

Molecular cloning, expression and modelling of cat allergen, cystatin (Fel d 3), a cysteine protease inhibitor

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Summary

Background Cats are an important source of indoor allergens. However, only two cat allergens, Fel d 1 and albumin, have been cloned and sequenced. IgE antibodies to Fel d 1 and albumin do not fully account for IgE responses to cat and there is good immunochemical evidence that cats produce other allergens.

Objective To identify and define the molecular structure of the other potential cat allergens.

Methods A cat skin cDNA library was screened using pooled serum obtained from five asthmatic patients which contained high levels of IgE antibody to cat dander. Selected cDNA clones were screened by plaque immunoassay and one cDNA clone, encoding cystatin, was expressed in *E. coli*. The three dimensional structure of cat cystatin was modelled using the SWISS-MODEL computer program.

Results Three positive cDNA clones (A, B and C) were identified, two of which were fully sequenced. Clones A and C encoded the same 98 amino acid residue sequence which showed 79% and 75% homology with bovine and human cystatin A, respectively. The cat cystatin sequence contained the conserved cysteine protease inhibitor signature and two of three lipocalin motifs. By plaque immunoassay, 60–90% of cat allergic sera had IgE ab to the expressed cystatin clones. The cysteine protease inhibitor motif was also partially conserved in dog allergen sequences, Can f 1 and Can f 2, which are lipocalins. The recombinant protein was expressed in *E. coli* as an 11-kDa protein, corresponding to the predicted MW of cat cystatin. The three-dimensional structure of cat cystatin was modelled on human cystatin structures.

Conclusion A newly identified allergen, cystatin (Fel d 3), has been cloned from cat skin and is a member of the cysteine protease inhibitor family.

Keywords: bronchial asthma, cat allergy, IgE, cystatin, cysteine protease inhibitor

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Introduction

The domestic cat is an important source of indoor allergens. Immediate hypersensitivity responses to cat allergens can be demonstrated by skin prick test, RAST and bronchial challenge tests. The major cat allergen, Fel d 1, has been cloned and both the cDNA and genomic DNA sequences of

Fel d 1 have been determined [1,2]. Fel d 1 is produced in cat sebaceous and salivary glands [3–5]. Large amounts of Fel d 1 have also been demonstrated in cat anal glands and it has been suggested that Fel d 1 production by sebaceous glands in male cats is under hormonal control [6–8]. Fel d 1 is a 17-kDa glycoprotein composed of two chains (chain 1 and chain 2) secreted as a 35–39 kDa homodimer.

Approximately 90% of cat-allergic patients make IgG and IgE abs to Fel d 1. Depletion studies have demonstrated that more than 50% of the RAST activity and 66% of the

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histamine release activity of cat hair and dander extract could be absorbed by Fel d 1 [9,10]. We also recently reported an excellent correlation between the amount of IgE abs to Fel d 1 using chimeric ELISA and the level of IgE abs to cat dander assessed using the CAP system [11]. Fel d 1 has been considered to be an excellent marker of both the immune response to cat allergens and of environmental exposure to cats.

There is good evidence that cats produce several other allergens, in addition to Fel d 1. In the 1980s, a number of cat allergens, including albumin, were identified by crossed radioimmuno-electrophoresis and by isoelectric focusing [12–15]. Immunoabsorption studies demonstrated that significant allergenic activity (30–40%) remained in cat dander extracts after absorption with Fel d 1 [13]. A 30-kDa cat allergen (Fel d Bd4.1/30K) was reported to elicit IgE responses in 50% of cat-allergic patients and was used as a marker for cat immunotherapy [16,17]. Although these allergens were identified immunochemically, their amino acid sequences were not determined and, with the exception of Fel d 1 and albumin, no other cat allergens have been clearly defined. The aim of the present study was to identify other potential cat allergens by screening a cat skin cDNA library using human IgE antibodies.

Materials and methods

Sera from patients allergic to cat dander

Five sera with high levels of IgE ab to cat dander (CAP class: 5–6) were obtained from patients with asthma enrolled in a previous Japanese prevalence study [11]. These sera were pooled and used for screening the cat skin cDNA expression library. Thirty-six cat-allergic sera (cat dander CAP class: 3–6) were obtained from other patients enrolled in the prevalence study and a further 13 sera from patients with allergy to cat were obtained from Dr Peyton Eggleston (Johns Hopkins University, Baltimore, MD, USA). The 49 sera were used for plaque immunoassay and were pre-absorbed with *E. coli* lysate extract (Promega, Madison, WI, USA) before each analysis. Control sera were obtained from nine individuals who were not allergic to cat.

*Cat (*Felis domesticus*) skin cDNA library construction and screening*

Cat skin was obtained by skin biopsy and stored in liquid nitrogen prior to mRNA extraction. The mRNA was extracted from 0.3 g cat skin using the PolyAtract System 1000 (Promega, Madison, WI, USA) and 20 µg of mRNA was used as a template for cDNA synthesis. The cat skin cDNA library was prepared in the Uni-ZAPTM expression system (Stratagene, La Jolla, CA, USA) and had an

amplified titre of 1.7×10^{10} pfu/mL. The library was screened with IgE ab in a 1 : 2 dilution of serum pooled from the five cat-allergic patients. Positive plaques were detected using alkaline phosphatase-labelled goat anti-human IgE (1 : 500) followed by colour development with BCIP/nitro blue tetrazolium (KPL, Gaithersburg, MD, USA) [18,19]. Three positive cDNA clones (clone A, B and C) were identified and further screened by plaque immunoassay using 49 individual sera from cat-allergic patients.

In vivo excision of pBluescript from Uni-ZAPTM XR and insert sequencing

The pBluescript phagemids containing cloned cDNA inserts were excised from the Uni-ZAPTM XR vector and plated with fresh *E. coli* cells to produce colonies (ExAssist/SOLR system, Stratagene). The size of inserts was assessed by double digestion with EcoRI and XhoI. Double stranded cDNAs were isolated and sequenced using an ABI Prism 1000 Model 377 automated DNA sequencer (Biomolecular Research Facility, University of Virginia, Charlottesville, VA, USA). The nucleotide sequence (clone A/C) was submitted to the GenBank with the accession number AF238996.

Sequence analysis

Nucleotide sequences were analysed using the GCG sequence analysis package (Wisconsin package, Accelrys, Madison, WI, USA). Protein sequences were compared with the PIR, SwissProt, GenBank, EMBL, EST, GSS data bases using FASTA and BLAST.

Expression of cat cystatin

Selected cDNAs were ligated into the NcoI and XhoI sites of the pET-22b(+) vector (Novagen) which contains the pelB leader to direct synthesized protein into the periplasmic space of *E. coli* and adds a C-terminal histidine tag to the protein. The pET-22b/cystatin construct was transformed into BL21GOLD(DE3) expression cells and protein synthesis was induced for 3 h at 30 °C using 1 mM IPTG. Recombinant cystatin was obtained from the periplasmic space by osmotic shock and was further purified by elution from a His-Bind resin in 100 mM imidazole. Purity and molecular weight were assessed by silver-stained SDS-PAGE (PhastSystem: Pharmacia Biotech, Piscataway, NJ, USA).

Comparative modelling of cat cystatin

The structure of cat cystatin was modelled using the automated comparative protein modelling server SWISS-MODEL [20–22]. The model is based upon the

1	ATG	ATA	CCT	GGA	GGC	TTA	AGT	GAA	GCC	AAA	CCT	GCC	ACT	CCA	GAA	45
	M	I	P	G	G	L	S	E	A	K	P	A	T	P	E	
46	ATC	CAA	GAG	ATT	GCT	AAC	GAG	GTT	AAA	CCT	CAG	CTT	GAA	GAA	AAA	90
	I	Q	E	I	A	N	E	V	K	P	Q	L	E	E	K	
91	ACA	AAT	GAG	ACT	TAC	CAA	AAA	TTT	GAA	GCT	ATA	GAG	TAC	AAA	ACT	135
	T	N	E	T	Y	Q	K	F	E	A	I	E	Y	K	T	
136	CAA	GTG	GTT	GCC	GGA	ATA	AAT	TAC	TAC	ATT	AAG	GTG	CAA	GTA	GAT	180
	Q	V	V	A	G	I	N	Y	Y	I	K	V	Q	V	D	
181	GAT	AAT	AGA	TAT	ATT	CAC	ATT	AAA	GTA	TTC	AAA	GGT	CTT	CCA	GTA	225
	D	N	R	Y	I	H	I	K	V	F	K	G	L	P	V	
226	CAA	GAT	TCA	TCT	TTG	ACA	CTT	ACT	GGT	TAC	CAG	ACT	GGC	AAA	AGC	270
	Q	D	S	S	L	T	L	T	G	Y	Q	T	G	K	S	
271	GAG	GAC	GAT	GAG	CTG	ACA	GGC	TTT	TAG							297
	E	D	D	E	L	T	G	F	***							

Fig. 1. Nucleotide and deduced amino acid sequence of cat skin clone A/C. The stop codon TAG is indicated by asterisks.

coordinates of recombinant human cystatin A (1cyu. pdb), human stefin A (1dvc. pdb) and human stefin B (1 sf. pdb) [23–25].

Results

Molecular cloning of cat cystatin

The cat skin cDNA library was screened using pooled IgE

antibodies to cat dander and three positive clones (A–C) were identified and sequenced. Nucleotide sequencing showed that clone A and C contained exactly the same 294-bp open reading frame, encoding a 98-amino acid residue protein (MW *c.*11 kDa), followed by the stop codon (TAG)(Fig. 1). These clones showed IgE antibody binding by plaque immunoassay in 60–90% of sera from 49 cat-allergic patients (results based on blind assessments by two different investigators) (Fig. 2). A third cDNA

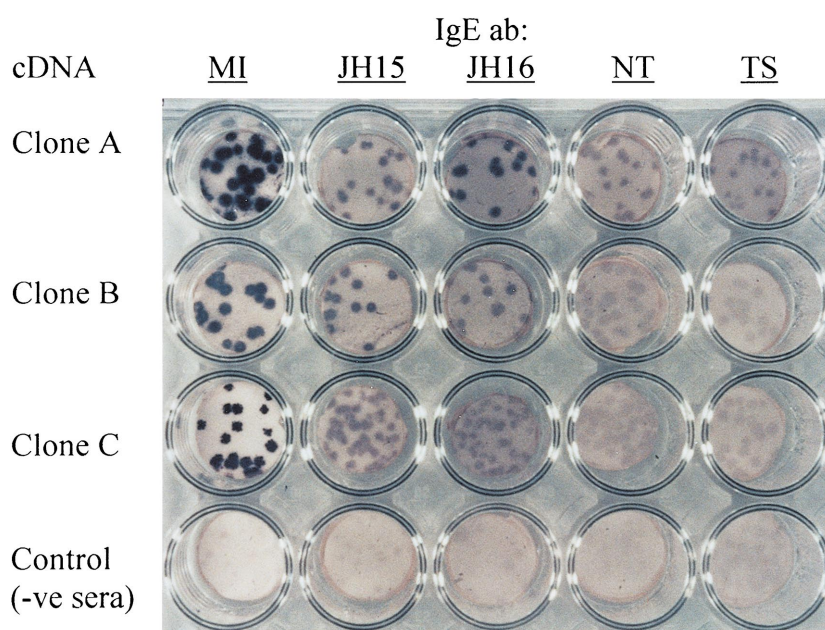


Fig. 2. Plaque immunoassay screening of cat skin cDNA clones A, B and C. The results show IgE ab from five sera (MI, JH15, JH16, NT and TS) binding to the three cDNA clones.

	1	10	20	30 ***	40	50
Cat cystatin	MIPGGLSEAK	PATPEIQEIA	NEVKPQLEEK	TNETYQKFEA	IEYKTQVVAG	
Bovine cystatin A	MIPGGLTEAK	IATPEIQEIA	NMVKPQLEEK	TNETYEEFTA	IEYKSQVVAG	
Human cystatin A	MIPGGLSEAK	PATPEIQEIV	DKVKPQLEEK	TNETYGKLEA	IVQKTQVVAG	
	60	70	80	90	98	
Cat cystatin	INYYIKVQVD	DNRYIHIKVF	KGLPVQDSSL	TLTGYQTGKS	EDDELTGF	
Bovine cystatin A	INYYIKIQTG	DNRYIHIKVF	KGLPQQSHSL	ILTGYQTGKT	KDDELAGE	
Human cystatin A	TNYYIKVRAG	DNDYMHKLVF	KGLPGQNE DL	TLTGYQTGKS	EDDELTGF	

Fig. 3. Alignment between cat cystatin, bovine cystatin A and human cystatin A. Amino acids in bold indicate homology with bovine cystatin A and human cystatin A. Sequences in italics (*IPGG*, residues 2–5 and *QVVAG*, residues 46–50) indicate two of three domains responsible for binding to cysteine proteases. The cysteine protease inhibitor signature sequence is underlined and a putative N-glycosylation site is indicated by asterisks.

clone (B) reacted with *c.*25% of sera, but could not be fully sequenced and was not further studied.

Selected plaque immunoassay results on five cat-allergic patients are shown in Fig. 2.

Sequence similarity searches revealed that clones A/C showed 79% and 75% homology with bovine and human cystatin A, respectively (Fig. 3). The cat cystatin sequence contained a conserved cysteine protease inhibitor motif (55: **TQVVAGINYYIKVQ**) and residues from the first two (*IPGG* and *QVVAG*) of three domains responsible for binding to cysteine proteases. The sequence also contained one potential N-linked glycosylation site (Fig. 3).

Sequence homology between cat cystatin and dog allergens (Can f 1 and Can f 2)

The cloning of cat cystatin prompted us to investigate

sequence homologies between cystatin and dog allergens, Can f 1 and Can f 2. These allergens show 58% and 23% sequence identity with human von Ebner's gland protein (VEGh), a lipocalin that has been shown to have cysteine protease inhibitor activity [26,27]. Both Can f 1 and Can f 2 are lipocalins and the extensive sequence homology between Can f 1 and VEGh suggests that Can f 1 may also function as a cysteine protease inhibitor. Comparison of the cat cystatin, VEGh and dog allergen sequences showed limited homology (Fig. 4). Cat cystatin and VEGh contain the QxVxG motif that is essential for inhibition of papain. In Can f 1, the glutamine residue is replaced by valine (VAVSG), but Can f 1 also contains an adjacent tryptophan residue located near the N-terminus (at position 35) that is conserved in the sequences of cystatins C, D and S [reviewed in 27]. The conserved QxVxG motif was not found in Can f 2 (Fig. 4).

Cat cystatin	45	TQVVAGINYYIKVQ	58
Stefin B	45	SQVVAGTNYFIKVF	58
VEGh	28	IQDVSGTWY.LKAM	40
Can f 1	28	TVAVSGKWI.LKAM	40
Can f 2	30	LEELSGRWH.SVAL	42

Fig. 4. Alignment of cysteine protease inhibitor motifs in cat and dog allergens. Conserved residues of the cysteine protease inhibitor signature sequence are shown in bold type, and indicate that the motif is highly conserved in cat cystatin and stefin B. There is less homology with the lipocalins (VEGh and Can f 1) and almost no homology with Can f 2. The conserved tryptophan residue that is thought to play a role in the cysteine protease inhibitor activity of lipocalins is underlined [27].

Expression of cat cystatin in E. coli

PCR-amplified DNA encoding cat cystatin was ligated into the pET-22b(+) vector and expressed as a C-terminal His-tagged protein in the periplasmic space of *E. coli*. Recombinant cat cystatin was obtained from bacterial lysates by affinity purification (yield *c.* 1–5 mg per litre of culture). The purified protein migrated as a single band of predicted *c.*11 kDa on an 8–25% gradient SDS-PAGE gel (Fig. 5).

Molecular modelling

The molecular structure of cat cystatin was modelled on the X-ray crystal structures of recombinant human cystatin A, human stefin A and human chain I of stefin B (Fig. 6)

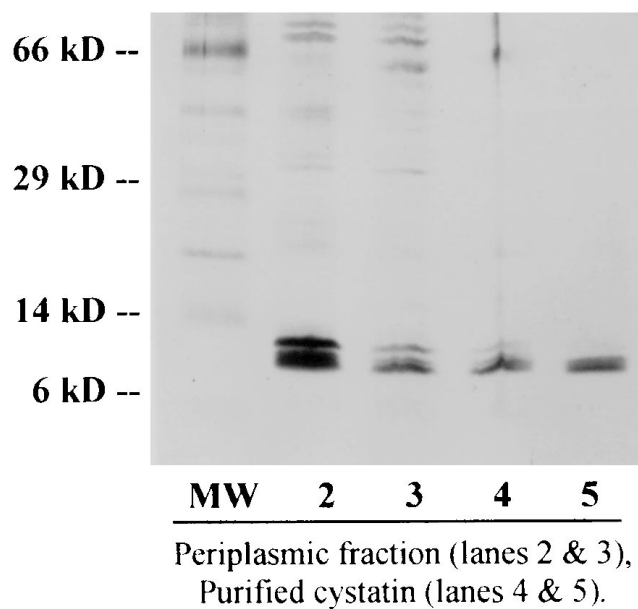


Fig. 5. SDS-PAGE analysis of recombinant cat cystatin expressed in *E. coli*. MW, molecular weight markers; lane 2 and 3, different concentrations of periplasmic fraction; lane 4 and 5, different preparations of purified cat cystatin.

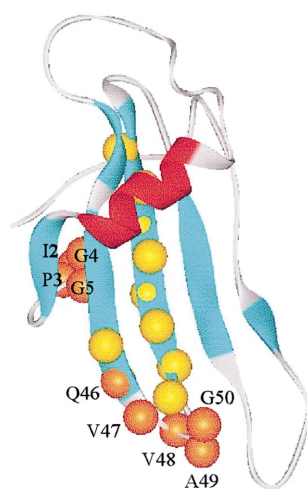
[23–25]. The cat cystatin molecule includes a two and a half turn α -helix and a five-stranded anti-parallel β -pleated sheet interconnected by a hairpin loop and an additional carboxy terminal strand. Stefin B is a cysteine protease inhibitor whose atomic coordinates were obtained from the

three-dimensional structure of recombinant human stefin B complexed with the hydrolase (sulphydryl proteinase) papain (papaya fruit latex) [25]. The three-dimensional structure of stefin B also includes a five-stranded β -sheet wrapped around a longer non-straight five turn α -helix, and an additional carboxy-terminal strand running along the convex side of the sheet [25]. The conserved residues of cat cystatin occur in equivalent positions to those in stefin B (Fig. 6).

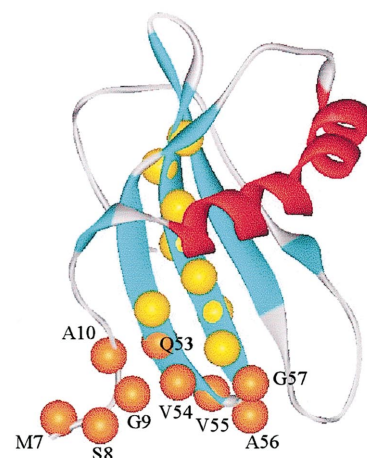
Discussion

Previous immunochemical studies provided good evidence for the existence of other cat allergens [12–15]. However, apart from Fel d 1, the only cat allergen that had been cloned was albumin [28]. The present results show that screening a cat skin cDNA library using IgE antibodies allowed us to successfully clone, sequence and express the cat cystatin allergen and to determine its structure using molecular modelling techniques. The allergen contains the conserved 14 amino acid residue cysteine protease inhibitor signature sequence which is critical for the biological function of cystatins. Cysteine proteases participate in intracellular protein degradation and are controlled by cystatins, which are endogenous protease inhibitors. The physiological function of cystatins is to protect against the potentially harmful effects of free cysteine proteases by rapidly trapping the enzymes in tight complexes. The cystatin superfamily is divided into three subgroups, stefins, cystatins and kininogens. Stefins (Family I cystatins) are small, acidic proteins of *c.*100 amino acids

Fig. 6. Cat cystatin modelled on the crystal structure of human stefin B (cystatin B). Ribbon representation showing the short α -helix (red), five anti-parallel β sheets (blue) and the hairpin loops (grey). The cat cystatin model was built on the X-ray crystal coordinates of three human cystatins using SWISS-MODEL [25]. The cysteine protease inhibitor motif is indicated by yellow and orange spheres in the first hairpin loop with the orange spheres representing residues that are directly involved in forming complexes with the protease (also labelled with the amino acid residue numbers).



Cat cystatin
(Fel d 3)



Human stefin B

residues, which lack disulphide bonds. Cystatins (Family II cystatins) are somewhat larger proteins of c.115 amino acids, having two disulphide bonds [29–32]. The cat cystatin allergen belongs to the stefin family because it is a 98 amino acid residue protein, without cysteine residues, which shows 80% sequence identity to bovine and human cystatin A.

The allergenic importance of cat cystatin was demonstrated by plaque immunoassay which showed that 60–90% of sera from cat-allergic patients contained IgE ab to the protein. Results of the plaque immunoassays were assessed blind by two different investigators. Recombinant cystatin was expressed as an 11-kDa protein in the pET-22b expression system. However, the prevalence of IgE reactivity to the recombinant cystatin, assessed by solid phase ELISA, was much lower than that obtained by plaque immunoassay, being c.10% in 50 sera tested (data not shown). These results suggest either that the recombinant protein may be incorrectly folded in the pET system or that the prevalence of IgE reactivity may be overestimated by plaque immunoassay in which the allergen is expressed as a fusion protein. Cat cystatin has provisionally been designated as *Felis domesticus* allergen 3 (Fel d 3) by the WHO/IUIS Sub-committee on Allergen Nomenclature.

Most animal allergens belong to the lipocalin family of ligand binding proteins or are albumins [10,33–35]. Amino acid sequence comparisons have shown that both Can f 1 and Can f 2 are members of lipocalin super-family [26,35]. Although the lipocalins and the cystatins are different protein families, they both include proteins that inhibit cysteine proteases. The human Von Ebner's gland protein is a lipocalin that inhibits papain, has homology with Can f 1 and Can f 2, and shares the QxVxG cysteine protease inhibitor motif with cystatins [40]. The results show some degree of conservation between the cysteine inhibitor signature motif in Fel d 3 and that of dog allergens, especially Can f 1 (Fig. 4). The three-dimensional structure of the complex of papain and stefin B showed that the interaction between both molecules is tripartite, including part of the amino terminus, the QVVAG loop and a minor contribution from the second hairpin loop [24]. Since the conserved residues of Fel d 3 occur in equivalent positions to those in stefin B, and the model for Fel d 3 is similar to the three-dimensional structure of stefin B, we expect that Fel d 3 will have a similar interaction with cysteine proteases. It is more difficult to predict from sequence comparisons whether Can f 1 is a cysteine protease inhibitor. Can f 1 has a critical substitution of glutamine by valine in the protease binding motif (VAVSG) which may affect its biological function (Fig. 4). Although Can f 1 and Can f 2 have been sequenced, neither allergen has been expressed in recombinant form. Further assessment of the biological activity of Fel d 3 and Can f 1 will require

expression of these allergens (or use of natural allergens) for comparison in functional studies.

The results are also relevant to the recent body of evidence implicating a role for cysteine proteases in enhancing IgE responses, especially the dust mite cysteine protease allergen, Der p 1. The proteolytic activity of Der p 1 has been shown to cleave CD23, the low affinity IgE receptor on B cells and to cleave CD25, a subunit of the IL-2 receptor [36–38]. Der p 1 increased the permeability of the bronchial mucosa to macromolecules and enhanced the production of pro-inflammatory cytokines [39,40]. As a result of these findings, it has been suggested that the protease activity of Der p 1 critically enhances its allergenicity and may partly explain why Der p 1 is such a potent allergen [41,42]. On the other hand, these findings may be tempered by the observations that cats, and possibly dogs, produce cysteine protease inhibitors that are themselves allergens. It is not unusual for environmental exposure to animal allergens to exceed 100 µg/g and these levels of inhibitor might be expected to reduce the potential proteolytic activity of Der p 1. Recent evidence suggests that pet exposure during the first year of life is associated with lower prevalence of allergic sensitization and symptoms of rhinitis and asthma in school children [43]. However, it is not known whether exposure to high levels of animal allergens has any effect on sensitization to mite allergens, which might be expected from our results if cysteine protease activity is an important prerequisite for sensitization to mites.

Screening of cat skin cDNA libraries, and libraries prepared from other cat tissues, will enable other cat allergens to be identified and the repertoire of IgE responses to cat to be fully determined. Cat is one of the few mammalian allergens for which a lipocalin allergen has not so far been identified and the libraries could certainly be screened to identify putative cat lipocalin allergens using PCR techniques. The advantages of using recombinant cat allergens for allergy diagnosis and treatment have recently been reviewed [44,45]. Current data suggest that recombinant Fel d 1 and cat albumin have a diagnostic efficacy of 94%, which could be improved using one or more additional allergens, such as cat cystatin [44]. The present results will allow the effects of cysteine protease inhibitor allergens on allergenicity to be investigated and could also lead to the development of new diagnostic and therapeutic reagents.

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