

ORIGINAL ARTICLE

Evaluation of two new recombinant guinea-pig lipocalins, Cav p 2 and Cav p 3, in the diagnosis of guinea-pig allergy

C. Hilger¹, K. Swiontek¹, S. Kler¹, C. Diederich², C. Lehnert³, L. Vogel⁴, S. Vieths⁴ and F. Hentges^{1,3}

¹Laboratory of Immunogenetics and Allergology, CRP-Santé, Luxembourg, Luxembourg, ²Department of Veterinary Medicine, University of Namur, Namur, Belgium,

³National Unit of Immunology-Allergology, Centre Hospitalier of Luxembourg, Luxembourg and ⁴Department of Allergology, Paul-Ehrlich Institut, Langen, Germany

Clinical & Experimental Allergy

Summary

Background Guinea-pigs, classical laboratory animals now often kept as pets, regularly elicit important allergic reactions. Two guinea-pig allergens have been described previously to some extent; however, biomolecular and immunological data are lacking.

Objective To identify major guinea-pig allergens, produce them as recombinant proteins and define their allergenic potential and clinical importance in allergic patients.

Methods Protein extracts were prepared from various guinea-pig tissues and allergens were purified by ion exchange chromatography. The N-termini of two immunoglobulin E (IgE)-reactive proteins were determined and degenerate primers were designed for cDNA amplification by RT-PCR. Allergenic properties of recombinant proteins were assayed by immunoblotting, ELISA and mediator release assays. Specific IgE were quantified in the sera of 26 guinea-pig-allergic patients.

Results Major IgE-reactive proteins were detected in submaxillary and harderian gland extracts. Two proteins were identified and the cDNAs were cloned. The 17 kDa protein expressed in the harderian gland corresponds to the previously described Cav p 2. The 19 kDa protein, Cav p 3, is a new allergen expressed in the submaxillary gland. Recombinant Cav p 2 and Cav p 3 were recognized by IgE antibodies from 65% and 54% of guinea-pig-allergic patients, respectively. Both proteins demonstrated equivalent allergenic activity in the mediator release assays.

Conclusion and Clinical Relevance Two major guinea-pig allergens, Cav p 2 and Cav p 3, have been characterized and produced as recombinant proteins. Combined to guinea-pig serum albumin, the new allergens proved to be valuable in the component-resolved diagnosis of guinea-pig allergy.

Keywords allergen, guinea-pig, guinea-pig serum albumin, harderian gland, lipocalin, submaxillary gland

Submitted 4 August 2010; revised 28 January 2011; accepted 31 January 2011.

Correspondence:

Dr Christiane Hilger, Laboratory of Immunogenetics and Allergology, CRP-Santé, 84 Val Fleuri, L-1526 Luxembourg. E-mail: christiane.hilger@crp-sante.lu

Introduction

Animal allergens are an important cause of inhalant allergic diseases. In regions of dry or cold climate with low levels of house dust mites, allergens of domestic pets are the major sensitizing agents and an important risk factor for asthma [1]. About one-third of United States and European house-

holds have mammalian pets. Cats and dogs are the preferred indoor animals, followed by rabbits and guinea-pigs. Mice represent about half of all laboratory animals. They are followed in number by rats, rabbits and guinea-pigs [2]. About one-third of animal workers develop work-related allergic symptoms [3]. In a large Japanese epidemiological study, allergic symptoms were reported in 26% of workers exposed to mice, compared with 25% for rats, 30% for cats and 31% for guinea-pigs [4]. As about two-thirds of the subjects were handling more than one species, these figures might only be indicative for each species. Allergic symptoms typically include asthma, rhinitis and conjunctivitis, as well as contact urticaria or eczema.

Data submission:

The cDNA sequences coding for Cav p 2 and Cav p 3 have been submitted to the EMBL database (FN256284 and FN 256285). Data will be kept confidential until acceptance of the manuscript.

The majority of known respiratory allergens derived from mammals belong to the lipocalin protein family [5]. Lipocalins are a group of proteins ubiquitously present in animals, plants and bacteria [6]. They share common biological properties such as binding and transport of small hydrophobic molecules. The primary sequence of lipocalins is highly variable, but they have a common tertiary structure, which is characterized by a central, eight-stranded β -barrel forming an internal ligand-binding pocket [7].

Guinea-pig allergens have been detected in dust, dander, fur, urine and saliva [8]. Two allergens of 20 and 17 kDa, Cav p 1 and Cav p 2, have been purified from hair extract [9, 10]. The 15 N-terminal amino acids have been determined and the presence of a GxW motif in their protein sequence allowed assigning the two allergens to the lipocalin protein family. Up to now, no purified or recombinant guinea-pig allergens are available for research or diagnostic purposes. However, the characterization and availability of major allergens are important prerequisites for setting up a successful immunotherapy.

In the present study, we have characterized two allergens from *Cavia porcellus*, the guinea-pig. The corre-

sponding cDNAs were cloned and recombinant allergens were expressed in *Escherichia coli*. One of the allergens corresponds to the previously described Cav p 2.0101; the other one is a new allergen that was designated Cav p 3.0101 by the WHO/IUIS Allergen Nomenclature Subcommittee. Specific immunoglobulin E (IgE) antibodies to the recombinant proteins were quantified using the sera of a cohort of 26 guinea-pig-allergic patients who had become sensitized in a domestic setting. The specificity of allergens was determined using sera of cat- and dog-allergic patients.

Methods

Patients and sera

For allergen characterization, sera of 27 persons with inhalant allergy to guinea-pig and medium to high (18 over 100 kU/L) specific IgE were selected from the outpatient population of the Luxembourgish National Immunology-Allergology Unit (Table 1). The age of the patients ranged from 4 to 70 years (mean age 35, median 34). All

Table 1. Guinea-pig-allergic patients

Patient	Age	Sex	Clinical symptoms	Guinea-pig epithelium e6 kU _A /L	Cat epithelium and dander e1 kU _A /L	Dog dander e5 kU _A /L	Pets at home	Cav p 2 kU _A /L	Cav p 3 kU _A /L	GPA kU _A /L
1	45	F	A, R, C	> 100	24.0	8.2	g.-pig, cat, dog	9.7	4.7	6.8
2	48	M	A, R, E	> 100	ND	ND	g.-pig	34.9	0.0	ND
3	70	F	A	> 100	ND	ND	g.-pig	5.9	6.0	ND
4	31	F	A,R	> 100	0.0	8.1	g.-pig*, dog	ND	ND	ND
5	46	F	A, R	> 100	NI	0.4	g.-pig	117.0	58.2	ND
6	47	F	A	91.0	9.3	3.3	g.-pig	25.4	43.1	ND
7	28	F	A	51.7	0.0	0.0	g.-pig, cat	0.0	0.0	11.3
8	32	F	A	47.5	0.0	0.0	g.-pig	12.2	6.8	ND
9	47	F	A, R	77.4	7.4	0.8	g.-pig, cat	0.0	0.0	0.8
10	40	M	R, C	74.6	0.0	21.1	g.-pig	33.7	1.0	ND
11	50	M	A, R, C	90.0	0.6	4.0	g.-pig	16.9	0.0	ND
12	15	M	A, R, U	44.0	0.5	0.7	g.-pig, cat, dog	0.0	0.3	0.0
13	52	F	Rs ns	25.0	ND	0.0	NI	3.3	0.7	0.2
14	28	M	A	23.1	4.9	3.6	g.-pig	0.0	0.0	0.0
15	35	F	A	62.3	10.6	2.1	g.-pig*, cat	0.0	0.0	0.0
16	23	M	A	23.4	> 100	> 100	dog	0.0	0.0	2.0
17	4	F	Rs ns	24.0	0.1	2.3	NI	4.9	3.2	1.0
18	51	F	A, R	43.0	0.0	1.5	g.-pig	0.0	0.0	0.4
19	33	F	A, R	34.0	76.0	90.0	no animal	0.0	0.0	1.7
20	40	F	A, R	> 100	21.0	48.0	g.-pig	280.0	0.0	1.4
21	24	F	R	58.0	4.0	9.1	g.-pig cat, rabbit	6.8	2.7	2.1
22	34	F	A	> 100	4.3	28.0	g.-pig	74.0	66.0	13.2
23	4	M	Rs ns	39.0	0.0	0.0	NI	9.1	0.9	0.7
24	19	F	R	18.0	1.7	0.9	g.-pig	0.0	0.0	1.7
25	23	F	R, C	72.0	1.5	0.8	g.-pig	14.8	5.6	3.4
26	41	F	A	47.0	0.0	0.0	g.-pig	0.9	3.2	2.5
27	28	F	A, R	86.0	0.0	1.9	g.-pig	31.0	0.0	0.4

*Occasional contact with guinea-pig.

A, asthma; R, rhinitis; C, conjunctivitis; U, urticaria; E, eczema; Rs ns, respiratory symptoms not specified; NI, no information; ND, not done; g.-pig, guinea-pig.

had positive skin prick tests to a commercial guinea-pig (hair/dander) extract (Stallergenes, Anthony Cedex, France) and/or specific IgE to guinea-pig epithelium (e6) quantified by ImmunoCAP (Phadia, Uppsala, Sweden). Twenty of the patients were women. At the time of diagnosis, 20 patients had a guinea-pig at home and two more persons had occasional exposure to guinea-pigs. Twenty patients had asthma and in 10 of these, guinea-pigs were the only allergic trigger that could be linked to the asthma attacks. Fourteen patients had rhinitis, four conjunctivitis, one urticaria, one eczema and three non-specified respiratory symptoms.

IgE to recombinant guinea-pig allergens were determined by ELISA in 26 patients. Sera of 23 non-allergic persons with low total IgE (range 2–10 kU/L) were collected to serve as controls. Another control group consisted of 22 patients with specific IgE to pollen or mite extracts, but negative to animal dander, total IgE range 16–370 kU/L. A group of cat- and dog-allergic patients ($n=16$, mean IgE to cat dander 54.2 kU_A/L (range 6.6–>100), mean IgE to dog dander 26.6 kU_A/L (range 0.7–>100) was recruited to test the specificity of the recombinant allergens. Specific IgE were determined by ImmunoCAP using e1 (cat dander), e5 (dog dander), e6 (guinea-pig epithelium) and e220 [cat serum albumin (CSA)]. The study was approved by the National Committee for Medical Research Ethics (Ref. 201001/06).

Source material

All extracts were derived from postpubescent male Dunkin Hartley guinea-pigs. Harderian and submaxillary glands were harvested immediately after sacrifice and frozen at -20°C for protein extraction or conserved in RNAlater (Qiagen, Hilden, Germany) for RNA extraction. Hair was cut using scissors. Animal handling met the European guidelines for experimental animals.

Preparation of protein extracts

Unwashed hair was extracted with phosphate-buffered saline (PBS) containing protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche, Mannheim). Gland tissue was frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle and suspended in lysis buffer (50 mM TRIS-HCl pH 8, 150 mM NaCl, 1% triton X-100, protease inhibitors).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE under reducing conditions. Immunoblotting was performed as described [11], with the following modification: patient sera were diluted 1/10 in blocking buffer containing 2% cold water fish gelatine (CWFG; Sigma-Aldrich, Bornem, Belgium).

Proteins were assayed for the presence of glycosylation using a commercial kit (ProQ Emerald 300, Pierce, Erembodegem, Belgium). 2D-gels were performed with immobilized pH gradient strips according to the manufacturer's instructions (Bio-Rad, Nazareth, Belgium).

Protein sequencing

The N-terminal sequence analysis of purified proteins was performed by automated Edman degradation on a Procise 49X HT Protein sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

DNA and protein sequence analysis, database searches and multiple alignments were performed using Heidelberg Unix Sequence Analysis Resources (HUSAR, DKFZ, Heidelberg, Germany). Secondary structures were predicted using 2DSweep [12].

Molecular cloning of complementary deoxyribonucleic acid encoding guinea-pig allergens

Total RNA was extracted from the submaxillary and harderian glands using the RNeasy lipid tissue kit according to the manufacturer's instructions (Qiagen). cDNA was obtained using the SMART RACE cDNA amplification kit (Clontech, St-Germain-en-Laye, France).

Degenerate primers were designed based on the N-terminal amino acid sequences obtained from purified Cav p 2 and Cav p 3. 3'RACE was performed on RNA extracted from the submaxillary gland and PCR amplification products were cloned into pCR2.1-TOPO (Invitrogen, Merelbeke, Belgium) for sequence analysis. Sequences obtained using the primers Cavp2b (5' AAGTNCNGGNAATTGGCG 3') and Cavp3h (5' GARATHAAYGGNCAATGGAATAC 3') allowed to design specific reverse primers for amplification of the 5' end by 5'RACE. The open reading frame coding for the mature protein was cloned into the expression vector pQE60 (Qiagen).

The Cav p 2 open reading frame was also amplified from the harderian gland using gene-specific primers. The cDNA was cloned and compared with the Cav p 2 cDNA obtained from the submaxillary gland by DNA sequence analysis.

Expression and purification of recombinant (r)Cav p 2 and rCav p 3

cDNAs coding for mature proteins Cav p 2 and Cav p 3 were expressed in *E. coli* Rosetta-gami cells (Merck, Darmstadt, Germany) as recombinant proteins with a C-terminal hexahistidine tag. Bacteria were induced at an optical density of 0.6 at 600 nm using 1 mM isopropyl- β -D-thiogalactopyranoside (Roth, Karlsruhe, Germany) for 4.5 h at 30°C . Bacteria were collected by centrifugation

and resuspended in lysis buffer (20 mM NaH_2PO_4 , 500 mM NaCl, 10 mM imidazol pH 7.4). Cells were lysed by adding lysozyme at 1 mg/mL for 30 min at 4 °C. Benzonase (Merck) was added for 30 min at room temperature to reduce sample viscosity. The recombinant proteins were purified by immobilized metal ion affinity chromatography (HisTrap HP, GE Healthcare, Buckinghamshire, UK) under native conditions according to the instructions of the manufacturer. Cav p 2 and Cav p 3 were eluted by an imidazol gradient (20 mM NaH_2PO_4 , 500 mM NaCl, 10–500 mM imidazol pH 7.4) at 50 and 185 mM imidazol, respectively. Protein yield was 21.6 mg/L for rCav p 2 and 2.2 mg/L bacterial culture for rCav p 3. Fractions were pooled, dialysed against PBS and concentrated by centrifugation using Ultrafree concentrators (Millipore, Billerica, MA, USA). The purity of the recombinant proteins was analysed by SDS-PAGE and silver staining. Identity was confirmed by N-terminal sequencing.

Quantification of immunoglobulin E antibodies by enzyme-linked immunosorbent assay

Specific IgE were quantified by ELISA as described [11] using purified rCav p 2, rCav p 3 and guinea-pig serum albumin (GPA) at a concentration of 5 µg/mL PBS. Patient sera were serially diluted into blocking buffer containing 2% CWFG. The cut-off was determined as the mean optical density at 405 nm for the negative controls plus 3 SD. In order to give a specific IgE titre instead of optical density for each patient's serum, we used as a reference standard the serum of a patient with a known titre of IgE specific for CSA (Sigma), determined by Phadia CAP-System (Phadia). This serum was serially diluted and added to CSA-coated wells. A standard curve was generated that allowed calculating specific IgE titres to guinea-pig allergens.

Enzyme-linked immunosorbent assay and ImmunoCAP inhibition experiments

ELISA inhibition experiments were performed by incubating the sera with 100 µg of Cav p 2 or Cav p 3/mL for 2 h at room temperature before adding to the plate. IgE to guinea-pig epithelium (e6) were inhibited by adding CSA to the serum at a final concentration of 1 mg/mL and incubating at 4 °C overnight.

Mediator release from rat basophilic leukaemia cells

The stimulation of rat basophilic leukaemia (RBL)-2H3 cells, transfected with the α -chain of the human high-affinity IgE receptor (clone RBL-703/21), was performed as described [13]. Human sera were added to the cells in a final dilution of 1/10–1/40 and incubated overnight. Antigen-specific release was quantified by measuring β -hexosaminidase activity and expressed as the percentage

of total release that was obtained by lysis of the cells. Results were corrected for spontaneous release (serum added without allergen).

Purification of Cav p 2 and Cav p 3 from guinea-pig hair and submaxillary gland

Cav p 2 was purified from guinea-pig hair and Cav p 3 from the submaxillary gland. Protein extracts in PBS were dialysed into 20 mM TRIS-HCl, pH 8 and separated by anion exchange chromatography (RESOURCE Q column, GE Healthcare). Bound proteins were eluted by a linear gradient of 0–0.5 M NaCl. Fractions were analysed by SDS-PAGE, followed by silver staining and by immunoblotting with patient or mouse serum. Fractions containing Cav p 3 were further purified by gel filtration using Superdex 75 (GE Healthcare). Pure GPA was obtained as a by-product during Cav p 3 isolation.

Production of mouse sera to rCav p 2 and rCav p 3

Polyclonal mice sera were raised in BALB/c mice by an intraperitoneal injection of either 50 µg purified rCav p 2 or rCav p 3 suspended in $\text{Al}(\text{OH})_3$ (Sigma). Three boosts at a 3-week interval were administered subsequently. Sera were harvested 3–10 days after the last boost by an intracardiac blood puncture. Animal experiments were performed according to national regulations and approved by the Ministry of Health (Ref. RM/SD816/05).

Results

Detection of immunoglobulin E reactive proteins in salivary and lacrimal gland extracts

IgE antibodies of guinea-pig-allergic patients detected intense protein bands in hair extract in the 15–24 kDa molecular weight range (data not shown). A number of organs were screened for allergen expression. Submaxillary and harderian gland extracts showed strong IgE binding in the 15–20 kDa range for several patients (Figs 1a and b). Submaxillary gland extract was separated by 2D-SDS-PAGE and an IgE-reactive spot at 19 kDa with a pI of 5.2 was isolated and subjected to N-terminal sequencing. The amino acid sequence obtained was GQTLDPSEINGQxxT. The extract of the harderian gland was also subjected to 2D-SDS-PAGE and an immunoreactive spot at 17 kDa corresponding to a pI of 4.3 was isolated. N-terminal sequencing provided the sequence DSDYSKVPGNWRTI, which proved to be identical to the sequence of a previously identified protein, Cav p 2, isolated from hair [9].

Preparation of recombinant and native Cav p 2 and Cav p 3

Degenerate primers were designed on the basis of the N-terminal sequences. DNA fragments were amplified from

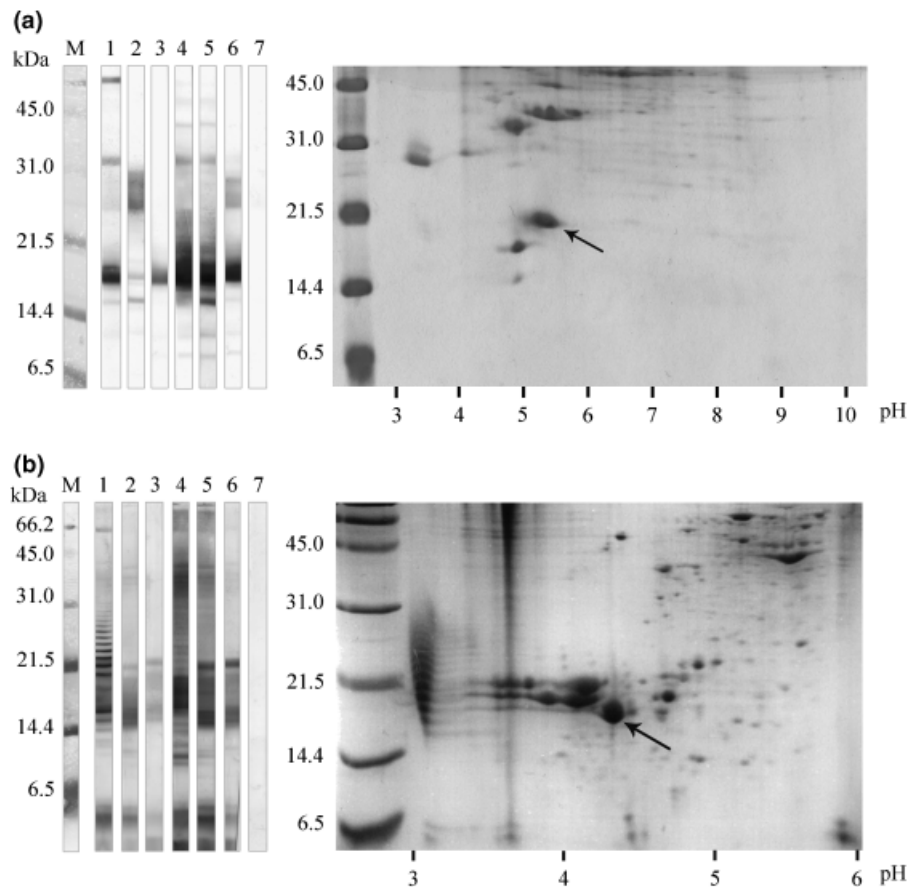


Fig. 1. Allergen detection in protein extract of guinea-pig submaxillary (a) and harderian gland (b). Proteins were separated by 20% SDS-PAGE and analysed in IgE immunoblots with patient sera. Submaxillary gland (45 µg) and harderian gland (150 µg) extracts were analysed by 2D gels. Arrows designate the spots analysed by N-terminal sequencing. M, Coomassie-stained molecular weight marker; lanes 1–6, patient's sera; 7, negative control.

the submaxillary gland. The combination of the 5' and 3' end amplifications provided the complete cDNA sequences. cDNA FN256285 encodes a new *C. porcellus* allergen, which was designated Cav p 3.0101 by the WHO/IUIS Allergen Nomenclature Sub-Committee. Analysis of the deduced amino acid sequence detected a putative secretory signal peptide with a predicted cleavage site corresponding to the site determined by N-terminal sequencing. Cleavage of the signal peptide results in a 155 amino acid mature protein with a predicted molecular weight of 17.5 kDa and an isoelectric point of 5.15.

Sequence analysis of Cav p 2 cDNA (FN256284) predicted a secretory signal peptide and a cleavage site corresponding to the N-terminus of Cav p 2. The mature protein has a length of 154 amino acids, a predicted molecular weight of 17.1 kDa and an isoelectric point of 4.27. There are no putative asparagine-linked glycosylation sites on Cav p 2 or Cav p 3. The absence of glycosylation was confirmed for purified native Cav p 2 and Cav p 3 using a commercial glycoprotein staining kit (data not shown).

Native Cav p 2 was isolated from guinea-pig hair by anion exchange chromatography. Cav p 3 was purified

from submaxillary gland extract by anion exchange chromatography, followed by gel filtration. Cav p 2 runs as a broad 17–19 kDa band under reducing conditions (Fig. 2a). Cav p 3 migrates as a single 19 kDa band (Fig. 2a). A 66 kDa band was copurified with Cav p 3. This band reacts strongly with patient's serum (Fig. 2b). The protein could be identified as GPA using a mouse anti-GPA antibody and N-terminal sequencing (data not shown). Running Cav p 2 and Cav p 3 under non-reducing and reducing conditions induces a mobility shift, suggesting that the cysteines in the protein form disulphide bonds.

Recombinant proteins were expressed in *E. coli* with a C-terminal hexahistidine tag without a signal peptide. The theoretical molecular weight of the recombinants is about 1.2 kDa higher than that of the native molecules due to the tag (Fig. 2a).

Cav p 2 and Cav p 3 are lipocalins

Amino acid sequences of Cav p 2 and Cav p 3 were aligned and compared with other, similar mammalian allergens present in protein databases (Fig. 3). Both proteins were

clearly assigned to the lipocalin protein family and more specifically to the odorant/pheromone-binding protein family. The lipocalin protein signature GxW was found in the N-terminal region of both proteins. Another motif, YxxxYxG, almost completely conserved on lipocalins [14], is found at positions 75–81. Nine β -strands are predicted for Cav p 2 and Cav p 3, which are all consistent with the β -strand positions determined for Bos d 2 [15]. A helix is located between H and I, corresponding to the conserved α -helical C-terminal structure of lipocalins [7]. Significant amino acid identities were found between Cav p 2 and Bos d 2 (39%), Equ c 1 (36%), Fel d 4 (34%), Rat n 1 (31%) and Mus m 1 (30%). Similarly, Cav p 3 aligned well with Bos d 2 (40%), Fel d 4 (32%), Equ c 1 (30%) and Rat n

1 (27%). Cav p 2 and Cav p 3 share 43% identical amino acid positions. Cys⁵⁹ and Cys¹⁵² are conserved in all seven lipocalins. From resolved lipocalin structures, they are known to form an intramolecular disulphide bridge [15, 16]. Another disulphide bridge, located in the loop between strands B and C in Bos d 2 [15], could also be formed by Cys⁴⁰ and Cys⁴⁴ found in Cav p 2 and Cav p 3. An additional cysteine is present in Cav p 2 at position 89.

Reactivity of patient sera with native and recombinant Cav p 2 and Cav p 3

Native Cav p 2 and Cav p 3 are recognized by IgE antibodies of serum from patient No 1 (Fig. 2b). An additional IgE-reactive band was detected in the purified Cav p 2 sample at 24 kDa and identified as Cav p 2 by N-terminal sequencing. Conformational changes are likely to be the explanation for this migration pattern. Recombinant rCav p 2 and rCav p 3 are recognized by IgE antibodies of patient no 1 (Fig. 2b) and by the respective mouse sera (Fig. 2c). Patient and mouse serum detected a 24 kDa band, which is hardly visible in the silver-stained rCav p 2 preparation.

In order to evaluate the clinical relevance of the new allergens, 26 sera of the 27 guinea-pig-allergic patients were tested for IgE reactivity to rCav p 2 and rCav p 3 by ELISA (Fig. 4). From 24 patients, detailed clinical data were available (Table 1). Seventeen out of 26 patients (65%) had IgE antibodies to rCav p 2, 14/26 (54%) had IgE antibodies to rCav p 3 and 50% had IgE antibodies to both rCav p 2 and rCav p 3. Nineteen patients were tested for specific IgE to GPA and 10 of these had IgE > 1 kU_A/L.

The ability of rCav p 2 and rCav p 3 to induce basophil degranulation was studied using a rat basophil cell line expressing the human high-affinity IgE receptor and sera

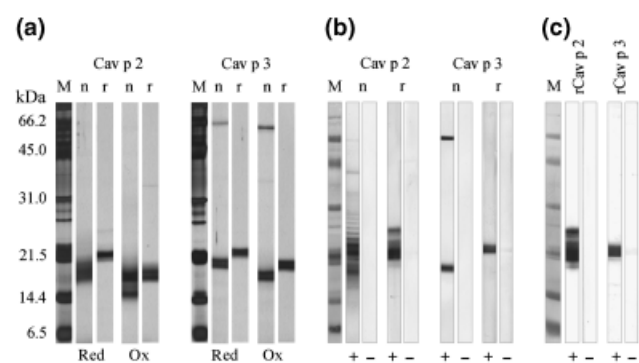


Fig. 2. Analysis of native and recombinant Cav p 2 and Cav p 3 by SDS-PAGE and immunoblot. (a) Native (n) and recombinant (r) Cav p 2 and Cav p 3 (200 ng) were analysed by 15% SDS-PAGE under reducing (red) and non-reducing (ox) conditions and silver-staining. (b) Native (n) and recombinant (r) proteins (1 µg) were separated under reducing conditions and analysed by immunoblot using patient serum No 1. (c) rCav p 2 and rCav p 3 are detected in immunoblot by mouse polyclonal sera directed against rCav p 2 and rCav p 3, respectively. M, molecular weight marker; lanes n, native; lanes r, recombinant proteins; +, serum added; -, negative control.

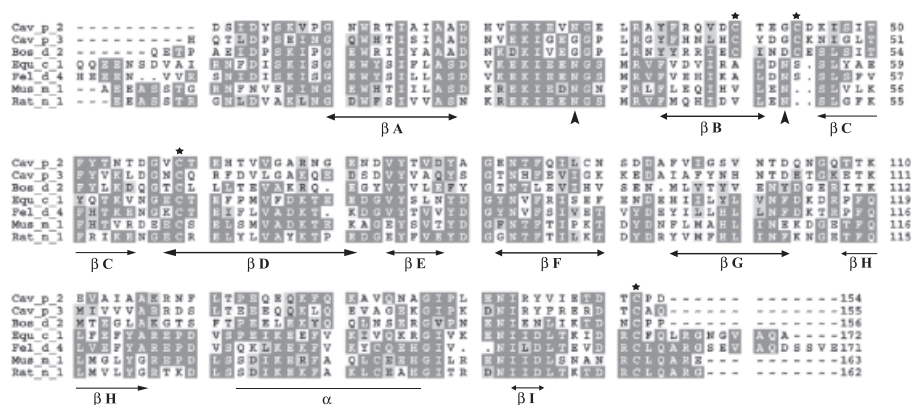


Fig. 3. Sequence alignment of Cav p 2 and Cav p 3 proteins with homologous mammalian lipocalin allergens. Cav p 2 and Cav p 3 were aligned with Bos d 2 (Q28133), Equ c 1 (Q95182), Fel d 4 (Q5vfh6), Mus m 1 (P02762) and Rat n 1 (P02761) using Clustal W. Residues identical to the consensus are shaded in dark grey and residues similar to consensus in light grey. The location of the eight β -strands (A–H) composing the central barrel and the additional β -strand at the C-terminus (I) correspond to the ones determined on the crystal structure of Bos d 2 (1bj7). They are indicated by → indicates an α -helix, * conserved cysteines, ▲ predicted glycosylation sites at N²⁸ (Equ c 1, Fel d 4, Rat n 1) and N⁴³ (Equ c 1, Fel d 4). The numbering of the cysteines and glycosylation sites corresponds to the numbering of the Cav p 2 sequence.

from four guinea-pig-allergic patients. Both lipocalins induced a comparable release with saturation reached at 10 ng/mL (Fig. 5). Serum of patient no 20, who had specific IgE to rCav p 2, but not to rCav p 3, was not able to induce basophil degranulation in the presence of rCav p 3. No mediator release was obtained when using serum of a mite-allergic patient (data not shown).

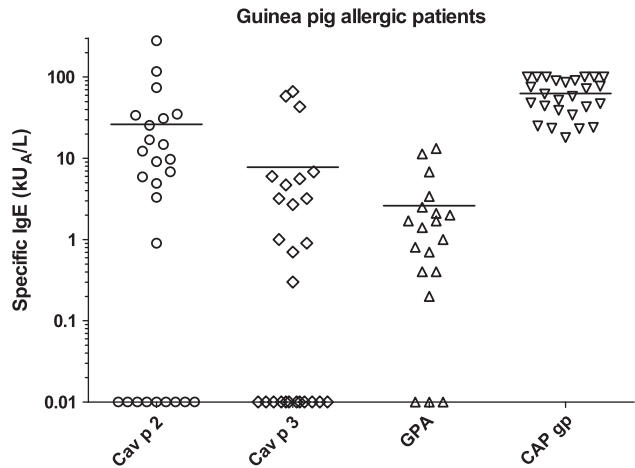


Fig. 4. Quantification of IgE specific for rCav p 2, rCav p 3 and guinea-pig serum albumin (GPA) by ELISA. rCav p 2, rCav p 3 and GPA were coated to a microplate. IgE antibodies against rCav p 2 and rCav p 3 were determined for 26 guinea-pig-allergic patients. IgE antibodies against GPA were quantified for 19 of them. A line marks the mean kU_A/L for each allergen. CAP values were limited to 100 kU_A/L. The cut-off for positivity (mean of 23 controls + 3 SD) was determined to be 0.12 kU_A/L for rCav p 2 and 0.01 for rCav p 3 and GPA.

Analysis of cross-reactivity and specificity of Cav p 2 and Cav p 3

The sera of six patients presenting IgE antibodies to rCav p 2 and rCav p 3 were tested for IgE cross-reactivity between the two allergens using saturating quantities of inhibitor (Table 2). The addition of rCav p 2 as an inhibitor did not substantially change IgE binding to rCav p 3. The addition of rCav p 3 resulted in a 16–28% loss of IgE binding in four of the six patients.

IgE to rCav p 2 and rCav p 3 were sought in a group of pollen- or mite-allergic patients. No reactivity to rCav p 2 or rCav p 3 was detected in these patients. In the pollen/mite-allergic group, the mean IgE to rCav p 2 and rCav p 3 were 0.07 and 0.009 kU_A/L, respectively (data not shown). When comparing the group of guinea-pig-allergic patients with a group of cat- and dog-allergic patients, IgE to rCav p 2 and rCav p 3 were negative, with one exception.

Table 2. ELISA inhibition

Patient No.	% IgE binding to rCav p 2 in the presence of rCav p 3*	% IgE binding to rCav p 3 in the presence of rCav p 2*
1	104	99
3	82	98
5	96	96
6	83	98
8	72	85
22	84	111

*The percentage of IgE binding was calculated from the mean OD values measured with or without addition of the inhibitor allergen.

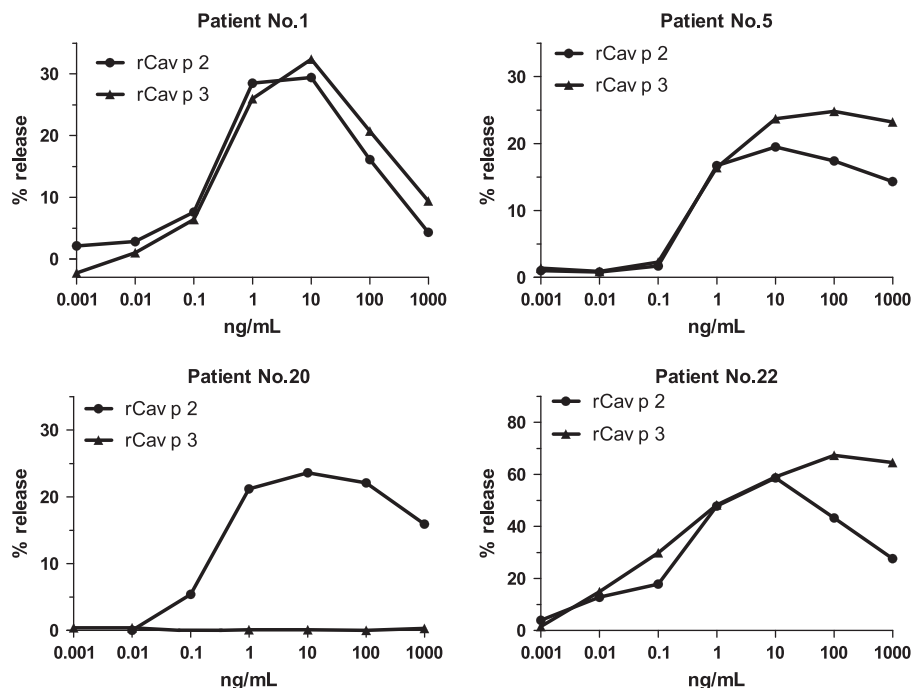


Fig. 5. Mediator release from RBL cells induced by rCav p 2 and rCav p 3. RBL-703/21 cells were sensitized with sera from four guinea-pig-allergic patients and incubated with different concentrations of rCav p 2 and rCav p 3.

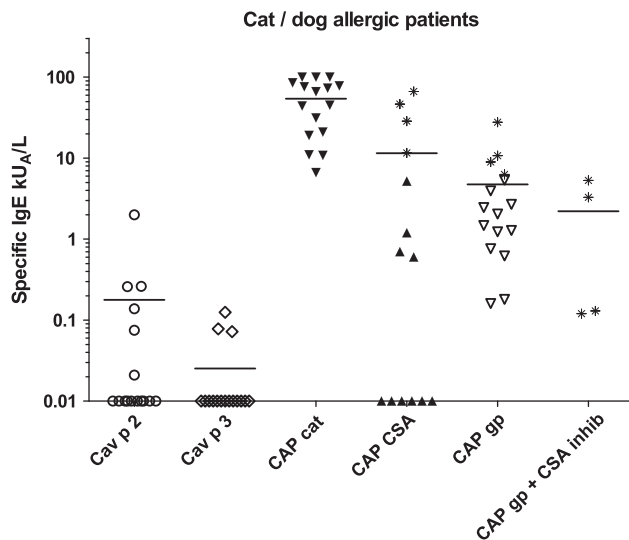


Fig. 6. Determination of specific immunoglobulin E (IgE) to guinea-pig epithelium and allergen components in cat- and dog-allergic patients. IgE antibodies against rCav p 2 and rCav p 3 were determined for 16 patients allergic to both cat and dog. Allergen-specific IgE were also quantified by ImmunoCAP e1 (cat), e6 (guinea-pig) and e220 [cat serum albumin (CSA)]. Specific IgE to guinea-pig epithelium e6 were inhibited with CSA in four cat-allergic patients (CAP gp + CSA inhib). These data points are marked with *. A line marks the mean kU_A/L for each allergen. CAP values were limited to 100 kU_A/L.

When using the Mann–Whitney test, *P*-values were highly significant (*P* = 0.0017 and *P* = 0.0032, respectively).

For control purposes, we performed a commercial specific IgE test to guinea-pig epithelium in the group of cat- and dog-allergic patients. Surprisingly, this assay was positive in 14 out of the 16 patients (range 0.6–27.7) (Fig. 6). We then tested IgE reactivity to CSA as we suspected a cross-reactive molecule to be responsible for this reactivity. Eight out of 16 cat- and dog-allergic patients had IgE to CSA. CAP inhibitions were performed with CSA in those four patients with the highest IgE antibody titre to CSA. Specific IgE to guinea-pig epithelium declined in all four patients from 9.0 to 0.1, 27.7 to 3.3, 10.7 to 5.3 and 6.3 to 0.1.

Discussion

Guinea-pigs have a high allergenic potential. They were shown to induce sensitization and allergic symptoms in a high percentage of exposed laboratory animal workers [4]. Allergic symptoms are often severe. In our study of patients highly sensitized to guinea-pigs due to domestic exposure, 83% of the patients had asthma. In 10 of the asthmatic patients (50%), complaints were concordant with a single exposure to guinea-pigs. This is higher than in animal handlers, where respiratory symptoms were found in about 28% of persons [4]. Possibly, the duration of daily exposure, which is longer when guinea-pigs are kept at home, plays a role. 20 of the 27 patients were

women. The important prevalence of women might be in part due to the fact that women tend to be exposed for longer durations to guinea-pig dander (four were housewives and one did the cleaning in a home having guinea-pigs). Symptoms of severe acute laryngitis have been reported upon exposure to guinea-pigs in a non-work-related situation [17]. The overall prevalence of allergy to guinea-pigs is increasing as guinea-pigs are more frequently kept as pets. Biomolecular characterization of guinea-pig allergens is important to enable an optimal and detailed diagnosis.

In this study, we have cloned two cDNAs coding for guinea-pig allergens. The N-terminus, molecular weight and pI of one of these allergens are identical to the respective values of Cav p 2 reported by Fahlbusch et al. [9]. It was therefore designated Cav p 2.0101 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

The cDNA coding for the other allergen expresses a protein with some similarity to the 15 N-terminal amino acids described for Cav p 1 [10]. However, in our protein, five of these amino acids are different and six additional residues are preceding the N-terminus determined previously. The protein is assumed to be different from Cav p 1 and it is therefore designated Cav p 3.0101. Cloning of the cDNA coding for Cav p 1 will finally elucidate whether both allergens are different or isoallergens. According to the nomenclature, isoallergens are defined as allergens from a single species with 67% or greater amino acid identity [18].

Sequence alignments and database searches clearly identified both allergens as lipocalins. They possess the first of three possible lipocalin signature motives that is characteristic for outlier lipocalins.

Cav p 2 and Cav p 3 have been cloned from the submaxillary gland by RT-PCR using degenerate primers. The protein spot of Cav p 2 however was isolated from a harderian gland protein extract. No similar spot was identified in the submaxillary gland extract. We suppose that Cav p 2 is expressed at a low level in this tissue. We performed a Cav p 2 cloning experiment using RNA from the harderian gland to confirm that the protein is synthesized in this tissue. The cDNA sequences retrieved corresponded exactly to the clones obtained from the submaxillary gland (data not shown). Cav p 2 is the first allergen shown to be expressed in the harderian gland. The harderian gland is a lacrimal gland present in most terrestrial vertebrates. In rodents, the gland synthesizes lipids, porphyrins and indoles. Hormonal control and marked sex differences of the harderian gland might indicate a function as a producer of social odours [19].

The allergenic potential of both allergens was analysed *in vitro*. Mediator release was dose-dependent and reached a plateau around 10 ng/mL for each allergen. To evaluate the importance of the two cloned allergens in allergic reactions to guinea-pig, recombinant Cav p 2 and

Cav p 3 were used to quantify specific IgE of 26 guinea-pig-allergic patients by ELISA. IgE antibodies to rCav p 2 were detected in 65% of the patients; 54% had IgE antibodies to rCav p 3. Cross-reactivity between rCav p 2 and rCav p 3 was evaluated by cross-inhibition ELISA. A minor decrease in IgE binding was observed with some patient sera using high doses of rCav p 3. Although Cav p 2 and Cav p 3 seem to be independent allergens with low sequence identity (43%), limited cross-reactivity might be observed depending on the patient's IgE epitope profile. The prevalence of IgE directed to Cav p 2 and Cav p 3 is high, but IgE levels are not elevated. IgE titres to Fel d 4, the cat lipocalin, have been reported to be low [20]. Similarly, few patients react exclusively to the dog lipocalins Can f 1, Can f 2 or Can f 4 [21]. So far, lipocalins might not be able to generate strong IgE responses. The only mammalian respiratory allergen known to elicit dominant reactions is Fel d 1, an uteroglobin [22].

One-third of the patients did not react to any of these allergens. Fahlbusch et al. [10] reported that a majority of patients recognized the 20 kDa allergen Cav p 1. We hypothesize that a number of the Cav p 2- and Cav p 3-negative patients possibly recognize this allergen. In addition, about one-third of our patients had IgE specific for GPA. Mammalian serum albumins are minor allergens but they play an important role in IgE cross-sensitizations to different furred pets [23]. In contrast, the presence of IgE directed to Cav p 2 and Cav p 3 seems to be a specific marker for guinea-pig sensitization. In a group of 16 cat- and dog-allergic patients, IgE to rCav p 2 and rCav p 3 were below 0.35, with one exception. In contrast, 14 of those patients were rated positive using a commercial assay against guinea-pig epithelium. When these sera were then tested for specific IgE to CSA, half of them were positive. Inhibition experiments confirmed that the positive results to the guinea-pig ImmunoCAP were probably due to cross-reactivity between CSA and GPA contained in the guinea-pig extract.

We have characterized two major guinea-pig allergens, Cav p 2 and Cav p 3, the first being secreted by the harderian gland and the second by the submandibular gland. Both are lipocalins and increase the already im-

pressive family of inhalant animal lipocalin allergens. The reason why animal lipocalins are the main inhalant allergen group from pets and farm animals characterized so far is still elusive. It can hardly be due to the identities of their protein sequences. Amino acid identities between different lipocalins vary between 25% and 40%. Whether the reason for the allergenicity lies in the nature of the ligands they carry and their biological function has still to be worked out. It could also simply be due to the fact that lipocalins often are secreted and can be taken up together with other stimulants that gear the immune response to an allergy-prone response. Another current working hypothesis is that endogenous human lipocalins prevent strong T cell responses to animal lipocalins due to thymic deletion of T cells with high-affinity receptors. The stimulation of the remaining T cells carrying low-affinity receptors would favour a Th2-deviated immune response [24].

A majority of patients sensitized to animal allergens have positive tests to more than one animal. Generally, it is not clear whether there are several independent sensitizations or whether the multiple positive tests are due to cross-reactive allergens as for instance mammalian serum albumins. Even if rodent pets such as guinea-pigs are increasingly implicated in inhalant allergies, sensitization to cat or dog is much more prevalent. It is therefore very relevant to clearly show that positive tests are due to a sensitization to guinea-pig allergens and not to cross-reactions with cat and dog allergens. This is not possible with difficult-to-standardize conventional extracts [25], which lack any analytical power.

The introduction of commercial microassays for specific IgE quantification requires the availability of representative allergens of each allergen source [26]. The use of Cav p 2 and Cav p 3, the lipocalin allergens isolated in this study, in combination with GPA are a first step towards a component-resolved diagnosis of guinea-pig allergy.

Acknowledgements

Conflict of interest statement: none of the authors has declared a conflict of interest.

References

- 1 Custovic A, Simpson A, Woodcock A. Importance of indoor allergens in the induction of allergy and elicitation of allergic disease. *Allergy* 1998; 53: 115–20.
- 2 European Commission. Fifth Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union. Report from the Commission to the Council and the European Parliament. COM, 2007:1–14.
- 3 Bush RK, Wood RA, Eggleston PA. Laboratory animal allergy. *J Allergy Clin Immunol* 1998; 102:99–112.
- 4 Aoyama K, Ueda A, Manda F, Matsushita T, Ueda T, Yamauchi C. Allergy to laboratory animals: an epidemiological study. *Br J Ind Med* 1992; 49:41–7.
- 5 Virtanen T, Kinnunen T. Mammalian allergens. *Clin Allergy Immunol* 2008; 21:201–18.
- 6 Grzyb J, Latowski D, Strzalka K. Lipocalins – a family portrait. *J Plant Physiol* 2006; 163:895–915.
- 7 Flower DR, North ACT, Sansom CE. The lipocalin protein family: structural and

- sequence overview. *Biochim Biophys Acta* 2000; **1482**:9–24.
- 8 Walls AF, Newman Taylor AJ, Longbottom JL. Allergy to guinea pigs: I. Allergenic activities of extracts derived from the pelt, saliva, urine and other sources. *Clin Allergy* 1985; **15**:241–51.
 - 9 Fahlbusch B, Rudeschko O, Schlott B et al. Further characterization of IgE-binding antigens from guinea pig hair as new members of the lipocalin family. *Allergy* 2003; **58**:629–34.
 - 10 Fahlbusch B, Rudeschko O, Szilagyi U et al. Purification and partial characterization of the major allergen, Cav p 1, from guinea pig *Cavia porcellus*. *Allergy* 2002; **57**:417–22.
 - 11 Hilger C, Bessot JC, Hutt N et al. IgE-mediated anaphylaxis caused by bites of the pigeon tick *Argas reflexus*: cloning and expression of the major allergen Arg r 1. *J Allergy Clin Immunol* 2005; **115**:617–22.
 - 12 del Val C, Ernst P, Falkenhahn M et al. ProtSweep, 2DSweep and DomainSweep: protein analysis suite at DKFZ. *Nucleic Acids Res* 2007; **35**:W444–50.
 - 13 Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005; **60**:1021–8.
 - 14 Tegoni M, Pelosi P, Vincent F et al. Mammalian odorant binding proteins. *Biochim Biophys Acta* 2000; **1482**:229–40.
 - 15 Rouvinen J, Rautiainen J, Virtanen T et al. Probing the molecular basis of allergy. Three-dimensional structure of the bovine lipocalin allergen Bos d 2. *J Biol Chem* 1999; **274**:2337–43.
 - 16 Lascombe MB, Gregoire C, Poncet P et al. Crystal structure of the allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J Biol Chem* 2000; **275**:21572–7.
 - 17 Zacharisen MC, Levy MB, Shaw JL, Kurup VP. Severe allergic reactions to guinea pig. *Clin Mol Allergy* 2005; **3**:14–7.
 - 18 Chapman MD, Pomes A, Breiteneder H, Ferreira F. Nomenclature and structural biology of allergens. *J Allergy Clin Immunol* 2007; **119**:414–20.
 - 19 Payne AP. The harderian gland: a tercentennial review. *J Anat* 1994; **185** (Part 1):1–49.
 - 20 Smith W, Butler AJ, Hazell LA et al. Fel d 4, a cat lipocalin allergen. *Clin Exp Allergy* 2004; **34**:1732–8.
 - 21 Mattsson L, Lundgren T, Olsson P, Sundberg M, Lidholm J. Molecular and immunological characterization of Can f 4: a dog dander allergen cross-reactive with a 23 kDa odorant-binding protein in cow dander. *Clin Exp Allergy* 2010; **40**:1276–87.
 - 22 van Ree R, van Leeuwen WA, Bulder I, Bond J, Aalberse RC. Purified natural and recombinant Fel d 1 and cat albumin in in vitro diagnostics for cat allergy. *J Allergy Clin Immunol* 1999; **104**:1223–30.
 - 23 Spitzauer S, Pandjaitan B, Soregi G et al. IgE cross-reactivities against albumins in patients allergic to animals. *J Allergy Clin Immunol* 1995; **96**:951–9.
 - 24 Virtanen T, Kinnunen T. Adaptive immunity as a determinant of allergenicity. *J Allergy Clin Immunol* 2009; **124**:171–2; author reply 72.
 - 25 van Ree R. Indoor allergens: relevance of major allergen measurements and standardization. *J Allergy Clin Immunol* 2007; **119**:270–7; quiz 78–9.
 - 26 Harwanegg C, Laffer S, Hiller R et al. Microarrayed recombinant allergens for diagnosis of allergy. *Clin Exp Allergy* 2003; **33**:7–13.