

ORIGINAL ARTICLE

Engineering of major house dust mite allergens Der p 1 and Der p 2 for allergen-specific immunotherapy

J. A. Asturias*, I. Ibarrola*, M. C. Arilla*, C. Vidal†, A. Ferrer‡, P. M. Gamboa§, J. Viñuela¶, M. L. Sanz||, C. Andreu‡ and A. Martínez*

*Bial-Aristegui, Research & Development Department, Bilbao, Spain, †Servicio de Alergología, Hospital de Conxo, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago, Chile, ‡Servicio de Alergia, Hospital 'Vega Baja' de Orihuela, Alicante, Spain, §Servicio de Alergia, Hospital de Basurto, Bilbao, Spain,

¶Laboratorio de Inmunología, Hospital de Conxo, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago, Chile and ||Departamento de Alergología e Inmunología Clínica, Clínica Universitaria de Navarra, Pamplona, Spain

Clinical & Experimental Allergy

Summary

Background Specifically designed recombinant allergens with reduced IgE reactivity are promising candidates for a more defined, effective, and safer specific immunotherapy (SIT).

Objective We sought to obtain hypoallergenic hybrid molecules which could potentially be applied to house dust mite (HDM) allergy treatment.

Methods Two hybrid molecules (QM1 and QM2) derived from the two major *Dermatophagoides pteronyssinus* allergens, Der p 1 and Der p 2, were engineered by PCR, produced in *Escherichia coli*, and purified. The overall IgE-binding capacity of the hybrids was compared with their single components by Western blot, specific IgE, skin prick test (SPT), and IgE-inhibition assays. T cell proliferation assay were performed to confirm their retention of T cell reactivity. Immune responses to the hybrid molecules were studied in BALB/c mice.

Results The IgE reactivity of both hybrid proteins was strongly reduced as evaluated by *in vitro* methods. Furthermore, *in vivo* SPTs performed on 106 HDM-allergic patients showed that the hybrid proteins had a significantly lower potency to induce cutaneous reactions than the individual components. Hybrid molecules induced higher T cell proliferation responses than those produced by an equimolecular mixture of Der p 1 and Der p 2. Immunization of mice with the hybrid proteins induced Der p 1- and Der p 2-specific IgG, which inhibited the binding of allergic patients' IgE to these natural allergens.

Conclusion QM1 and QM2 hybrids exhibited less IgE-binding activity but preserved immunogenicity and fulfilled the basic requirements for hypoallergenic molecules suitable for a future SIT of HDM allergy.

Keywords blocking antibodies, genetic engineering, house dust mite allergy, hybrid proteins, IgE epitope, recombinant hypoallergens

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Correspondence:

Dr Juan A. Asturias, Bial-Aristegui, Research & Development Department, Alameda Urquijo, 27, 48008-Bilbao, Spain. E-mail: juan.asturias@bial.com

Introduction

IgE-mediated hypersensitivity affects almost 25% of the population in industrialized countries. Although allergic symptoms can be mitigated using anti-inflammatory drugs and immunosuppressive agents, specific immunotherapy (SIT) is the only allergen-specific approach for the treatment of type I allergy and for preventing its progression to severe disease manifestations [1]. Successful clinical outcomes are well documented but several problems can be associated with allergen extract-based SIT, including difficulties on extract standardization, the risk of inducing life-threatening anaphylactic side-effects

and induction of new IgE sensitizations to proteins in allergen vaccines [2, 3]. Therefore, despite being used in clinical practice since early last century, new approaches for successful and safer SIT are required. The use of recombinant allergens can overcome many of these problems and presents the advantage of making possible the production of genetically modified molecules. These molecules could contain reduced IgE-binding epitopes (hypoallergens), while preserving essential motifs for T cell recognition and for induction of protective antibody responses [4, 5]. Native allergens cross-link IgE and induce mast cell and basophil degranulation. Their uptake by antigen-presenting cells (APCs) (i.e. specific B cells) is

mediated and facilitated by the interaction of conformational epitopes with specific IgE and leads to higher Th2 cytokines and IgE production [1]. In contrast, modified allergens that lack IgE binding or effector cell degranulation capacities might avoid these pathways, while preferentially targeting APCs that utilize phagocytosis or pinocytosis for antigen uptake (i.e. monocytes, macrophages, and dendritic cells) independently of the allergen structural features [1, 6]. This mechanism induces a balanced Th0- or Th1-like cytokine production by T cells and lower IgE and higher IgG production by B cells [6, 7].

House dust mite (HDM) allergy is one of the most common allergies world-wide which affects more than 50% of allergic patients [8] with *Dermatophagoides pteronyssinus* (DPT) and *D. farinae* being the most important sources of allergens in house dust in Europe and the United States. Examination of DPT extracts has indicated that over 30 different proteins can induce IgE antibody in HDM-allergic patients and 21 of these allergens are described and show cross-reactivity with allergens from other mite species (<http://www.allergen.org>) [9]. Der p 1 and Der p 2 represent the most important allergens among HDM allergens because they are recognized by the vast majority of HDM-allergic patients and 50–100% of IgE reactivity to the whole DPT extract was directed towards these allergens [10–13]. Der p 1 is a glycosylated cysteine protease of 25 000 Da containing three disulphide bridges which form covalent bonds between residues C4–C117, C31–C71, and C65–C103. The crystal structure of Der p 1 shows that the protein is composed of dimers of two globular domains formed by the N- and C-terminal sequences connected by a flexible outside loop [14, 15]. T cell epitopes seem to be located in this central loop [16] while B cell epitopes are described throughout the molecule [17, 18]. Der p 1 and other HDM allergens have proteolytic activity and could act as Th2 cell adjuvants cleaving CD23 and CD25 [19]. Der p 2 is a non-glycosylated protein of 14 000 Da that contains three disulphide bonds and is composed of two anti-parallel β -pleated sheets [20]. T cell epitopes were located in all regions of the protein and IgE epitopes were shown to be conformational [21–23].

The generation of recombinant hypoallergenic proteins using hybrid technology consists on assembling the most relevant disease-eliciting antigens within a single molecule which has hypoallergenic properties and, moreover, strongly increases its immunogenicity [24]. Based on this concept, we generated two recombinant chimeric proteins of Der p 1 and Der p 2 allergens to obtain a vaccine prototype for SIT against HDM allergy.

Methods

Patients

One hundred and six patients with HDM allergy (49 females and 57 males; mean age, 27.8 years; age range,

18–69 years) and 40 control subjects (26 females and 14 males; mean age, 37.4 years; age range, 14–54 years) were included in the study. The diagnosis of HDM allergy was based on clinical history, positive skin prick test (SPT), and specific IgE to DPT extract class ≥ 2 (ImmunoCAP System; Phadia, Uppsala, Sweden). Control subjects comprised 20 non-atopic subjects and 20 allergic individuals sensitized to different allergenic sources unrelated to DPT, as demonstrated by negative SPT responses.

The study was approved by the Research Ethics Committee of the Vega Baja Hospital of Orihuela, Basurto Hospital of Bilbao, and the Research Ethics Committee of Galicia, Consellería de Sanidade, Santiago de Compostela. All patients and control subjects provided oral and written informed consent.

Dermatophagoides pteronyssinus extract and purified natural allergens

DPT extract was prepared as previously described [22]. Purification of natural Der p 1 and Der p 2 was performed as previously described [25] with some modifications. Briefly, 7 g of DPT mites/faeces were rapidly extracted in PBS and the supernatant was separated by ammonium sulphate precipitation into two fractions at 60% and 95% saturation. For Der p 1 purification, the 60% saturation pellet was dissolved and subjected to gel permeation chromatography in a Superdex SX200 column (GE Healthcare, Buckinghamshire, UK). Positive fractions in blot were pooled, dialysed against 20 mM Tris pH 8.5 and injected in a High Trap Q column (GE Healthcare) equilibrated with the same buffer. nDer p 1 was obtained in the unbound fraction. For Der p 2 purification, the 95% saturation pellet was dissolved, dialysed against 50 mM Tris pH 8.0 and applied in a High Flow 16/10 Q column (GE Healthcare) and eluted with the same buffer supplemented with 1 M NaCl. Bound fraction was dialysed against 20 mM AcNa pH 5.5 and injected in a High Trap SP column (GE Healthcare). Protein eluted with 200 mM NaCl was concentrated in an Amicon ultrafiltration device and applied to a Superdex SX75/300 column in a Smart System (GE Healthcare). nDer p 2 was found in the 16 kDa protein fraction.

Construction of the recombinant hybrid molecules

Der p 1.0124 and Der p 2.0115 (according to the IUIS-WHO Allergen Nomenclature Subcommittee) were cloned by RT-PCR using primers based on reported sequences. We generated two hybrid proteins based on Der p 1.0124 and Der p 2.0115 and primers described in Table 1 (Fig. 1). The first hybrid, called QM1, is composed of almost the whole sequences of both major allergens. A Der p 2-fragment from residues 5 to 123 at the N-terminus, introducing point mutations on Cys8 and Cys119 to Ser to avoid

forming the described disulphide bridge, and a Der p 1-fragment from residues 4 to 222 (end) at the C-terminus were joined. The other hybrid called QM2, was constructed as follows: the N-terminal half of Der p 2 from residues 1 to 73 amplified by primers P7 and P8 was cloned into the *Bam*HI/*Eco*RI sites of pBluescript KS (Stratagene, Edinburgh, UK); the Der p 1 fragment from residue 5 to 222 was amplified by P9 and P10 and cloned into the *Pst*I and *Avr*II sites of the previous construction; and finally, the

C-terminal half of Der p 2 from residue 74 to 129 was amplified by primers P11 and P12 and cloned into the *Avr*II y *Eco*RI of the later construction. The completed *Bam*HI/*Eco*RI QM2-cassette was cloned into the expression plasmid pKN172 [26]. For cloning purposes the dipeptide Pro-Arg was included between Der p 1 and Der p 2 (Fig. 1).

Expression and purification of the hybrid proteins

All DNA constructs were cloned into the expression plasmid pKN172 [26] and transformed into *Escherichia coli* BL21(DE3). Cells were grown in 1 L of LB-medium containing 100 µg/mL ampicillin until the log phase was reached. Then, recombinant proteins were expressed by induction with 0.6 mM isopropyl-β-thiogalactopyranoside, and after 3 h at 37 °C, cells were harvested by centrifugation. Cells were resuspended in 50 mL of 50 mM Tris buffer, pH 8.0, 1 mM DTT and treated with 100 µg/mL lysozyme for 30 min at 37 °C. After a 2-min sonication, 1% Triton X-100 was added and incubated at room temperature for 30 min with gentle agitation and then, centrifuged at 10 000 g for 15 min. Inclusion bodies were washed twice with 2 M urea, 0.2% Triton X-100, and after a 1-min sonication, centrifuged again. Pellet containing purified inclusion bodies was resuspended in 10 mL of 6 M guanidinium chloride and 0.5% 2-mercaptoethanol, shaken for 1 h at 4 °C. For QM2, inclusion bodies were first reduced with 50 mM DTT for 1 h and alkylated in presence of 0.2 M 2-iodoacetamide for 1 h. Excess of reagent was neutralized with 0.2 M 2-mercaptoethanol. Both hybrid proteins were dialysed overnight against 6 M urea 25 mM, Tris pH 8.5 and refolded successively against 3, 1.5, 0.75, 0.38, and 0.19 M urea, for 90 min each. Final dialysis was performed overnight at 4 °C in water for QM2 and in 5 mM cysteine, 1 mM cystine, 50 mM Tris pH 8.0 for QM1 to complete an oxidative refolding. Further purification of

Table 1. Primers for preparation of the hybrid molecules.

Primer no.	Nucleotide sequence (5' → 3')
P1	CGGGATCCCGTCCATCATCGATCAAA <i>Bam</i> HI
P2	CGGAATTCTTAGAGAATGACAACATATGG <i>Eco</i> RI
P3	CGGGATCCGATCAAGTCGATGTCAAAG <i>Bam</i> HI
P4	CGGAATTCTTAATCGCGGATTTTAGC <i>Eco</i> RI
P5	CGGGATCCGTCAAAGATAGTGCCAATC <i>Bam</i> HI
P6	ACGGATCTGCAGGTAGCAATAGCACTGGCCAA <i>Pst</i> I
P7	CGGGATCCGATCAAGTCGATGTCAAAG <i>Bam</i> HI
P8	CCGAATTCCTAGGCTGCAGCCATTTGGATCGAT <i>Eco</i> RI <i>Avr</i> II <i>Pst</i> I
P9	ACTGACAGGCTCGTCCATCATCGATCAAAAC <i>Stu</i> I
P10	CACCTAGGGAGAATGACAACATATGG <i>Avr</i> II
P11	CACCTAGGCATTACATGAAAAGCCCA <i>Avr</i> II
P12	CGGAATTCTTAATCGCGGATTTTAGC <i>Eco</i> RI

Restriction enzyme sites are underlined. Point mutations in the Der p 2 sequence to change Cys8 and Cys119 to Ser are marked in *italics bold*.

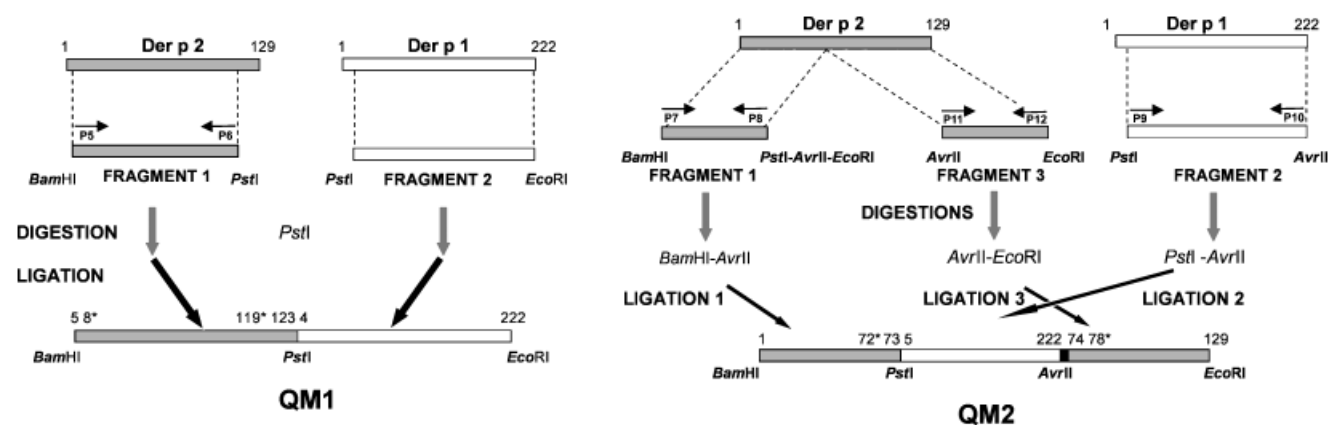


Fig. 1. Schematic construction of hybrid proteins. Numbering refers to amino acid position and primers are indicated by arrows. Complete sequence data are available as supporting information (Fig. S1). The nucleotide sequences for Der p 2.0115, Der p 1.0124, and the hybrid proteins QM1 and QM2 have been deposited in the GenBank database under accession numbers FM177223, FM177224, FM177225, and FM177226, respectively.

soluble fractions was performed by gel permeation chromatography (Superdex SX200 column). Protein content of each extract was determined by the Bradford's method (Bio-Rad, Palo Alto, CA, USA) using bovine serum albumin (BSA) as standard. The content of lipopolysaccharide in the preparations was determined with the *Limulus* amoebocyte lysate test (Endosafe rapid LAL single test, Charles River Laboratories, Charleston, SC, USA) according to the manufacturer's instructions.

Electrophoresis and Western blotting

SDS-PAGE was performed using the Laemmli method under reducing conditions in 12% polyacrylamide gels. Proteins (2 µg/lane) were stained with Coomassie Blue or were detected by Western blotting as previously described [27] using a serum pool (diluted 1/5) from all of the HDM-allergic patients included in the study.

Determination of specific immunoglobulin E levels and inhibition assays

Specific IgE levels to DPT extract and purified proteins were evaluated by means of ELISA. Multiwell plates (Greiner Microtiter, Greiner-BioOne, Frickenhausen, Germany) were coated overnight at room temperature with DPT extract (1 µg/well containing 63 ng of Der p 1 and 18 ng of Der p 2) or purified proteins (100 ng/well) and incubated for 90 min at 37 °C with patient sera at several dilutions. For IgE inhibition, the serum pool (diluted 1/20) from 30 HDM-allergic patients enrolled in the study with specific IgE to DPT extract higher than 50 IU/mL was pre-incubated at 4 °C overnight with different concentrations of natural and hybrid proteins. Bound IgE in both assays was determined as previously described [28].

Skin prick test

SPT was performed as previously described [29] with a commercial DPT extract (125 µg/mL, Bial-Aristegui, Bilbao, Spain) containing 7.9 µg/mL Der p 1 and 2.2 µg/mL Der p 2 (quantified by Indoor Biotechnologies, Charlottesville, VA, USA), natural allergens at 1, 10, and 100 µg/mL, and the hybrid molecules (QM1 and QM2) at 5, 50, and 500 µg/mL. Weal areas > 7 mm² were considered positive.

Peripheral blood mononuclear cells proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from 23 HDM-allergic patients by Ficoll (Lymphoprep, Nycomed AB, Lidingö, Sweden) density gradient centrifugation from heparinized venous blood samples. After viability estimation by trypan blue exclusion, 10×10^6 /mL PBMC were labelled with carboxyfluorescein succinimidyl ester (Molecular Probes Inc., Eugene, OR, USA) at 5 µM

during 10 min at 37 °C/5% CO₂, neutralized with 50% fetal calf serum another 5 min and washed twice with RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum. Labelled PBMCs were resuspended in complete medium (RPMI-1640, gentamycin 50 µg/mL, 2 mM glutamine supplemented with 5% human AB sera) and plated at a concentration of $1-2 \times 10^6$ cells in 24-well flat bottom plates. Afterwards, antigens were added and incubated for 7 days at 37 °C/5% CO₂. Cells were analysed on a BD FCAS-calibur flow cytometer with four colour fluorescence capabilities (Becton-Dickinson, Franklin Lakes, NJ, USA). Negative controls using buffer alone and positive controls using Pokeweed Mitogen (Invitrogen, Carlsbad, CA, USA) at the same concentration of allergens (10 µg/mL) were performed for every patient. At least 10 000 events were analysed and results are expressed as percentage of gated events in Cell Quest Software (Becton-Dickinson). The T cell proliferation assays were set up by testing PBMCs from five HDM-allergic patients using DPT extract and nDer p 1 and nDer p 2 (nD1D2) purified protein at final protein concentrations of 0.1, 1, and 10 µg/mL. Purified proteins followed a dose-response curve and comparative analysis were performed at maximum response 10 µg/mL.

Circular dichroism and dynamic light scattering analysis

Far-UV (190–250 nm) circular dichroism (CD) spectra at pH 7.0 and 20 °C were recorded with a Jasco J-810 spectropolarimeter (Jasco Europe, Cremella, Italy). Protein concentration was 0.035 mg/mL in 20 mM sodium phosphate buffer and 40 scans were accumulated. Aggregation was measured under apparent equilibrium conditions as an increase in the average particle size measured by dynamic light scattering (DLS) in a ZEN1600 Zetasizer (Malvern Instruments, Malvern, UK). Samples were measured and analysed using the standard operating procedures provided by the manufacturer.

Immunization of mice

Six-week-old female BALB/c mice (Haarlam, Barcelona, Spain) were immunized intraperitoneally (i.p.), five times each 15 days, with 10 µg of either the equimolar mix of purified nD1D2, QM1 or QM2 adsorbed to aluminium hydroxide. Six mice were used for each protein and sera were obtained 10 days after the last boost via bleeding from the submandibular vein, pooled and stored at –20 °C until use.

Inhibition of immunoglobulin E binding to nDer p 1 and nDer p 2 by mice immunoglobulin G against hybrid molecules

ELISA plates (Greiner) were coated (100 ng/well) overnight with nD1D2 in PBS, pre-incubated with a 1/20 dilution of anti-nD1D2, anti-QM1, and anti-QM2 serum

pools of six mice. A pre-immune serum pool was used as immunization control. After washing, plates were incubated with individual sera (diluted 1/20 and 1/50) from HDM-allergic patients or a pool of sera (diluted 1/50) from 30 HDM-allergic patients and bound IgE antibodies were detected with an HRP-labelled anti-human IgE monoclonal antibody (Southern, Birmingham, AL, USA) diluted 1/2000 and *o*-phenyldiamine (Sigma). The blocking capacity was expressed as a percentage signal of wells with no mouse serum added.

Statistical analysis

Statistical data analysis was performed using the Wilcoxon test included in the SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Characteristics of the recombinant hybrid molecules

The first hybrid protein, called QM1, is composed of almost the whole sequence of Der p 1 and Der p 2 major allergens. The N-terminus of QM1 is a fragment from Der p 2 (residues 5–123), with point mutations on C8 and C119 to avoid formation of the disulphide bridge, and the C-terminus is a fragment from Der p 1 (residues 4–222). This protein was expressed as a 34.9 kDa protein (Fig. 2) with a final yield of 120 mg/L of bacterial culture. The other hybrid called QM2, is composed of three parts: the N-terminal half of Der p 2 from residues 1 to 73; almost the whole Der p 1 as in QM1 and the C-terminal half of Der p 2 from residue 74 to the end. Hybrid QM2 was expressed as a 39.7 kDa protein with a final yield of 43.4 mg/L culture (Fig. 2). Both proteins appeared in the insoluble inclusion body fractions but could be solubilized with guanidinium

chloride. After refolding by dialysis, both proteins remained soluble at a concentration below 1 mg/mL. SDS-PAGE analysis showed a degree of protein purity $> 95\%$ (Fig. 2). The endotoxin content was < 0.1 EU/ μ g of recombinant protein. QM1 and QM2 showed similar CD spectra to that of nDer p 2, indicating a β -sheet conformation but the spectra of the hybrids did not change after heating at 90 °C (data not shown), exhibiting a characteristic behaviour of aggregated proteins. To investigate these results further, DLS analysis was performed showing that QM1 and QM2 formed microaggregates of approximately 10 and 60 molecules, respectively.

Reduced immunoglobulin E binding of recombinant hybrid proteins

Western blotting with a serum pool from HDM-allergic patients showed a decrease of IgE reactivity when comparing QM1 reactivity to the strong IgE binding with natural allergens Der p 1 and Der p 2. In addition, the shuffled hybrid protein QM2 showed no detectable IgE-binding capacity (Fig. 2).

One hundred and six individual sera from HDM allergics were tested with the hybrid proteins by ELISA. nD1D2 reacted with 99% of the sera (105 out of 106) but only seven out of 106 sera (6.6%) exhibited IgE reactivity to QM2 higher than 0.35 IU/mL. Eighty-eight sera (83%) reacted to QM1 (median, 17.01 IU/mL with lower and upper 95% confidence limits of 9.9 and 22.2, respectively) (Fig. 3a). QM1 IgE-binding capacity was lower ($P < 0.001$) than that of nD1D2 (median, 16.5 IU/mL; 95% confidence limits 10.7–29.2). Strong reduction of QM1 IgE reactivity was found compared with the whole extract (median, 39.35 IU/mL; 95% confidence limits 18.8–50.9) ($P < 0.001$). The amount of purified proteins coated was 100 ng, while the content of Der p 1 and Der p 2 in the coated extract was 63 and 18 ng, respectively. DPT extract IgE-binding capacity was higher ($P < 0.001$) than that of nD1D2 but both showed a good correlation (Spearman's $\rho = 0.875$; $P < 0.001$). Complete data of individual patient values are available as supporting information (Table S1).

Further analysis of IgE reactivity was carried out by means of IgE-inhibition assays using the pool of 106 sera. Both hybrid proteins showed a strongly reduced ability to inhibit IgE binding to immobilized nD1D2 than that observed using nD1D2 as an inhibitor (95% inhibition) (Fig. 3b). nD1D2 was able to reach 50% of IgE inhibition at 3 ng/mL, while 1500 ng/mL of QM1 were necessary to reach the same inhibition. QM2 gave a similar IgE reactivity to that of the control protein (BSA).

Reduced allergenic activity of recombinant hybrid proteins

The hypoallergenic nature of the two preparations was investigated *in vivo* by SPT in 106 HDM-allergic patients

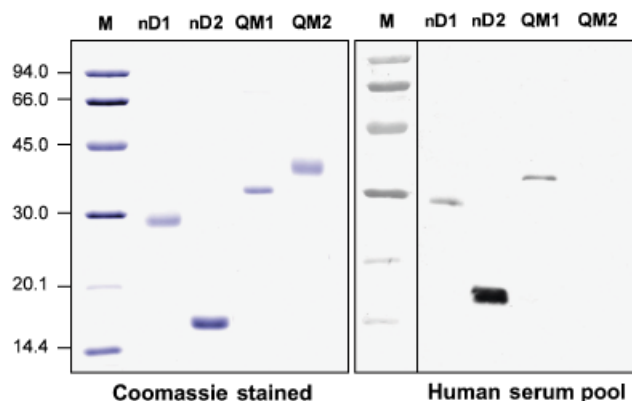


Fig. 2. Coomassie-stained SDS-PAGE and IgE Western blotting of nDer p 1 (nD1), nDer p 2 (nD2), QM1, and QM2 (2 μ g/lane) incubated with the serum pool from house dust mite-allergic patients.

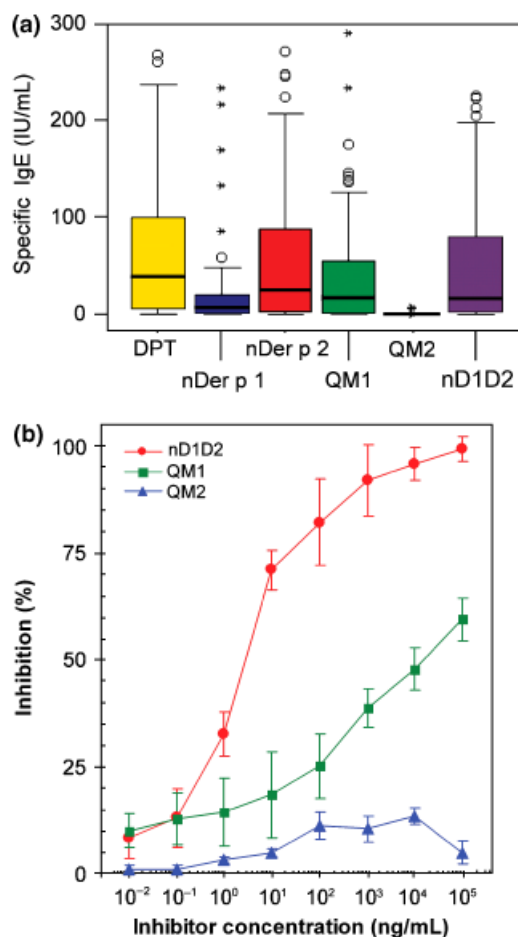


Fig. 3. (a) Box-plot representation of duplicate specific IgE means from individual patient sera ($n = 106$) to DPT extract, of nDer p 1, nDer p 2, nD1D2, QM1, and QM2. The results are shown as box-plots where the edges of each box mark the 25th and 75th percentiles and the lines indicate the median values. The bars extending up and down from each box show the largest observed value that is not an outlier. Open circles and stars indicate outliers and extremes of each patient group. Both groups of IgE reactivity to hybrid molecules (QM1 and QM2) showed statistically significant differences ($P < 0.001$) to DPT, nDer p 1, nDer p 2, and nD1D2 groups. (b) IgE-inhibition curves. The binding of human IgE from the sera pool to nD1D2 was inhibited using: nD1D2, QM1, and QM2. Values represent the mean $\pm$ SD ($n = 3$).

(Fig. 4). The whole group of patients showed positive skin reactions to the whole DPT extract and 105 out of 106 (98.1%) were positive to Der p 1 or Der p 2. On the other hand, only five out of 106 (4.7%) reacted with QM2 at its highest concentration (500 μ g/mL) while 16 (15.1%) and 86 (81.1%) patients reacted with QM1 at concentrations of 50 and 500 μ g/mL, respectively. Nevertheless, all positive responses elicited by QM1 at 500 μ g/mL (median, 40.33 mm^2 ; 95% confidence limits 35.05–47.88) had lower reactivity ($P < 0.001$) than those elicited by Der p 1 and Der p 2 at 100 μ g/mL (median values of 53.96 and 78.86 mm^2 , respectively) but higher than Der p 1 at 10 μ g/mL (median, 26.48 mm^2). The reactivity of QM1, at 500 μ g/mL, was not statistically different from reactivity of

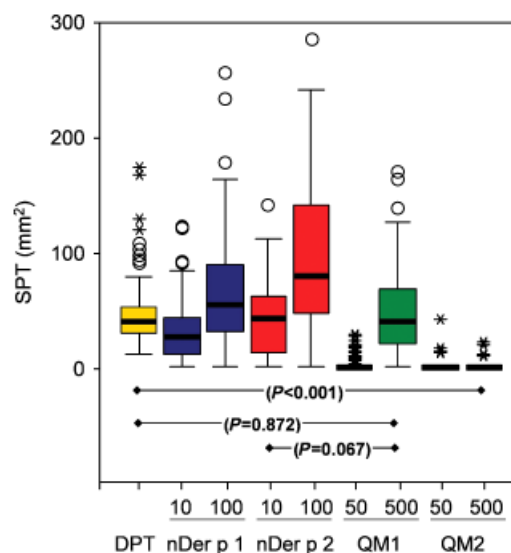


Fig. 4. Skin prick test results from individual patients ($n = 106$) with DPT extract, nDer p 1 and nDer p 2 (both at 10 and 100 μ g/mL), and QM1 and QM2 (both at 50 and 500 μ g/mL) are shown. Individual values are given as means of duplicate weal surface areas.

nDer p 2 at 10 μ g/mL ($P = 0.067$) or from DPT extract ($P = 0.872$) (median values of 41.90 and 39.55 mm^2 , respectively). No adverse side-effects were observed in any of the subjects tested and no skin reactions could be elicited with the purified allergens in control individuals indicating the high specificity of the test.

T cell reactivity of recombinant hybrid proteins

To investigate the T cell stimulating capacity of the fusion proteins, specific proliferation of PBMCs was performed. The assays were set up by testing PBMCs from five HDM-allergic patients using purified proteins at different concentrations and comparative analyses were carried out at maximum response (10 μ g/mL). Specific proliferation of PBMCs from 23 HDM-allergic patients showed that QM1 (median, 15%; 95% confidence limits 10–25%) induced a significantly higher proliferation ($P < 0.05$) than that induced with nD1D2 (median, 9%; 95% confidence limits 5–13%) (Fig. 5). The proliferation induced with QM2 (median, 13%; 95% confidence limits 8–17%) was comparable to that of nD1D2 ($P = 0.940$). Furthermore, upon comparison of both hybrid proteins, QM1 induce a higher proliferation than QM2 ($P < 0.005$).

QM1- and QM2-induced antibodies inhibit patients' immunoglobulin E binding to natural allergens

Antisera raised by immunization of mice with nD1D2, QM1, and QM2 were tested for reactivity with nD1D2, showing that immunization with both hybrids lead to

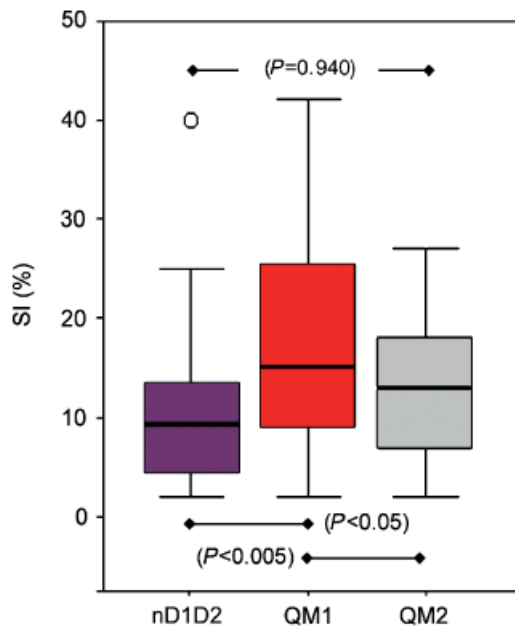


Fig. 5. Induction of T cell proliferation. Peripheral blood mononuclear cells from individual patients ($n = 23$) were stimulated with $10 \mu\text{g/mL}$ of nD1D2, QM1, and QM2. Individual values are given as means of triplicate stimulation index percentages (SI%).

higher nD1D2-specific IgG antibody levels compared with immunization with nD1D2 (Fig. 6a).

In order to examine whether specific mouse antibodies raised by hybrid proteins immunization were able to block the binding of mite-allergic patients' IgE to nD1D2, a competitive ELISA was performed with individual sera from nine HDM-allergic patients. The inhibition obtained with mouse anti-nD1D2 antibodies was between 41% and 72% (mean 56%) while mouse anti-QM1 and anti-QM2 antibodies inhibited serum IgE binding to nD1D2 between 43% and 82% (mean 60%) and between 0% and 45% (mean 20%), respectively (Table 2). The inhibition obtained with mouse anti-nD1D2 antibodies was slightly lower (although not statistically different) than that obtained with mouse anti-QM1 antibodies.

Additionally, the mouse blocking antisera was evaluated using a pool of sera from 30 HDM-allergic patients (Fig. 6b). Mouse IgG raised against QM1 and QM2 inhibited serum IgE binding to nD1D2 by 71% and 26%, respectively, whereas the inhibition obtained with mouse anti-nD1D2 antibodies or pre-immune sera was 65% and 17%, respectively (Fig. 6b). The partial inhibition of IgE reactivity by QM2-induced IgG was due to the very low inhibition of specific IgE to Der p 2. The ability of anti-QM2 IgG antibodies to interfere with patients' IgE binding to Der p 2 was much lower than that of anti-QM1 sera (13% and 85%, respectively). On the contrary, the inhibition of IgE binding to Der p 1 was comparable in both mouse antisera (inhibition with anti-QM2: 52%; with anti-QM1: 52%) (Fig. 6b).

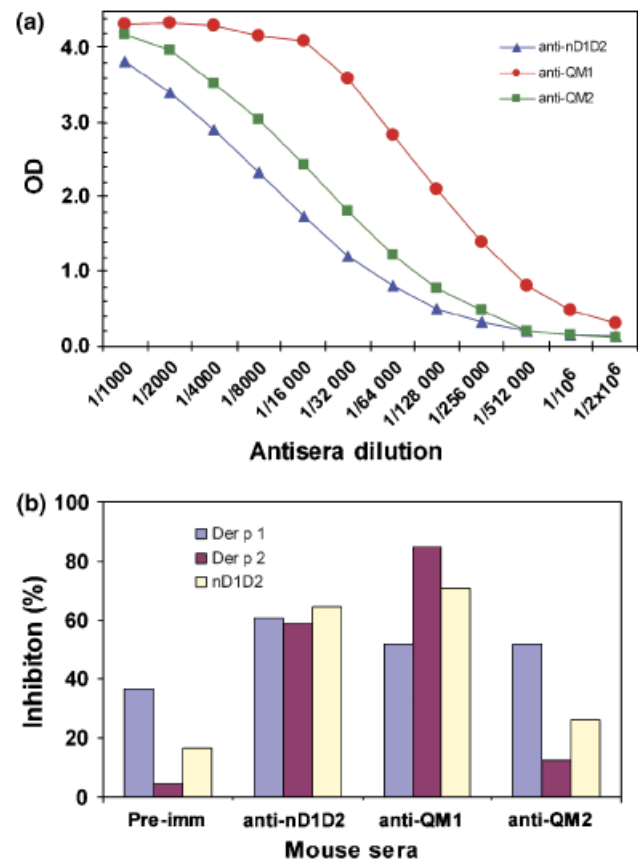


Fig. 6. (a) Titration of antisera raised by immunization of mice with nD1D2, QM1, and QM2. Different dilutions of antisera were tested for reactivity to nD1D1. The mean optical density (OD) corresponding to bound IgG antibodies are displayed for each serum dilution. (b) Inhibition of human IgE binding to nD1D2 and its components, Der p 1 and Der p 2 after pre-incubation with nD1D2-, QM1-, and QM2-specific mouse IgG antibodies.

Discussion

In this study, we have demonstrated the successful generation of two low IgE-binding activity hybrid molecules consisting of the two major allergens of *D. pteronyssinus*, Der p 1 and Der p 2. Both hybrids have a reduced activity for IgE binding and cross-linking while retaining T cell stimulatory capacity. Therefore, both molecules have to be considered as candidates for SIT replacing the commonly used whole natural allergen extracts. Highly heterogeneous immune responses have been observed in patients undergoing SIT based on allergen extracts [30, 31] mainly due to the complex composition of whole allergenic extracts used [32]. Specifically, SIT studies with whole HDM extracts have several well-known disadvantages such as the induction of new IgE reactivities and potential harmful effect in patients with related food allergies [3, 33]. Furthermore, several HDM allergens (Der p 1, 3, 6, and 9) are proteases which could act as Th2 cell adjuvants cleaving CD23 and CD25 exacerbating the overall bronchoconstrictive effect detected in asthmatic lungs [19].

Table 2. Percent inhibition of patients' immunoglobulin E binding to nD1D2 by nD1D2-, QM1-, and QM2-specific mouse immunoglobulin G antibodies

Patient	Anti-nD1D2	Anti-QM1	Anti-QM2
6	51	56	0
28	52	57	19
34	69	77	36
38	41	49	11
41	41	43	24
49	64	53	21
50	60	57	9
100	54	65	18
104	72	82	45
Mean	56	60*	20 [†]

Patient numbering refers to Table S1.

*Not statistically significant difference with anti-nD1D2 group ($P = 0.139$).

[†]Statistically significant differences ($P < 0.01$) with anti-nD1D2 and anti-QM1 groups.

nD1D2, nDer p 1 and nDer p 2.

Recombinant allergens have become available for the formulation of defined and safer allergy vaccines [4, 34] and the use of hybrid technology can overcome the problems of poor immunogenicity of certain allergens as well as simplify production and registration procedures [24]. The first important step is the selection of the targets for hybrid technology. Selection criteria may include the frequency of sensitization, clinical relevance, the magnitude of IgE responses, and the extent to which IgE epitopes are represented in a given allergen source [35]. nD1D2 is able to inhibit up to 90% of the total IgE-binding activity of the DPT extract (data not shown) which is at the top of the range of 50–100% of the IgE inhibition previously described [10], but far from the data of Weghofer et al. [36] who describe that a mixture of six purified mite allergens (Der p 1, 2, 5, 7, 8, and 10) are needed to bind an average of 76% of DPT-specific IgE antibodies. This discrepancy could be due to the low number of patients ($n = 18$) tested in that study, therefore we used a high number of patients ($n = 106$) from different locations of continental climates (inland and coast areas, Santiago de Compostela and Bilbao, respectively) and Mediterranean climates (Orihuela). A recent report from Thomas' group [13] described that by using solid-phase microtitre analysis, Der p 1 and Der p 2 bound 50–65% of the IgE antibody against HDM. Nevertheless, they suggested that these allergens could provide a formulation suitable for SIT because the large responses to them could regulate the entire allergen response. Very recently, two new successful vaccine strategies to treat birch pollen allergy focused on Bet v 1 because it is the main elicitor of this allergy and the unique allergen for almost 60% of birch-allergic patients [37, 38].

Different approaches have been undertaken to identify B cell epitopes on these two major HDM allergens suggesting the conformational nature of their epitopes [22]. Recently, specific mimotopes from these allergens were matched on the 3D structure of Der p 1 and Der p 2 confirming the conformational nature of their B cell epitopes [21]. On the basis of these studies, two strategies were applied to develop hypoallergenic allergen derivatives. In the case of QM1, the Der p 2 structure was disturbed by destroying the C8–C119 disulphide bond and the mutated allergen was fused to Der p 1. Site-directed mutagenesis of disulphide bonds of Der p 2/Der f 2 has been shown to reduce IgE binding [39–41] by degeneration of protein secondary structure [42]. Considering that QM1 contains 10 cysteine residues, it could be predicted that the proportion of native-like folded protein would be lower than that of its separate native components. Folding of cysteine-containing proteins is difficult in *E. coli* due to the reducing state of the bacterial cytoplasm resulting frequently in the accumulation of protein aggregates that have to be solubilized [43]. The second approach to obtain a hypoallergenic variant was to generate chimeras by gene rearrangement [24]. The reduction of allergenic activity was achieved in QM2 by dividing Der p 2 into two defined fragments and rejoining the two parts together with Der p 1. QM1 and QM2 remained soluble but formed microaggregates similar to the hypoallergen Bet v 1 trimer [44] or the hypoallergenic C8–C119 mutant of Der f 2 which formed soluble polymers of around 100 molecules [41].

The reduced allergenic activity of QM1 could be the result of microaggregation, steric hindrance, and/or unfavourable charge interactions hiding some IgE epitopes required for efficient cross-linking. The latter possibility would be in agreement with our findings that QM1 was able to cross-link FcεRI-bound IgE or inhibit IgE to nD1D2 less efficiently than nD1D2 as shown by IgE inhibition and prick test. This hybrid molecule showed in IgE-inhibition studies >5000-fold reduction of IgE binding with respect to nD1D2 (Fig. 3b) although this difference was less pronounced when *in vivo* SPT results were compared. Skin allergenicity of QM1 at 500 µg/mL was similar to that elicited by nDer p 2 at 10 µg/mL or the whole DPT extract containing 7.9 and 2.2 µg/mL of Der p 1 and Der p 2, respectively. Similar results were also found in the hypoallergen Q1 from *Parietaria judaica* [29] and the Bet v 1 trimer [44]. The second hybrid molecule, QM2, showed a complete abolishment of *in vitro* IgE reactivity as assessed by Western blot, ELISA, and IgE inhibition when compared with QM1 and elicited positive cutaneous response in only 4.7% of patients. Also, the basophil-activating capacity of QM2 was much reduced compared with that of QM1 (M. L. Sanz, unpublished results).

A prerequisite for hypoallergens when used as candidates for SIT includes a reduced IgE-binding activity when

compared with the corresponding allergens as well as the presence of T cell epitopes and the capacity to induce 'blocking' antibodies [1, 45]. T lymphocytes are crucial cellular components in the down-regulation of IgE responses during SIT [6, 7]. T cell reactivity of these hybrid molecules was analysed by stimulation of PBMC showing that QM1, composed of the complete sequences of Der p 1 and Der p 2, produced a higher allergen-specific T lymphocyte activation than QM2, suggesting that both hybrids could be processed differently by the APCs or that the cleavage point chose for disruption of the Der p 2 structure in the later hybrid could compromise a T cell epitope. The latter possibility could be supported by the finding of a putative T cell epitope within residues 61–86 [23] because intact T cell epitopes are required to enable the induction of specific T cell tolerance or anergy against the allergen [1]. Immunization of mice showed the immunogenicity of both hybrids by their capacity to induce IgG antibody responses. IgE binding from HDM-allergic patient sera to nD1D2 was inhibited by IgG induced with each hybrid although QM1-induced IgG showed a better inhibitory capacity compared with IgG induced with QM2. The only partial inhibition of IgE reactivity by QM2-induced antiserum has been also found with a Phl p 2-mosaic [46]. There, authors suggested that it could be due to the destruction of IgE epitopes and thus no IgG could be induced against the original IgE epitopes. This could be the case on QM2 as IgG-blocking activity of IgE binding to Der p 2 is really low and the IgE reactivity of this hybrid was almost abolished. The low ability of mouse anti-nD1D2 antibodies to inhibit IgE binding to itself (60–65%; Table 2 and Fig. 6b) was similar to that reported in the case of Der p 2-specific IgE [47]. These differences between the specificity of murine IgG and human IgE responses to mite allergens could be influenced in part by the different mode of immunization as it has been reported before [48]. In mice, which are immunized by i.p. injections containing adjuvants, antigens are extensive processed while in humans, which are sensitized by inhalation of minute quantities of allergen without adjuvants, either limited allergen processing or a different form of processing occurs [48]. Both QM1 and QM2 exhibited higher T cell stimulating capacity and induced stronger protective antibody responses than the separated molecules, as found in grass pollen hybrids [49, 50].

Recent clinical trials on SIT using recombinant allergens from grass or birch pollens have demonstrated that such treatments were safe, induced IgG1 response, and reduced IgE against the corresponding natural allergens [38, 39, 51, 52]. The hybrid allergens strategy has the advantage that a single molecule contains immunogenic information from two or more major allergens [24]. The results presented in this study suggest that both QM1 and QM2 hypoallergenic derivatives could provide a suitable formulation for successful immunotherapy against HDM

allergy thereby avoiding the disadvantages of whole extract-based treatment. QM1, the less hypoallergenic hybrid, could be used for sublingual immunotherapy and the strong hypoallergenic one (QM2) for subcutaneous immunotherapy. Nevertheless, studies with animal models and clinical trials must be performed to assess safety and efficacy of treatment with these molecules.

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Conflict of interest: Some of the authors are employees at the biopharmaceutical company Bial-Aristegui.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid sequences of rDer p 1, rDer p 2, QM1, and QM2 recombinant proteins. Sequences from Der p 1 and Der p 2 are marked in blue and red, respectively. Amino acid residues introduced by restriction enzymes sites are double underlined and point-mutation changes are boxed.

Table S1. *In vivo* and *in vitro* pattern of sensitization to DPT extract, nDer p 1, nDer p 2, mixed natural purified allergens (nD1D2), and hybrid molecules.

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Fig. C1. Amino acid sequences of rDer p 1, rDer p 2, QM1, and QM2 recombinant proteins. Sequences from Der p 1 and Der p 2 are marked in blue and red, respectively. Amino acid residues introduced by restriction enzymes sites are double underlined and point-mutation changes are boxed.

Der p 1

```
actaaagcctgcagatcatcaatggaatgtccacactgaatctgatttgggaacaaatgcgc
T N A (C) S I N G N A P A E I D L R Q M R
actgcaactccgacttgcattgcaaggagcctgttggttcattgttggccttctctcgtgtt
T V T P I R M Q G G (C) G S C W A F S G V
ggcgcaactggaatcagcttatttggcttaccgtaaatcaatcattggtcttgcgtaacaa
A A T E H S A Y L A Y R N Q S L D L A E Q
gaatgtctcgttattgtctcccaacagcgttataatgttgatcacatccacgtgttatt
E L V D (C) A A S Q N G (C) H G D T I P R G I
gaatatacctcaacataatgtgtgtcgcgaagaagctactatcgatcgttgcacgagaa
E Y I Q H N G V Q E S Y Y R Y V A R E
caatcatccgcagcaccacatgcacaagcttccgtatctcaaatattgcacaatttac
Q S (C) R R R P N A Q R F G I S N Y (C) Q I Y
ccacaaatgcacacaaaattcgtgaagcttggctcgaacccacagcgttattgcgcgtc
P P N A N K I R E A L A Q T H S A I A V
aatattggcatcaaaatttagacgcttccgtcattatgatggcgcaacaatcattcaa
I I G I K D L D A F R H Y D G R T I I Q
cgcgataatggttaccacacaaactatcacgctgtcacaacttgggttgcagtaacgca
R D N G Y Q P N Y H A V N I V G Y S N A
cagggtgtcgattatgtgactgcgaacacagttgggataccaattggggtgataatggt
Q G V D Y W I V R N S W D T N W G D N G
taaggattatttgcgcacaatcgattgatgatggaagaatccatattggttc
Y G Y F A A N I D L M M I E E Y P Y V V
attctctaa
I L -
```

QM1 (338 residues)

```
gtcaaaagtagtgcaaatcatgaaatcaaaaagtttggtagcaggatgccaatggttca
V K D (C) A N H E I K K V L V P G C H G S
gaacacatgtatcattcctggtgtaacacattccaattggaagcgttcttgcgaagcaca
E P C I I H R G K P F Q L E A V F E A N
caaaactcaaaaacgcgtgaataatgaaatcaaaatgcaatcgtggtttgaaagttgat
Q N S R T A K I E I R A S I D G L E V D
gtctccgctatcgatccaaatgcagtcattatgaaatgcgcacaaatgcgaagacaa
V P G I D P N A C H Y M K C P L V K G Q
caatatagtatttaaatatacagaagatttgcgaacaaatgcgaacaaatgcgaagatt
Q Y D I K Y T W N V P K I A P K S E N V
gtgcacactgttcaagcttattggtgataagtggttcttggcgaactgatttgcacgtc
V Y T V K Y V N G S D N G V L A A T (C)
aagtatacaggaatgtcctcagtgaaatcgatttgcgcacaaatgcgaactgcacatcc
S I N G N A P A E I D L R Q M R E V T P
attcgttcgaagagcgttgcatttgcattgttgcgttctctctgttgcgcgaactgaa
I R M Q G G C G S C W A F S G V A A T E
tcagcttatttggcttaccgtatcattcattggtcttgcgtgacacgaatgattcgat
S A Y L A Y R N Q S L D L A E Q E L V D
tgtctgtcccaacacggttgcattggtgataccttccacgctgatttgaatcatccaa
C A S Q H G C N G D T I P R G I E Y I Q
cataatgtgtcgcgcacaaagctactatcattcgttgcgcgaacacattcgtgcga
H N G V V Q E S Y Y R Y V A R E Q S C R
cgacacaaatgcacacagcttgcgttctcacaactattgcacaaatgcacacaaatgca
R P N A Q R F G I S N Y C Q I Y P P N A
aacaacaaatcgtgaagcttgcgttccaaacccacagcgtcattgctcattattggaac
N K I R E A L A Q T H S A I A V I I G I
aagaatttagaacttccgtcattatgagggcgacacacattcaccgcgaacattggt
K D L D A F R H Y D G R T I I Q R D N G
taccacaaacacattcagcgttcacacattgttgcgttgcagacgaacagcgttcgat
Y Q P N Y H A V N I V G Y S N A Q G V D
tattggatcgtcagcaaacagttgggtatcccaatgggggtgataatggttaccgttattt
Y W I V R N S W D T N W G D N G Y G Y F
gctgcgcacatcgatttgcattgattgaagaatataccatgttgcattctctaa
A A N I D L M M I E E Y P Y V I L -
```

Der p 2

```
gatcaagtcgagtgatgcaaaagatttgcgaatcatgaaatcaaaaagtttggtagcagg
D Q V D V K D (C) A N H E I K K V L V P G
ttccatggttccagaacccatgattcatcgttgtaaacattccaattgggaagcgtgt
(C) H G S E P (C) I I H R G K P F Q L E A V
ttcgaagccacacaaactcgaacacgcgttaaaattgaaatcaaaatgcaatcgtggt
F E A N Q N S K T A K I E I K A S I D G
ttagaagttgatgttcccgatgcgaacacgattgcattatgataaattgcaatg
L E V D V P G I D P N A (C) H Y M K K (C) P L
gttaagagacacaaatgatattaaatatacatggaatgttccgaataattgcacacaaa
V K G Q Q Y D I K Y T W N V P K I A P K
ctgaaatgttgcgtcactgcttgaagttatgggtgataatggttcttgcgttgcgt
S E N V V V T V K V M G D N G V L A (C) A
attgctactcatgctaaaatccgcgattaa
I A T H A K I R D -
```

QM2 (349 residues)

```
gatcaagtcgagtgatgcaaaagatttgcgaatcatgaaatcaaaaagtttggtagcagg
D Q V D V K D C A N H E I K K V L V P G
tgccatggttccagaacccatgattcatcgttgtaaacattccaattggaagcgtt
C H G S E P C I I H R G K P F Q L E A V
ttcgaagccacacaaactcgaacacgcgttaaaattgaaatcaaaatgcaatcgtggt
F E A N Q N S K T A K I E I K A S I D G
ttagaagttgatgttcccgatgcgaacacgattgcattatgataaattgcaatg
L E V D V P G I D P N G C S I N G N A
gttgaatcgtatttgcgaacaaatgcgaacgtcactccacttgcattgcaagagcgt
R E I D L R Q M R T V T P I R M Q G C
ggttcatgttgggttctctcgttgcgcgaactgaatcagcttatttgcgttaccgt
G S C W A F S G V A A T E S A Y L A Y R
aatcaatcattggtatttgcgtgaacgaatgctgatttgcgttcccaacacggttgc
N Q S L D L A E Q E L V D C A S Q H G C
atggttgatcaccattccacgttgcatttgaatatacctcaacataatggttgcgtcgaaga
H G D I P R G I E T I Q H N G V Q E
aactactatgatcgttgcgcgaacacacacgcgcgaacacaaatgcgaacggttgc
S Y Y R Y V A R E Q S C R R P N A Q R F
ggatctcacaactattgcacaaatttaccacacaaatgcacacaaatcgtgaagcttgc
G I S N Y C Q I Y P P N A N K I R E A L
gctcaaacccacagcgtcatttgcgtcattattggcatcaaaagatttagacgttccgt
A Q T H S A I A V I I G I K D L D A F R
cattatgttggcgaacacacacacacacacacacacacacacacacacacacacacacac
E Y D S A T I I G R D N G Y P N Y H A
gtcaacatgttgcgttgcacgaacacacacacacacacacacacacacacacacacacac
V N I V G Y S N A Q G V D I W I V R N S
tggtgataccaattgggtgataatggttgcgttgcgttgcacacacacacacacacacacac
N D T N W G D N G Y G Y F A A N I D L M
tcatttgaagatatacattggttgcatttccatcagcagcattcagcagcagcagcagcagc
E Y D S A T I I G R D N G Y P N Y H A
gttaagagacacaaatgatattaaatatacatggaatgttccgaataattgcacacaaa
V K G Q Q Y D I K Y T W N V P K I A P K
ctgaaatgttgcgtcactgcttgaagttatgggtgataatggttcttgcgttgcgt
S E N V V V T V K V M G D N G V L A C A
attgttactcatgctaaaatccgcgattaa
I V T H A K I R D -
```

Fig. C1

Table C1: *In vivo* and *in vitro* pattern of sensitization to DPT extract, nDer p 1, nDer p 2, mixed natural purified allergens (nD1D2), and hybrid molecules.

	DPT extract		nDer p 1		nDer p 2		nD1D2	QM1			QM2		
Patient no.	SPT 125 µg/mL (mm ²)	IgE (IU/mL)	SPT 100 µg/mL (mm ²)	IgE (IU/mL)	SPT 100 µg/mL (mm ²)	IgE (IU/mL)	U/ml (IU/mL)	SPT 50 µg/mL (mm ²)	SPT 500 µg/mL (mm ²)	IgE (IU/mL)	SPT 50 µg/mL (mm ²)	SPT 500 µg/mL (mm ²)	IgE (IU/mL)
1	24.6	34.5	24.4	9.0	79.0	102.5	76.1	8.1	51.6	38.1	0.0	0.0	0.3
2	107.3	16.3	155.1	7.1	10.9	0.3	1.1	0.0	0.0	0.3	0.0	0.0	0.3
3	35.8	2.8	34.8	13.3	0.0	0.3	0.6	0.0	0.0	0.3	0.0	0.0	0.3
4	43.2	6.7	65.0	2.0	106.4	22.9	6.3	0.0	57.7	14.4	41.2	22.5	0.3
5	39.4	5.2	24.5	3.4	101.9	16.0	7.1	12.7	77.4	15.4	0.0	0.0	0.3
6	41.8	301	31.6	31.5	48.5	92.5	98.9	0.0	51.4	84.5	17.2	0.0	0.3
7	23.2	164	21.7	35.5	92.4	73.4	73.9	0.0	21.7	55.2	0.0	0.0	0.3
8	43.6	50.9	155.0	9.7	140.6	25.8	14.8	16.9	43.1	17.9	0.0	0.0	0.3
9	23.4	0.6	0.0	0.3	59.2	1.8	1.4	0.0	21.8	0.6	0.0	0.0	0.3
10	11.3	0.8	32.8	0.3	0.0	0.3	0.5	0.0	0.0	0.3	0.0	0.0	0.3
11	61.3	1	23.7	0.3	77.5	9.2	2.6	0.0	47.9	4.4	0.0	0.0	0.3
12	44.4	41.9	104.1	10.3	51.4	48.6	37.0	0.0	107.2	26.2	0.0	0.0	0.3
13	28.2	48.1	26.7	6.2	53.3	25.6	16.2	0.0	26.6	35.0	0.0	0.0	0.3
14	61.3	51.8	85.0	7.0	147.2	84.1	68.3	10.3	83.4	43.1	0.0	0.0	0.3
15	45.4	268	37.5	47.7	66.7	224.7	171.7	19.2	71.9	85.1	0.0	0.0	0.3
16	18.2	0.5	0.0	0.3	29.8	2.0	1.7	0.0	20.5	0.8	0.0	0.0	0.3
17	25.6	15.2	37.7	5.9	55.2	20.8	10.7	0.0	31.8	14.0	0.0	0.0	0.3
18	73.0	13.8	38.6	5.9	188.9	44.3	19.2	22.9	138.0	34.7	0.0	0.0	5.64
19	22.3	7.8	36.4	11.1	100.8	25.2	7.4	0.0	20.1	16.1	0.0	0.0	0.3
20	41.4	0.5	15.5	5.3	103.6	10.0	3.4	10.1	40.2	12.5	0.0	0.0	0.3
21	31.6	0.8	14.4	0.3	83.5	1.0	1.2	0.0	24.4	0.5	0.0	0.0	0.3
22	50.9	4	76.2	0.3	90.8	2.7	2.5	2.5	43.5	0.5	0.0	0.0	0.3
23	21.8	1.3	33.9	0.3	40.6	10.8	2.1	0.0	25.9	5.8	0.0	0.0	0.3
24	27.0	2.9	25.0	1.6	0.0	0.3	0.8	0.0	0.0	1.6	0.0	0.0	0.3
25	31.2	0.4	0.0	0.3	0.0	0.9	0.2	0.0	0.0	1.7	0.0	0.0	0.3
26	15.4	3.9	24.1	1.8	69.0	5.5	3.5	28.7	17.4	4.9	0.0	11.0	0.88
27	27.2	0.6	20.0	0.3	44.5	3.9	2.0	0.0	41.1	3.4	0.0	0.0	0.3
28	23.2	41.5	27.8	11.8	38.1	46.2	18.0	0.0	21.4	27.7	0.0	0.0	0.3
29	29.9	7.6	59.4	2.3	88.4	13.2	9.6	9.7	40.4	4.0	0.0	0.0	1.22
30	18.6	57.9	25.6	6.8	35.9	114.4	67.2	0.0	27.3	50.5	0.0	19.4	0.3
31	11.8	0.5	13.6	0.3	36.0	2.8	2.3	0.0	11.2	3.3	0.0	0.0	0.3
32	20.5	5.7	34.6	0.3	52.0	1.6	2.0	0.0	18.1	0.5	0.0	0.0	0.3
33	20.2	2	30.7	0.3	0.0	0.3	1.0	0.0	0.0	0.3	0.0	0.0	0.3
34	45.9	74.2	103.7	7.8	216.4	41.2	33.5	9.3	75.6	30.0	0.0	0.0	0.3
35	32.8	104	41.5	3.2	71.3	271.4	225.9	0.0	48.1	123.1	0.0	0.0	0.3
36	46.7	17.8	44.4	2.9	125.7	22.0	16.9	0.0	80.6	20.2	0.0	0.0	0.3
37	73.9	14.5	256.9	0.3	314.9	13.6	15.5	0.0	112.2	8.9	0.0	0.0	0.3
38	75.6	142	157.2	38.8	151.2	81.7	139.6	0.0	116.5	50.1	0.0	0.0	0.48
39	37.6	100	43.6	25.8	84.4	50.2	50.8	0.0	56.5	22.2	0.0	0.0	0.3
40	33.4	67.4	34.8	14.7	13.2	0.3	1.1	0.0	0.0	0.3	0.0	0.0	0.3
41	40.2	98.7	112.1	18.5	95.5	67.8	89.0	0.0	65.6	60.2	0.0	0.0	0.3
42	49.0	100	131.6	14.9	88.3	116.9	130.6	0.0	46.4	65.9	0.0	0.0	0.3
43	26.4	128	52.6	47.5	171.6	85.3	109.1	0.0	28.9	81.3	0.0	0.0	0.3
44	65.4	43.6	63.7	2.5	115.0	27.6	17.7	0.0	66.0	14.9	0.0	0.0	0.3

45	48.0	33.4	82.1	2.1	116.7	30.6	17.2	0.0	40.2	21	0.0	0.0	0.3
46	23.4	5.6	14.3	0.3	96.1	24.6	6.3	0.0	23.8	9.9	0.0	0.0	0.3
47	39.7	18.8	46.5	0.3	96.7	32.0	19.4	0.0	0.0	10.7	0.0	0.0	0.3
48	52.2	213	178.6	35.8	181.8	100.3	100.3	6.9	62.9	87	0.0	0.0	0.3
49	41.3	96.2	151.8	12.0	419.6	88.3	78.5	27.4	138.0	64.2	0.0	0.0	0.3
50	45.3	100	107.8	31.1	179.6	86.7	80.4	0.0	39.0	43.5	0.0	0.0	0.3
51	21.5	0.7	0.0	0.3	38.7	0.3	0.8	0.0	0.0	0.3	0.0	0.0	0.3
52	64.3	20.4	110.8	3.7	36.8	0.3	1.1	0.0	0.0	0.3	0.0	0.0	0.3
53	50.1	40.5	107.4	4.9	36.3	0.3	1.4	0.0	0.0	0.3	0.0	0.0	0.3
54	120.2	96.2	144.8	7.1	285.8	62.7	59.4	0.0	84.3	53.3	13.3	11.4	0.3
55	29.3	30.2	101.0	0.3	107.8	12.4	11.0	0.0	39.1	2.9	0.0	0.0	0.3
56	14.4	3.5	0.0	0.3	0.0	0.3	0.4	0.0	0.0	0.3	12.6	10.4	0.3
57	34.7	100	29.3	27.1	46.8	123.9	108.8	0.0	49.9	71.4	0.0	0.0	0.3
58	29.7	40.2	47.7	4.6	25.2	4.1	1.1	0.0	0.0	0.3	0.0	0.0	0.3
59	33.2	379	98.4	85.7	201.5	248.3	223.8	0.0	68.6	139.2	0.0	0.0	0.3
60	32.9	45.6	77.8	4.6	70.9	23.4	11.4	0.0	41.5	20.2	0.0	0.0	0.3
61	50.9	137	63.7	29.4	148.3	59.1	69.9	0.0	35.5	59.8	0.0	0.0	0.3
62	30.1	170	63.5	24.5	101.3	111.6	95.1	0.0	44.4	82.3	0.0	0.0	1.07
63	30.5	18	43.5	0.9	143.9	11.1	11.7	0.0	44.4	2.2	0.0	0.0	0.3
64	34.7	16.4	10.9	2.7	62.0	35.3	22.4	0.0	28.9	17.9	0.0	0.0	0.3
65	27.3	8.7	67.2	9.3	0.0	0.4	0.5	0.0	0.0	0.3	0.0	0.0	0.3
66	44.1	81.7	38.6	8.9	54.2	68.7	60.0	0.0	35.0	42.2	0.0	0.0	0.3
67	34.2	59.2	72.9	8.1	171.3	34.3	22.3	0.0	37.5	26.3	0.0	0.0	0.3
68	66.9	35.6	163.1	10.1	49.0	21.2	17.1	0.0	40.4	22.7	0.0	0.0	0.3
69	14.1	3	25.5	0.3	78.7	1.1	1.4	0.0	32.2	0.5	0.0	0.0	0.3
70	37.9	3	86.5	0.3	97.3	1.1	2.2	0.0	46.3	0.3	0.0	0.0	0.3
71	48.1	3.9	21.1	0.3	235.2	5.4	4.4	0.0	63.1	3.15	0.0	0.0	0.3
72	53.1	100	163.0	24.3	219.1	141.9	107.9	18.1	126.4	125.4	0.0	0.0	0.3
73	33.4	332	26.2	58.8	103.9	245.3	212.6	0.0	61.5	137.0	0.0	0.0	0.3
74	30.6	4.2	96.1	0.3	0.0	0.3	0.4	0.0	0.0	0.3	0.0	0.0	0.3
75	30.0	5.9	98.2	0.6	5.2	0.3	0.4	0.0	0.0	0.3	0.0	0.0	0.3
76	70.5	61.9	89.1	15.3	167.9	43.3	37.8	8.3	95.6	30.6	0.0	0.0	0.3
77	31.1	238	87.5	20.1	116.5	153.0	136.5	0.0	79.1	90.0	0.0	0.0	0.3
78	45.5	151	110.1	27.3	169.1	101.6	90.2	0.0	88.4	113.4	0.0	0.0	0.3
79	39.3	84.7	36.6	27.3	152.3	83.4	72.3	3.6	66.4	54.9	0.0	0.0	6.74
80	51.5	173	70.8	36.9	242.1	105.5	76.8	6.8	20.3	45.1	0.0	0.0	0.3
81	36.8	3.4	0.0	0.3	94.5	3.7	2.9	0.0	21.1	0.7	0.0	0.0	0.3
82	167.9	62.1	363.1	9.3	337.0	86.3	56.7	8.8	164.1	33.5	0.0	0.0	0.3
83	51.4	400	89.3	368.8	196.6	722.7	768.2	0.0	104.9	525.5	0.0	0.0	0.3
84	29.4	19.7	38.6	3.8	58.2	9.4	6.0	5.8	39.7	2.9	0.0	0.0	0.3
85	58.2	47	57.8	12.1	180.7	23.7	17.6	0.0	35.2	11.9	0.0	0.0	1.00
86	19.4	300	58.9	216.0	210.5	392.1	182.0	0.0	27.7	120.5	0.0	0.0	0.3
87	174.0	400	133.0	668.3	68.4	729.3	621.2	14.2	170.1	175.0	0.0	0.0	0.3
88	44.4	10.5	55.7	4.0	11.1	0.3	0.6	0.0	0.0	0.3	0.0	0.0	0.3
89	54.3	36.5	95.4	9.8	54.3	19.8	12.3	0.0	35.0	9.7	0.0	0.0	0.3
90	57.3	63.8	53.8	27.6	130.9	129.2	81.6	0.0	71.1	141.9	0.0	0.0	0.3
91	91.0	176	233.9	32.1	133.7	179.3	114.5	0.0	111.5	27.2	0.0	0.0	0.3
92	39.9	38.5	54.8	6.0	58.0	18.5	12.2	0.0	61.2	9.9	0.0	0.0	0.3
93	96.8	32.7	71.1	8.3	94.5	19.5	11.1	23.2	82.4	10.4	0.0	0.0	0.3
94	34.1	2.3	40.6	0.3	56.0	1.5	1.3	0.0	36.2	1.2	0.0	0.0	0.3
95	90.3	218	85.9	133.4	149.2	425.2	205.2	0.0	53.1	339.3	0.0	0.0	0.3
96	33.5	21.3	33.4	5.0	9.5	0.4	0.8	0.0	0.0	0.3	0.0	0.0	0.3
97	52.3	45.8	55.3	3.6	58.2	46.3	29.2	0.0	34.8	21.5	0.0	0.0	0.3
98	93.3	400	36.7	683.7	189.8	304.9	377.7	0.0	75.6	146.0	0.0	0.0	0.3

99	49.6	41.5	90.3	11.3	15.8	0.3	0.8	0.0	0.0	0.3	0.0	0.0	0.3
100	33.1	322	34.1	169.2	54.3	206.7	167.0	19.2	67.6	291.0	0.0	0.0	0.3
101	52.3	162	37.1	19.7	64.6	205.6	122.9	0.0	87.8	82.4	0.0	0.0	0.3
102	78.7	14.2	54.2	4.4	13.9	0.43	0.9	0.0	0.0	0.3	0.0	0.0	0.3
103	102.9	262	62.6	234.5	54.5	132.0	106.2	0.0	48.1	99.6	0.0	0.0	0.3
104	129.5	90.4	58.3	18.4	47.6	165.7	197.1	0.0	69.6	233.9	0.0	0.0	0.3
105	37.6	17.9	39.1	5.9	69.2	17.1	12.0	0.0	35.3	10.6	0.0	0.0	0.3
106	43.6	6.3	85.2	1.4	51.1	3.9	3.0	18.4	68.6	1.9	0.0	0.0	0.3