

Letter to the Editor

Identification of a new major dog allergen highly cross-reactive with Fel d 4 in a population of cat- and dog-sensitized patients

To the Editor:

Cat and dog allergens have been shown to be the most important causes of inhalant allergy to animals.¹ Because allergy to cat and dog are frequently associated, and as earlier reports have shown IgE cross-reactivity between cat and dog dander in sensitized patients,^{2,3} it is important to distinguish cosensitization from IgE cross-reactivity to cat and dog allergens. Several cat and dog allergens have been characterized,⁴ but the only molecules found to give clear-cut IgE cross-reactions are cat and dog serum albumins.² Additional cross-reactive allergens have been presumed based on inhibition studies using patient sera without IgE reactivity to serum albumin.^{2,5} Three lipocalins have been isolated from dog dander (Can f 1, Can f 2, and Can f 4).⁴ Two lipocalins have been characterized from cat (Fel d 4 and Fel d 7).^{4,6} Lipocalins represent the largest mammalian allergen family.⁴ They share common biological properties such as binding and transport of small hydrophobic molecules. Despite a high sequence diversity, they have a common tertiary structure characterized by an 8-stranded β -barrel.

Relying on the amino acid sequence of Fel d 4 and the quite homologous amino acid sequence of Equ c 1, we searched the National Center for Biotechnology Information protein database for highly similar proteins. An open reading frame derived from a genomic dog sequence (GenBank XM_850249; LOC481674) was predicted to code for a protein with a sequence identity of 67% to Fel d 4 and 57% to Equ c 1. The expressed sequence tag profile suggested the expression of the predicted lipocalin in salivary gland. Specific primers were designed and reverse transcription followed by PCR was performed on RNA extracted from dog submaxillary gland. The cloned cDNA corresponded to the predicted mRNA and confirmed all intron/exon boundaries. The dog lipocalin, designated Can f 6.0101, was expressed in *Escherichia coli* and purified by immobilized metal ion affinity chromatography under native conditions followed by anion exchange using a Resource Q column. The recombinant protein was used to immunize BALB/c mice. Mouse anti-Can f 6 sera detected 2 protein bands at 27 and 29 kDa in dog dander extract (data not shown). The cDNA corresponding to Fel d 4 (Swissprot Q5VFH6) was purchased from Eurofins (Ebersberg, Germany). Fel d 4 was expressed in *E. coli* and was purified by affinity chromatography.

Forty-four patients (mean age 33.5 years, 48% females) seen for diagnostic workup of inhalant or cutaneous allergy were selected for combined sensitization to cat and dog as shown by skin prick tests and *in vitro* specific IgE to cat and dog dander. Thirty-two patients had rhinitis and/or asthma related to cat and/or dog exposure. Five patients had atopic eczema possibly related to cat and/or dog exposure (see Table E1 in this article's Online Repository at www.jacionline.org). Sera were tested by ELISA for IgE reactivity to rFel d 4 and rCan f 6. Fifty-two percent of the patients had IgE antibodies to rFel d 4 (range = 1.4–51 kU_A/L), and 61% had IgE antibodies to rCan f 6 (range = 0.8–152 kU_A/L). IgE binding to rCan f 6 was significantly correlated with IgE binding to dog dander ($r = 0.6295$; $P < .0001$). Because of the

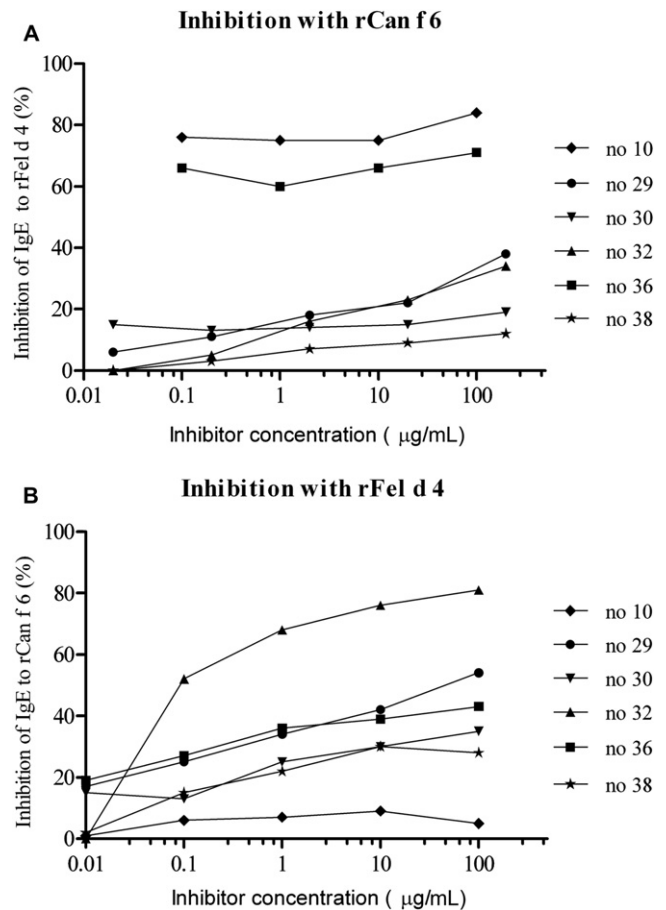


FIG 1. Analysis of IgE cross-reactivity between Fel d 4 and Can f 6 by ELISA inhibition to rFel d 4 (A) and rCan f 6 (B) on the solid phase. The sera of 6 patients were preincubated with rCan f 6 (Fig 1, A) or rFel d 4 (Fig 1, B) at increasing concentrations. The percentage of IgE binding was calculated from the mean OD values with or without addition of the inhibitor allergen.

high amino acid sequence identity and the striking correlation between IgE reactivity to rFel d 4 and rCan f 6 ($r = 0.7780$; $P < .0001$) (see Fig E1 in this article's Online Repository at www.jacionline.org), inhibition experiments were performed by using the sera of 6 patients with relevant titers of IgE to both lipocalins (Fig 1).

For patient 10, who had a dog at home and suffered from rhinitis, addition of 0.1 µg/mL rCan f 6 inhibited IgE reactivity to rFel d 4 to 76%. In the reciprocal experiment, addition of rFel d 4 had only a minor effect on reactivity to rCan f 6 (6% inhibition). This strongly suggests that this patient is sensitized to Can f 6 and IgE is cross-reacting with Fel d 4. On the other hand, for patient 32, an asthmatic cat owner, we could show sensitization to Fel d 4 and cross-reactive IgE to Can f 6. As this patient is negative for Can f 1 and Can f 2, this supports the assumption that she is primarily sensitized to cat dander. A dominant sensitization to Can f 6 was found for patient 36. For patient 38 and also patients 29 and 30 who both had inhalant symptoms when exposed to cat and dog, inhibitions and cross-inhibitions gave variable profiles reaching from 12% up to 54% inhibition. These findings suggested a cosensitization to Can f 6 and Fel d 4, resulting in

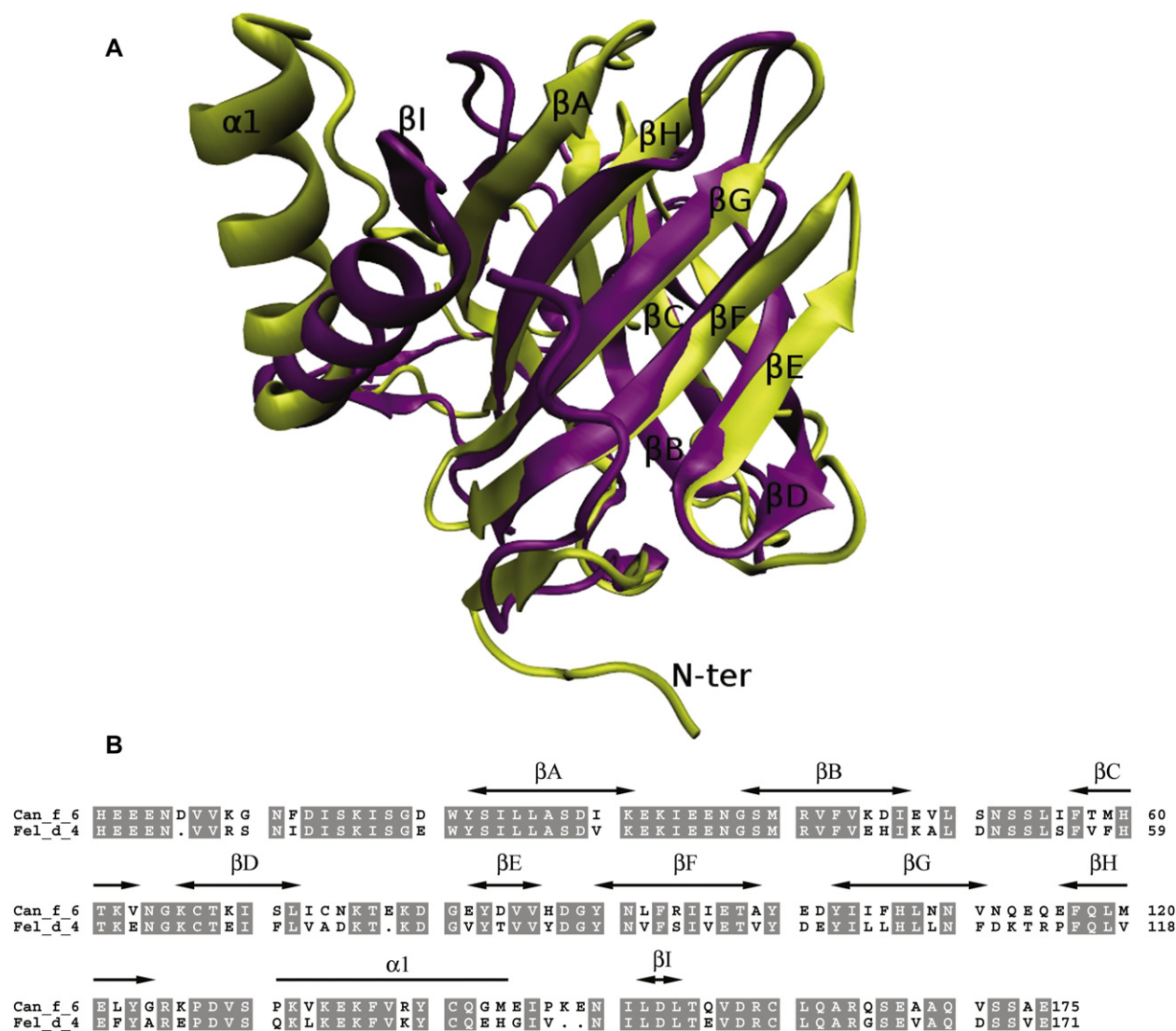


FIG 2. Structural comparison of Can f 6 and Fel d 4 lipocalins. **A**, Superimposition structure of Can f 6 (yellow) and Fel d 4 (purple). **B**, Sequence alignment: Shaded areas in the alignment show identical amino acids. The secondary structure elements are indicated above the alignment. β -Strands A-I and the terminal $\alpha 1$ helix are positioned relative to their localization on Can f 6.

IgE recognizing a variable number of specific as well as cross-reactive epitopes. The individual patient epitope recognition pattern is a function of the number of recognized epitopes as well as of IgE affinity. We cannot exclude that part of the variable IgE profile of the patients is due to sensitization to a cross-reactive lipocalin different from Can f 6 and Fel d 4. In order to assess IgE reactivity to other relevant cross-reactive allergens, IgE titers were determined to serum albumins. In our patient cohort, 47% had IgE to Fel d 2 and 33% to Can f 3. This high frequency of IgE directed to serum albumin probably results from the fact that we have selected patients reacting to both cat and dog.

Modeling of Can f 6 and Fel d 4 was performed by using Modeler 9v7⁷ and CHARMM on the basis of the crystal structure of Equ c 1 (PDB:1EW3) (Fig 2, A). The superimposition of both lipocalins shows that the molecular backbone building the β -barrel is highly conserved while connecting loops and the $\alpha 1$ helices are differently positioned. Identical amino acids build continuous

segments at the N- and C-terminal ends, β -strands A and B, as well as in the loop L1 connecting βA and βB (Fig 2, B). Thus, there are several areas where cross-reactive IgE could bind.

In addition to cat and dog serum albumins and the lipocalins Fel d 4 and Can f 6 now shown to be cross-reactive, possible cross-reactive allergens have to be sought for. IgE reactivity to Fel d 4 has been moderately inhibited by dog dander extract in a group of cat-sensitized patients.⁸ Limited IgE cross-reactivity has been shown between Can f 2 and Fel d 4 by using a high excess of inhibitor.⁹ The sequence identity between those 2 molecules is 25%, which is considerably lower than the identity between Can f 6 and Fel d 4 (67%). Sequence identity between Can f 1 and the recently described cat lipocalin Fel d 7⁶ is 63%, suggesting that IgE cross-recognition is quite likely.

In this study, we have cloned a new dog allergen and could show IgE sensitization in 61% of dog- and cat-sensitized patients. Moreover, we have shown that Fel d 4 and Can f 6 are

cross-reactive allergens capable of inhibiting IgE reactivity to each other at low competitor concentrations. Whether this cross-reactivity has clinical significance or is mainly an interesting *in vitro* finding has to be further established. Can f 6 and Fel d 4 show great homology to Equ c 1, the major horse allergen. This suggests that a larger family of IgE cross-reactive lipocalins might exist. These findings have to be taken into account when interpreting positive skin or *in vitro* IgE tests to cat and dog dander.

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REFERENCES

1. Salo PM, Calatroni A, Gergen PJ, Hoppin JA, Sever ML, Jaramillo R, et al. Allergy-related outcomes in relation to serum IgE: results from the National Health and Nutrition Examination Survey 2005-2006. *J Allergy Clin Immunol* 2011;127:1226-35.e7.
2. Cabanas R, Lopez-Serrano MC, Carreira J, Ventas P, Polo F, Caballero MT, et al. Importance of albumin in cross-reactivity among cat, dog and horse allergens. *J Invest Allergol Clin Immunol* 2000;10:71-7.
3. Reininger R, Varga EM, Zach M, Balic N, Lindemeier AD, Swoboda I, et al. Detection of an allergen in dog dander that cross-reacts with the major cat allergen, Fel d 1. *Clin Exp Allergy* 2007;37:116-24.
4. Virtanen T, Kinnunen T. Mammalian allergens. *Clin Allergy Immunol* 2008;21:201-18.
5. Spitzauer S, Pandjaitan B, Muhl S, Ebner C, Kraft D, Valenta R, et al. Major cat and dog allergens share IgE epitopes. *J Allergy Clin Immunol* 1997;99:100-6.
6. Smith W, O'Neil SE, Hales BJ, Chai TL, Hazell LA, Tanyaratrisakul S, et al. Two newly identified cat allergens: the von Ebner gland protein Fel d 7 and the latherin-like protein Fel d 8. *Int Arch Allergy Immunol* 2011;156:159-70.
7. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 1993;234:779-815.
8. Smith W, Butler AJ, Hazell LA, Chapman MD, Pomes A, Nickels DG, et al. Fel d 4, a cat lipocalin allergen. *Clin Exp Allergy* 2004;34:1732-8.
9. Madhurantakam C, Nilsson OB, Uchtenhagen H, Konradsen J, Saarne T, Hogbom E, et al. Crystal structure of the dog lipocalin allergen Can f 2: implications for cross-reactivity to the cat allergen Fel d 4. *J Mol Biol* 2010;401:68-83.

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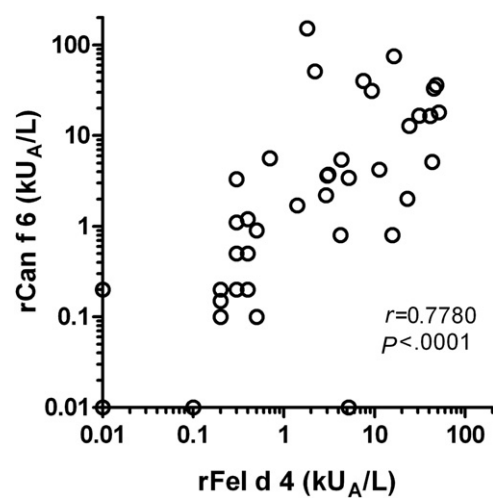


FIG E1. Comparison of IgE antibody binding to rCan f 6 and rFel d 4 in 44 patients allergic to cats and dogs.

TABLE E1. Cat- and dog-allergic patients

Patient no.	Gender	Age (y)	Symptoms upon exposure	Exposure to	Total IgE (kU/L)	Cat dander e1	nFel d 2 e220	rFel d 4	Dog dander e5	nCan f 3 e221	rCan f 6
1	F	45	A	Dog	378	9.34	5.3	0.3	61	13	1.1
2	M	22	E	Cat, dog	1269	>100	1.2	43	20	0.11	5.1
3	M	34	ns	None	274	44	<0.1	0.4	4.51	<0.1	0.2
4	F	38	ns	ns	598	66	46.5	0.5	12	22.2	0.1
5	M	32	A	Cat, dog	3802	85	66.4	2.2	>100	>100	51
6	M	49	A	Dog	2092	76	11.6	0.7	88	19.3	5.6
7	F	33	A	Cat, dog	140	15	<0.1	0.2	6.23	<0.1	0.1
8	M	56	R	Cat	1782	73	0.6	0.2	15	<0.1	0.2
9	F	20	E, A	Dog	3653	98	13	16.3	>100	17	75
10	F	23	R	Dog	450	9.75	nd	7.5	74	nd	40
11	M	30	R, C	Cat, dog	167	21	<0.1	0.2	13	<0.1	0.2
12	M	50	A	Cat, dog	3825	1.86	0.2	1.8	64	<0.1	152
13	F	51	R, A	Dog	460	2.71	0.7	0.4	18	2.53	0.5
14	M	53	ns	None	467	5.94	<0.1	0.3	18	<0.1	0.2
15	M	19	R, E	Cat	442	26	<0.1	0.2	4.48	<0.1	0.15
16	M	24	R, C, A	Cat, dog	2421	78	<0.1	0.3	18	<0.1	3.3
17	M	44	R	Dog	nd	70	10.2	0.2	13	6.65	0.2
18	F	26	R, C, A	Cat	410	95	<0.1	15.7	7.15	<0.1	0.8
19	M	17	R, A	Cat	4642	4.86	0.6	0.1	5.47	0.32	<0.1
20	F	39	ns	None	523	45	<0.1	<0.1	23	<0.1	<0.1
21	F	52	ns	None	222	21	28.6	<0.1	14	18.4	<0.1
22	F	23	E	Cat	1286	>100	<0.1	0.3	19	<0.1	0.5
23	M	6	ns	None	316	4.4	<0.1	5.2	0.56	<0.1	<0.1
24	F	21	A	Cat	118	14	2.3	2.9	13	1.8	2.2
25	M	51	A	Cat	246	11	<0.1	1.4	17	<0.1	1.7
26	F	17	ns	ns	183	37	<0.1	<0.1	2.09	<0.1	<0.1
27	M	25	ns	None	926	69	<0.1	<0.1	11	<0.1	0.2
28	F	54	R, A	Cat	968	>100	0.7	23	8.76	<0.1	2
29	M	18	A	Cat, dog	691	86	<0.1	48	80	<0.1	36
30	F	16	R, C, A	Cat, dog	765	7.13	<0.1	5.2	6.83	<0.1	3.4
31	F	27	R, C, A	Cat, dog	403	1.86	<0.1	0.5	2.97	<0.1	0.9
32	F	30	A	Cat	563	100	<0.1	51	9.02	<0.1	18
33	F	31	R	Dog	194	2.54	1.3	3	24	3.85	3.6
34	M	64	ns	ns	1531	>100	56.5	41	32	33	16.4
35	F	34	ns	ns	8620	39	0.1	4.3	31	<0.1	5.4
36	F	11	A	Cat, dog	381	7.54	<0.1	9.3	67	<0.1	31
37	M	17	R	Cat	158	34	18.8	4.2	5.96	11.5	0.8
38	M	53	A	Dog	1167	65	0.7	45	68	0.21	33
39	F	37	A	Cat	60	12	0.4	0.4	1.16	<0.1	1.2
40	M	42	A	Dog	55	1.46	<0.1	3.1	2.85	<0.1	3.7
41	M	42	A	Cat, dog	1743	63	<0.1	31	17	<0.1	16.5
42	M	21	A, R, E	Cat, dog	1114	>100	2.6	11.3	38	2.28	4.2
43	F	16	C, R	Dog	1471	26	<0.1	<0.1	24	<0.1	<0.1
44	M	62	A	Cat	4543	>100	>100	24.1	>100	>100	12.8

Allergen-specific IgE antibodies to rFel d 4 and rCan f 6 were quantified by ELISA, whereas all others were quantified by ImmunoCAP (Phadia, Uppsala, Sweden). Values are in kU_A/L. CAP values of more than 0.35 were considered positive. In ELISA, the cutoff for positivity (mean of 22 controls + 3 SD) was determined to be 0.87 for rFel d 4 and 0.71 for rCan f 6. Specific IgE titers were determined by using as a reference standard the serum of a patient with a known titer of specific IgE to nFel d 2. This serum was diluted and added to wells coated with nFel d 2. A standard curve was generated that allowed calculating specific IgE titers to Fel d 4 and Can f 6.

A, Asthma; C, conjunctivitis; E, atopic eczema; F, female; M, male; nd, not done; ns, not specified; R, rhinitis.