did not. Thus, the dissociated structure of an allergen does not seem to activate basophils or B cells sufficiently to induce IgE secretion or mediator release like histamine and leukotrienes. This may be exploited for immunotherapeutic approaches. Furthermore, Th2 cells are less stimulated by monomeric compared with hexameric HL-Per a 3 at least in T cells from *P. americana*-sensitized donors, suggesting a stimulatory bias in favour of a Th1 immune response. In this respect, the ratio IFN- γ : IL-4 was enhanced in T cells from all donors after co-culture with monomeric instead of hexameric HL-Per a 3-pulsed DC. Similar results concerning the importance of the structural features of an antigen were obtained by other groups: Akdis et al. [29] have shown that only correctly folded PLA molecules were recognized by antigen-presenting cells and could induce stimulation of Th2-type cytokines and IgE antibodies, and Korematsu et al. [30] have demonstrated that exclusive Th1 cell differentiation was induced by a mutant protein of the major mite allergen Der f 2, C8/119S, which has lost its characteristic β -barrel structure. In this respect, it has been shown recently that destruction of native conformation rather than oligomerization is important for reducing allergenicity [31]. However, genetically engineered recombinant derivatives of Bet v 1 (rBet v 1 fragments and trimer) showed reduced allergenic activity by inducing blocking IgG antibodies and have been successfully implicated in preliminary clinical vaccination studies [32, 33]. This leads to the assumption that oligomerization induced by synthetic peptide chemistry completely differs from naturally occurring three-dimensional structures. The reason for this difference may be

the presence of IgE antibodies that recognize the oligomeric protein that occurs naturally, but not synthetic constructs.

Taken together, immunotherapy with peptides containing T cell or B cell epitopes is a generally applicable strategy to reduce anaphylactic side-effects in future. Further knowledge about the structure of proteins, e.g. the quaternary structure of the major cockroach allergen Per a 3, will lead to improved understanding of its importance for induction and elicitation of allergic reactions as well its implication for immunotherapy [34, 35].

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References

- Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomes A, Chapman MD. Cockroach allergens and asthma. *J Allergy Clin Immunol* 2001; **107**:419–28.
- Sarpong SB, Hamilton RG, Eggleston PA, Adkinson NF Jr. Socio-economic status and race as risk factors for cockroach allergen exposure and sensitization in children with asthma. *J Allergy Clin Immunol* 1996; **97**:1393–401.
- Kang B, Vellody D, Homburger H, Yunginger JW. Cockroach cause of allergic asthma. Its specificity and immunologic profile. *J Allergy Clin Immunol* 1979; **63**:80–6.
- Helm RM, Pomes A. Cockroach and other inhalant insect allergens. *Clin Allergy Immunol* 2004; **18**:271–96.
- Gruchalla RS, Pongracic J, Plaut M *et al.* Inner city asthma study: relationships among sensitivity, allergen exposure, and asthma morbidity. *J Allergy Clin Immunol* 2005; **115**:478–85.
- Katial RK. Cockroach allergy. *Immunol Allergy Clin North Am* 2003; **23**:483–99.
- Chapman MD, Vailes LD, Hayden ML, Benjamin DC, Platts-Mills TA, Arruda LK. Structural and antigenic studies of cockroach allergens and their relevance to asthma. *Adv Exp Med Biol* 1996; **409**:95–101.
- Wu CH, Lee MF. Molecular characteristics of cockroach allergens. *Cell Mol Immunol* 2005; **2**:177–80.
- Chew FT, Shang HS, Pan QR. Cloning of group 2 allergen, aspartic protease precursor from American cockroach, *Periplaneta americana*. 2006. Unpublished AAX33727.
- Chew FT, Shang HS, Pan QR. Cloning of group 4 allergen, calycin from American cockroach, *Periplaneta americana*. 2006. Unpublished AAX33728.
- Chew FT, Shang HS, Pan QR. Cloning of group 5 allergen, GST homolog allergen from American cockroach, *Periplaneta americana*. 2006. Unpublished AAX33729.
- Chew FT, Shang HS, Pan QR. Direct submission (Per a 6 allergen). 2006. Unpublished AAX33730.
- Hindley J, Pomes A. Bla g 8: a calcium binding German cockroach allergen. 2006. Unpublished ABD47458.
- Hindley J, Wünschmann S, Satinover SM *et al.* Bla g 6: a troponin C allergen from *Blattella germanica* with IgE binding calcium dependence. *J Allergy Clin Immunol* 2006; **117**:1389–95.
- Santos AB, Chapman MD, Aalberse RC *et al.* Cockroach allergens and asthma in Brazil: identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. *J Allergy Clin Immunol* 1999; **104**:329–37.
- Wu CH, Lan JL. Cockroach hypersensitivity: isolation and partial characterization of major allergens. *J Allergy Clin Immunol* 1988; **82**:727–35.
- Jeng KC, Liu MT, Wu CH, Wong DW, Lan JL. American cockroach Cr-PI allergen induces lymphocyte proliferation and cytokine production in atopic patients. *Clin Exp Allergy* 1996; **26**:349–56.
- Wu CH, Lee MF, Liao SC, Luo SF. Sequencing analysis of cDNA clones encoding the American cockroach Cr-PI allergens. Homology with insect hemolymph proteins. *J Biol Chem* 1996; **271**:17937–43.
- Burmester T. Origin and evolution of arthropod hemocyanins and related proteins. *J Comp Physiol [B]* 2002; **172**:95–107.
- Wu CH, Lee MF, Wang NM, Luo SF. Sequencing and immunochemical characterization of the American cockroach per a 3 (Cr-PI) isoallergenic variants. *Mol Immunol* 1997; **34**:1–8.
- Telfer WH, Keim PS, Law JH. Arylphorin, a new protein from *Hyalophora cecropia*: comparisons with calliphorin and manducin. *Insect Biochem* 1983; **13**:601–13.
- Telfer WH, Kunkel JG. The function and evolution of insect storage hexamers. *Annu Rev Entomol* 1991; **36**:205–28.
- Lafargue B, Jaenicke E, Tenzer S *et al.* Cockroach allergens Per a 3 are oligomers. *J Biol Chem* 2007, submitted.
- Wu CH, Lee MF, Tseng CY. IgE-binding epitopes of the American cockroach Per a 3 allergen. *Allergy* 2003; **58**:986–92.
- Schöll I, Kalkura N, Shedziankova Y *et al.* Dimerization of the major birch pollen allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice. *J Immunol* 2005; **175**:6645–50.
- Lamkemeyer T, Zeis B, Decker H *et al.* Molecular mass of macromolecules and subunits and the quaternary structure of hemoglobin from the microcrustacean *Daphnia magna*. *FEBS J* 2006; **273**:3393–410.
- Averdam A, Markl J, Burmester T. Subunit sequences of the 4 × 6-mer hemocyanin from the golden orb-web spider, *Nephila inaurata*. *Eur J Biochem* 2003; **270**:3432–9.
- Gieras A, Focke-Tejkl M, Ball T *et al.* Molecular determinants of allergen-induced effector cell Degranulation. *J Allergy Clin Immunol* 2007; **119**:384–90.
- Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. *Eur J Immunol* 1998; **28**:914–25.
- Korematsu S, Tanaka Y, Hosoi S *et al.* C8/119S mutation of major mite allergen Derf-2 leads to degenerate secondary structure and molecular polymerization and induces potent and exclusive Th1 cell differentiation. *J Immunol* 2000; **165**:2895–902.
- Reese G, Ballmer-Weber BK, Wangorsch A, Randow S, Vieths S. Allergenicity and antigenicity of wild-type and mutant, monomeric, and dimeric carrot major allergen Dau c 1: destruction of

- conformation, not oligomerization, is the roadmap to save allergen vaccines. *J Allergy Clin Immunol* 2007; 119:944–51.
- 32 Vrtala S, Hirtenlehner K, Susani M *et al*. Genetic engineering of recombinant hypoallergenic oligomers of the major birch pollen allergen, Bet v 1: candidates for specific immunotherapy. *Int Arch Allergy Immunol* 1999; 118:218–9.
 - 33 Niederberger V, Horak F, Vrtala S *et al*. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004; 101 (Suppl. 2): 14677–82.
 - 34 Norman PS, Ohman JL Jr, Long AA *et al*. Treatment of cat allergy with T-cell reactive peptides. *Am J Respir Crit Care Med* 1996; 154:1623–8.
 - 35 Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006; 6:761–71.