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Available online December 9, 2013.
http://dx.doi.org/10.1016/j.jaci.2013.10.031

Microarrayed dog, cat, and horse allergens show weak correlation between allergen-specific IgE and IgG responses

To the Editor:

The beneficial role of pet exposure and the development of allergen-specific IgG antibodies induced by natural exposure is a controversial issue. Increased levels of IgG antibodies to Fel d 1 were found to be associated with decreased sensitization in children,[1] and higher levels of IgG/IgG$_4$ to mouse allergens were found to be associated with decreased symptoms in laboratory workers.[2] However, another study reported that exposure and high IgG levels to cat were not associated with a lower risk of allergic respiratory symptoms.[3] One possibility for this discrepancy may be that allergen-specific IgE and IgG responses are not synchronized and directed to the same allergens/epitopes. To address this question and to define the most frequently recognized animal allergens, we investigated allergen-specific IgE and IgG responses by using microarrayed allergens.

Sera from patients with allergic symptoms clearly attributable to cat exposure with and without concomitant allergy to dogs and horses, from allergic patients without allergy to animals, and from subjects without allergy were studied (see Table E1 in this article's Online Repository at www.jacionline.org). By using the ImmunoCAP ISAC technology (Thermo Fisher, Uppsala, Sweden, and Vienna, Austria), customized allergen microarrays containing in addition to Can f 1, Can f 2, Can f 3, and Can f 5, 2 recently described dog allergens, that is, Can f 4 and Can f 6, were prepared.[4] Fig E1 in this article's Online Repository at www.jacionline.org shows that recombinant Can f 4 (rCan f 4) and rCan f 6 exhibit correct molecular weight, are pure, and are folded. In addition, the chip also contained the cat allergens rFel d 1, natural Fel d 2 (nFel d 2), and rFel d 4 and the horse allergens rEqu c 1 and nEqu c 3. The simultaneous analysis of IgE and IgG responses toward 11 animal allergens showed that allergen-specific IgE and IgG responses were only poorly correlated (Fig 1 and Table I). High correlation between IgE and IgG antibodies was found only for Can f 4 ($r = 0.728$; $P < .001$), moderate correlations were observed for Can f 1 ($r = 0.581$; $P < .05$), Can f 2 ($r = 0.504$; $P < .05$), and Equ c 3 ($r = 0.550$; $P < .05$), and no correlations were observed for the other animal-derived allergens (Table I). Often, animal-allergic patients without IgE reactivity to certain allergen components mounted pronounced IgG responses toward these allergens (Fig 1 and Table I). Furthermore, allergic patients without animal allergy and nonallergic individuals exhibited specific IgG antibody responses toward animal allergens similar to those of animal-allergic patients (Fig 1). Almost for each tested allergen (ie, Can f 2, Can f 3, Can f 4, Can f 5, Can f 6, Fel d 1, and Fel d 2), we found allergic patients who showed selective IgE reactivity without detectable IgG antibodies (Fig 1 and Table I). Similar finding were made for house dust mite allergens (see Fig E2 in this article's Online Repository at www.jacionline.org). Moderate correlation between IgE and IgG was observed for only 2 of the 12 allergens, namely, Der p 2 and Der p 23 ($r = 0.560$, $P < .05$, and $r = 0.545$, $P < .05$, respectively; Fig E2).

In the case of a strictly sequential class-switch from allergen-specific IgG to IgE production, one would expect a good correlation between IgE and IgG responses but our results provide evidence for a direct switch from IgM to allergen-specific IgE without intermediate IgG response. Our results therefore may explain why natural allergen exposure does not always induce protective IgG responses leading to immunological tolerance as has been suggested for cat allergy because IgG is directed to other allergens/epitopes than is IgE.

In the group of dog-allergic patients (Table E1: patients 1-17), the frequencies of IgE reactivity to the individual dog allergens were as follows: Can f 1, 13 of 17 (76%); Can f 3, 10 of 17 (59%); Can f 5, 12 of 17 (71%); Can f 4, 10 of 17 (59%); Can f 2, 6 of 17 (35%), and Can f 6, 4 of 17 (23%) (Fig 1). In the group of cat-allergic patients, the frequencies of IgE reactivity to cat allergens (Table E1: patients 1-24) were as follows: Fel d 1, 24 of 24 (100%); Fel d 4, 15 of 24 (63%); and Fel d 2, 13 of 24 (54%). Using Equ c 1 and Equ c 3, only 6 of the 11 patients (Table E1: patients 1-4, 9-11, 15, 16, 21, and 23) reporting symptoms on contact with horses were identified, indicating that additional horse allergen components were needed. Each of the patients who had reported allergic symptoms on contact with dogs showed IgE reactivity to at least 1 of the microarrayed dog allergens and each of the patients who had reported symptoms on contact with cats reacted with at least 1 of the cat allergens present on the chip, indicating high sensitivity of the microarray for diagnosing cat and dog allergy. No IgE binding to microarrayed animal allergen components was detected in sera from nonallergic subjects or allergic patients with house dust mite and/or pollen allergy without animal allergy, indicating specificity of the microarray. Interestingly, IgE reactivity to dog and cat allergen extracts was found by ImmunoCAP measurements in allergic patients without clinical animal allergy (Table E1, patients 25 and 26). Our findings need to be confirmed in a larger population of patients to identify the most relevant animal allergens. Nevertheless, the chip should be useful for studying dog and cat allergen-specific IgE responses to follow the evolution of IgE responses in birth cohorts[5] and in populations from various countries.

Fig E3 in this article's Online Repository at www.jacionline.org shows that there is a sequence identity of 67% and 57% between the lipocalins Fel d 4 and Equ c 1 with the dog allergen Can f 6, respectively, and a very high sequence identity of more than 74% between the albumins from dog, cat, and horse (ie, Can f 3, Fel d 2, and Equ c 3). Dog, cat, and horse lipocalin allergens Can f 6, Fel d 4, and Equ c 1, which had previously been reported to be cross-reactive at the IgE level,[6] showed a significant correlation of IgE reactivities between Fel d 4 and Equ c 1 ($r = 0.557$, $P < .005$; see Table E2 in this article's Online

		rCan f 1		rCan f 2		rCan f 4		rCan f 5		rFel d 1		nCan f 3		nFel d 2		nEqu c 3		rCan f 6		rFel d 4		rEqu c 1	
group	patient	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG
	1	1.04	37.48	0.00	0.00	1.13	31.67	0.00	1.03	1.99	21.23	0.00	1.20	0.00	1.46	0.00	0.91	0.00	3.74	0.00	3.60	0.00	1.30
	2	37.31	11.38	0.00	0.00	19.28	2.50	4.58	0.98	29.67	55.75	66.15	63.88	78.38	96.08	26.67	44.52	2.39	1.64	16.42	17.38	0.79	5.50
	3	5.21	0.77	0.00	0.00	1.42	0.47	0.00	0.40	5.33	2.79	0.00	4.55	0.17	2.80	0.00	0.97	0.00	1.75	0.17	4.32	0.00	1.14
	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.46	5.10	2.07	4.27	3.53	2.93	3.57	0.00	0.00	0.00	3.46	1.69	36.95	2.95	1.77
DCA	5	9.01	8.16	2.06	0.00	5.93	5.77	0.41	0.84	7.69	3.62	2.64	0.00	0.00	0.26	0.00	0.24	0.00	0.82	3.42	4.19	4.50	1.91
	6	9.72	9.76	9.85	10.69	2.00	3.20	3.52	0.94	15.10	4.19	0.77	2.06	0.58	1.65	0.00	10.93	0.00	0.00	3.17	8.48	0.28	3.86
	7	9.33	7.85	5.25	2.66	0.00	0.00	0.14	2.59	6.83	10.71	1.57	3.79	2.23	1.58	0.00	0.60	0.00	1.51	0.58	4.35	0.00	0.00
	8	2.64	66.50	0.00	0.81	4.71	59.01	1.08	9.62	1.03	35.00	0.00	10.09	0.00	18.43	0.00	1.74	0.00	14.02	0.49	34.26	0.00	7.13
	9	0.00	1.97	0.00	4.66	0.00	0.00	3.62	11.46	14.14	7.49	0.00	0.00	0.00	0.00	0.00	0.00	0.31	5.58	8.90	22.17	0.56	9.93
	10	9.60	11.16	15.24	10.41	0.00	0.98	7.89	4.08	10.28	9.61	0.32	0.00	0.15	0.00	0.00	0.00	0.00	0.00	1.89	6.33	0.00	0.00
	11	2.67	5.50	0.00	0.00	0.00	0.26	0.22	1.93	14.05	2.34	0.00	2.21	0.00	1.99	0.00	0.90	0.00	0.93	1.75	2.97	0.00	6.40
	12	21.50	6.65	28.58	9.70	74.57	7.85	8.39	11.10	30.94	4.51	71.48	8.70	25.59	3.26	19.66	4.74	4.27	0.00	0.23	8.79	0.00	6.87
	13	2.35	2.70	0.00	0.00	0.00	0.42	0.50	0.83	1.45	0.91	0.00	0.59	0.00	0.00	0.00	0.48	0.32	1.26	0.16	1.92	0.14	3.17
	14	0.00	16.52	0.00	8.95	0.18	0.00	2.01	1.20	4.19	1.38	0.19	9.95	0.23	7.42	0.00	2.46	0.00	2.65	0.00	5.59	0.00	5.45
	15	2.07	7.61	1.62	0.00	0.87	4.67	0.60	15.68	2.03	4.36	2.61	29.54	1.20	24.27	0.38	12.37	0.00	0.00	0.56	14.68	0.00	10.19
	16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.15	2.30	5.69	1.56	4.62	1.26	0.12	0.95	0.00	0.86	0.00	7.91	0.00	0.00
	17	0.78	5.34	0.00	7.89	7.72	77.24	0.00	1.70	5.41	27.33	0.00	40.43	0.00	37.76	0.00	36.80	0.00	15.02	2.66	8.08	0.00	2.69
	18	0.00	0.00	0.00	0.00	0.00	0.00	5.20	44.84	5.98	39.06	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	7.79	0.00	1.09
	19	2.97	47.54	1.14	24.17	12.81	34.41	1.09	0.00	1.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.54	0.00	0.00	31.21	0.00	15.63
	20	0.00	2.13	0.00	2.98	0.00	3.25	0.00	1.38	27.45	8.79	0.00	0.57	0.60	0.71	0.00	1.36	0.00	1.21	0.00	18.36	0.00	6.36
CA	21	0.24	3.45	0.00	0.00	0.00	0.00	0.00	2.77	0.41	23.85	0.96	12.85	1.27	20.46	0.61	3.51	0.00	4.17	3.98	11.57	0.00	4.22
	22	0.00	1.55	0.00	0.00	0.00	0.55	0.00	1.47	2.13	7.94	0.00	0.88	0.00	0.80	0.00	0.68	0.00	3.52	0.00	17.19	0.00	6.96
	23	0.60	6.76	0.00	0.00	0.00	0.00	1.66	1.60	4.74	2.51	0.00	4.99	0.00	4.88	0.00	0.00	0.00	1.33	0.00	5.15	0.00	1.28
	24	0.00	4.50	0.00	1.90	0.00	3.19	0.00	5.41	2.25	7.51	0.00	4.06	0.00	1.44	0.00	1.56	0.00	3.38	0.00	5.85	0.00	2.88
	25	0.00	0.00	0.00	2.37	0.00	0.00	0.00	1.15	0.00	5.23	0.00	46.94	0.00	60.36	0.00	7.38	0.00	1.66	0.00	5.17	0.00	3.18
OA	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	27	0.00	1.35	0.00	2.52	0.00	0.50	0.00	0.82	0.00	2.14	0.00	0.79	0.00	1.07	0.00	1.61	0.00	1.75	0.00	15.81	0.00	6.17
	28	0.00	0.33	0.00	0.00	0.00	0.00	0.00	1.50	0.00	0.32	0.00	1.31	0.00	1.22	0.00	0.00	0.00	0.96	0.00	5.67	0.00	3.29
	29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.55	0.00	2.45	0.00	4.66	0.00	0.91	0.00	1.00	0.00	3.25	0.00	2.11
	30	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.98	0.00	0.00	0.00	0.00	0.00	0.36	0.00	1.80	0.00	2.73	0.00	3.56
NA	31	0.00	0.63	0.00	0.00	0.00	0.29	0.00	0.00	0.00	0.46	0.00	0.33	0.00	0.47	0.00	1.01	0.00	2.44	0.00	9.19	0.00	5.20
	32	0.00	0.63	0.00	0.67	0.00	0.00	0.00	0.45	0.00	0.71	0.00	1.56	0.00	0.89	0.00	0.45	0.00	1.82	0.00	12.97	0.00	4.21
	33	0.00	0.81	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.62	0.00	0.00	0.00	0.00	0.00	2.25	0.00	1.23	0.00	2.77
	34	0.00	0.45	0.00	0.68	0.00	0.00	0.00	0.56	0.00	0.92	0.00	0.28	0.00	0.35	0.00	0.47	0.00	0.96	0.00	4.63	0.00	1.73
	35	0.00	0.34	0.00	0.00	0.00	0.00	0.00	0.79	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.00	3.76	0.00	2.08
	36	0.00	3.89	0.00	2.93	0.00	0.92	0.00	0.56	0.00	2.29	0.00	1.78	0.00	1.54	0.00	3.73	0.00	4.52	0.00	27.80	0.00	5.23

IgE reactivity
- negative
- 0.1-0.9
- 1-14.9
- >15

IgG reactivity
- negative
- 0.1-0.9
- 1-14.9
- >15

FIG 1. Heat map of patients' IgE and IgG reactivity. IgE and IgG levels (inserts show color codes for the levels) specific for microarrayed dog (Can f 1-6), cat (Fel d 1, Fel d 2, and Fel d 4), and horse (Equ c 1 and Equ c 3) allergens are displayed for 4 groups of subjects (*DCA*, patients with symptoms to dog and/or cat; *CA*, cat-allergic patients; *OA*, allergic patients without allergy to animals; *NA*, nonallergic subjects). *n*, Natural; *r*, recombinant.

TABLE I. Correlations between allergen-specific IgE and IgG levels of animal-derived allergens

IgG \ IgE	Can f 1	Can f 2	Can f 3	Can f 4	Can f 5	Can f 6	Fel d 1	Fel d 2	Fel d 4	Equ c 1	Equ c 3
Can f 1	$r = 0.581$* $P = .003$										
Can f 2		$r = 0.504$† $P = .01$									
Can f 3			$r = 0.281$ $P > .05$								
Can f 4				$r = 0.728$* $P < .001$							
Can f 5					$r = 0.304$ $P > .05$						
Can f 6						$r = -0.168$ $P > .05$					
Fel d 1							$r = 0.100$ $P > .05$				
Fel d 2								$r = 0.337$ $P > .05$			
Fel d 4									$r = 0.154$ $P > .05$		
Equ c 1										$r = 0.048$ $P > .05$	
Equ c 3											$r = 0.550$* $P < .005$

*P values less than .005 were considered highly significant.
†P values less than .05 were considered significant.

Repository at www.jacionline.org) but not between the 2 other pairs of lipocalins (Can f 6: Fel d 4, $r = 0.163$, $P > .05$; Can f 6: Equ c 1, $r = 0.325$, $P > .05$). The weak IgE cross-reactivity among the lipocalin allergens was also evident by the fact that many patients displayed selective IgE reactivity to members of this allergen family. For example, 9 patients showed selective IgE reactivity to Fel d 4 but not to Equ c 1 and 11 patients showed IgE reactivity to Fel d 4 but not to Can f 6 (Fig 1; see Table E2).

Significant correlations in IgE reactivity were found between serum albumins from dog, cat, and horse (Can f 3 vs Fel d 2, $r = 0.788$, $P < .001$; Can f 3 vs Equ c 3, $r = 0.690$, $P < .001$; Equ c 3 vs Fel d 2, $r = 0.684$, $P < .001$; Table E2). However, for the albumins also, several patients were found with selective IgE reactivities toward certain albumins (Fig 1 and Fig E2). The poor associations of IgE reactivities between the lipocalins and albumins may also be due to IgG competing with IgE for chip-bound allergens.

In summary, we report a microarray containing 11 purified recombinant and natural allergens from dog, cat, and horse and its usefulness for the diagnosis of IgE sensitization to dogs and cats and the parallel analysis of allergen-specific IgG responses. The microarray not only allowed sensitive and specific detection of dog and cat allergen-specific IgE but also identified allergens that may be relevant components of vaccines and allowed to reveal species-specific sensitizations due to limited cross-reactivity of the allergen components. Furthermore, we discovered an interesting dissociation of allergen-specific IgE and IgG responses that indicates that nonsequential class-switch mechanisms are operative in animal allergy and may explain why naturally occurring allergen-specific IgG is not always protective.

We thank Prof Oswald Wagner for his support and Nadja Balic for ImmunoCAP testing, both from the Department of Laboratory Medicine, Medical University of Vienna.

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This study was supported by the SFB program F46 of the Austrian Science Fund (FWF) project F4605 and by research grants from Biomay AG, Vienna, Austria; the Christian Doppler Research Association, Austria; the FP7-funded EU project MeDALL; the Swedish Research Council; and the Stockholm County Council.

Disclosure of potential conflict of interest: C. Lupinek has received lecture fees from Thermo Fisher. M. van Hage has received lecture fees from Thermo Fisher Scientific, Novartis, and ALK-Abelló. R. Valenta has received research support from FWF Austrian Science Fund, Phadia, Thermo Fisher, and Biomay; and has received consulting fees from Phadia, Thermo Fisher, and Biomay. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online January 7, 2014.
http://dx.doi.org/10.1016/j.jaci.2013.10.058

Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: A 12-month follow-up analysis

To the Editor:

Treatment with the neutralizing monoclonal anti–IL-5 antibody mepolizumab for 12 months results in a significant reduction in exacerbation frequency and eosinophilic airway inflammation in subjects with refractory eosinophilic asthma.[1,2] Little is known about the effects of withdrawal of anti–IL-5 treatment. A theoretical risk of "rebound" worsening of eosinophilic airway inflammation associated with negative outcomes has been suggested[3] on the basis of *in vitro* observations that anti–IL-5 therapy is associated with upregulation of IL-5 synthesis by T_H2 cells, upregulation of IL-5R expression by eosinophils, and persistence of preformed IL-5 in complex with the drug for a variable period of time after cessation of therapy.[4]

As part of a follow-up analysis, subjects completing a 12-month study of mepolizumab in refractory asthma[1] were observed for 12 months with assessments every 3 months. The aims of this period of observation were to examine the kinetics of blood and sputum eosinophil counts, to examine the change in clinical markers of asthma control following cessation of therapy, and to investigate the temporal relationship between the change in biological and clinical control of disease. In addition, radiological markers of airway remodeling characterized by using helical thin-section computed tomography (CT) during the trial were assessed at the beginning and end of the 12-month follow-up period.

This was an unblinded, prospective, observational study. Systematic follow-up at 3-month intervals was maintained for study subjects in our Refractory Asthma Clinic for 12 months (see Fig E1 in this article's Online Repository at www.jacionline.org). All subjects provided written informed consent for the clinical trial, for recording analysis and reporting of data during the observation period and for an additional CT scan performed at the end of the observation period. The clinical trial was supported by an unrestricted grant from GlaxoSmithKline.

The final visit for treatment with either mepolizumab or placebo was assigned as the baseline visit for the follow-up analysis. This was the final visit during the study at which blood and sputum samples were collected. The 12-month follow-up period was calculated from this time point. All subjects received a 2-week course of oral prednisolone 30 mg once daily 2 weeks after this visit to optimize pulmonary function before a CT scan as part of the trial protocol. Details of the assessment and analysis are available in this article's Online Repository at www.jacionline.org.

Fifty-six subjects (27 assigned to mepolizumab) completed the original study. Characteristics of these subjects at the baseline visit for follow-up are summarized in Table E1 in this article's Online Repository at www.jacionline.org, and subject disposition is described in the Online Repository. The frequency of severe exacerbations in the 12-month follow-up observational period was 3.1 per subject in those previously treated with placebo and 3.9 per subject in those previously treated with mepolizumab (rate ratio, 1.25; 95% CI, 0.71-1.91; $P = .54$). There was a significant increase in the overall frequency of severe exacerbations from 0.56/patient at 0 to 3 months to 1.2/patient ($P = .007$) at 3 to 6 months for subjects who were treated with mepolizumab (rate ratio, 2.11; 95% CI, 1.76-2.54; $P < .001$) but not placebo (Fig 1). Changes to drug therapy are outlined in the Online Repository.

Significant between-visit increases in the blood eosinophil count were observed at 0 to 3 months ($P < .001$) and 3 to 6 months ($P = .004$; Fig 1; Table I) after stopping mepolizumab but not placebo. For the sputum eosinophil count, a significant rise occurred at 0 to 3 months ($P = .03$) but not thereafter (Fig 1; Table I). The Juniper Asthma Control Questionnaire (JACQ) rose by a mean of 0.59 points over the 12 months after the final treatment visit for subjects who received mepolizumab but not placebo (Fig 1; Table I). Most of the between-group difference could be attributable to a significant fall in JACQ ($P = .049$) occurring in the placebo group for the interval 9 to 12 months after the final treatment visit of the study (Fig 1). No significant differences in postbronchodilator FEV_1 or fraction of exhaled nitric oxide were seen (Fig 1; Table I).

A significant within-group increase in airway wall area per unit body surface area was identified in both groups (mean difference [95% CI]: placebo group, 1.2 mm^2/m^2 [0.1-2.2], $P = .03$; mepolizumab group, 1.0 mm^2/m^2 [0.2-1.8], $P = .01$). Participants who received mepolizumab had a significant increase in the total area of their measured airway (mean difference [95% CI], 1.4 mm^2/m^2 [0.2-2.6], $P = .03$]) (see Table E1).

Our study is the first to report changes that occur after stopping mepolizumab therapy in a study population likely to be relevant to the population treated with this agent. Cessation of mepolizumab was associated with a rise in the blood eosinophil count beginning soon after stopping therapy and continuing to baseline over 6 months. The frequency of severe exacerbations increased significantly after stopping mepolizumab, and 12 months after stopping mepolizumab, exacerbation frequency was not significantly different between subjects of the 2 study groups. The rise in exacerbations at 3 to 6 months after stopping mepolizumab was preceded by a rise in sputum and blood eosinophils, supporting suggestions that these events are related but have different time courses.[5-8]

The finding of increased asthma symptoms following cessation of mepolizumab was unexpected because symptoms were not modified significantly during the treatment period.[1] However, mean symptom scores for subjects receiving mepolizumab were lower than for subjects in the placebo group at the end of the treatment phase of the study. The rise in symptoms after stopping therapy may therefore in part represent regression to the mean. An increasing frequency of severe exacerbations will have also contributed to higher symptom scores at each routine visit. We consider it unlikely that the increase in symptoms after mepolizumab withdrawal was due to a rebound phenomenon because it was not associated with

METHODS

Patients' sera

Sera from 24 adult animal-allergic patients suffering from rhinitis, conjunctivitis, asthma, urticaria, and/or atopic dermatitis were analyzed. All patients had reported allergic symptoms attributable to contact with cat (patients 1-24, Table E1), and 1 subset exhibited allergic symptoms to cat and additionally to dog and/or horse (patients 1-17, Table E1). As controls, sera from 3 subjects with allergy to pollen or/and house dust mite but without allergy to animal dander and from 9 nonallergic subjects were analyzed. Control subjects were exposed to cats and dogs. All subjects were from Austria. The anonymized analysis of sera was approved by the ethics committee of the Medical University of Vienna, and subjects had signed approved consent forms. For each patient, a detailed case history summarizing allergic symptoms to animals and other allergen sources was established. In particular, patients were asked in detail about the occurrence, type, and intensity of allergic symptoms on contact with animals (ie, dog, cat, horse, rabbit, mice, guinea pig, cow, and sheep) (Table E1). None of the animal-allergic patients had received allergen-specific immunotherapy for animal allergies. Symptoms on contact with animals were recorded, and symptoms on contact with other allergen sources were also noted (Table E1). Animal-allergic patients were frequently cosensitized to house dust mites and grass/tree pollen. Each serum was tested regarding total IgE levels and for IgE reactivity to natural dog dander (e5) and cat dander (e1) allergen extracts as determined by the ImmunoCAP System (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). Table E1 summarizes the demographic, clinical, and serologic characteristics of the patients.

Expression and purification of recombinant dog allergens

Genes coding for the mature forms of Can f 4 (ACY38525.1) and Can f 6 (E2QYS2) was synthesized by using PCR-based assembly of oligonucleotides (GenScript, Piscataway, NJ) and inserted into the *NdeI/EcoRI* sites of pET-27b (Novagen, Darmstadt, Germany). Both genes contained sequences coding for a hexahistidine tag at the C terminus of the protein, and the gene sequences were optimized for *Escherichia coli* expression. The DNA sequences were confirmed by means of restriction enzyme analysis with corresponding restriction enzymes (Roche, Mannheim, Germany) and by automated sequencing of both DNA strands. *E coli* BL 21 DE3 (Stratagene, La Jolla, Calif) transformed with pET 27b-Can f 4 and pET 27b-Can f 6 were grown at 37°C for approximately 10 hours in a GFL 3033 incubator (GFL, Burgwedel, Germany) in Luria Bertani medium containing kanamycin (30 μg/mL) until a cell density (OD_{600nm}) of 0.5 for Can f 4 and 0.2 for Can f 6 was reached. Protein expression was induced by adding 0.5 mmol/L isopropyl-β-thiogalactopyranoside (Calbiochem, Merck, Darmstadt, Germany). Cells were harvested by centrifugation at 6000*g* for 10 minutes. rCan f 4 was purified under native conditions. For this purpose, cell pellets were lysed with an Ultra-Turrax (Janke & Kunkel-IKA Labortechnik, Staufen, Germany) in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). rCan f 6 was purified under denaturing conditions. In this case, cell pellets were lysed in lysis buffer A (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 mol/L Urea, pH 8). Both recombinant proteins were purified by Ni^{2+} metal-ion affinity chromatography according to the manufacturer's protocol (Qiagen, Hilden, Germany). Finally, purified Can f 4 was dialysed against PBS (pH 9) and rCan 6 was refolded by extensive dialysis against 10 mM NaH_2PO_4 (pH 7.5). The purity of the recombinant proteins was analyzed by using SDS-PAGE[E1] and Coomassie blue staining under reducing and nonreducing conditions. The molecular masses were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker, Billerica, Mass), and the protein concentration was determined by Micro-BCA Protein Assay Kit (Pierce, Rockford, Ill).

Circular dichroism analysis

The circular dichroism spectra of purified recombinant rCan f 4 and rCan f 6 were measured on a JASCO J-810 spectropolarimeter (Tokyo, Japan). Measurements were performed at protein concentrations of 0.1 mg/mL in a rectangular quartz cuvette with a path length of 0.2 cm. Spectra were recorded from 190 to 260 nm with a resolution of 0.5 nm at a scan speed of 50 nm/min and were derived from 3 scans. Final spectra were corrected by subtracting the baseline spectra obtained with the buffers alone. Results are displayed as the mean residue ellipticities (Θ) at given wavelengths. The secondary structure contents of the proteins were calculated with the secondary structure estimation programs CDSSTR and CONTIN.[E2]

Extended ISAC microarray

The animal allergen spectrum of ImmunoCAP ISAC (Thermo Fisher, Uppsala, Sweden) was expanded regarding rCan f 4 and rCan f 6. Allergens were spotted in triplicates, and IgE standards were added as for ImmunoCAP ISAC (Thermofisher). The animal allergen repertoire of the chip comprised dog allergens (rCan f 1, rCan f 2, nCan f 3, rCan f 4, rCan f 5, and rCan f 6), cat allergens (rFel d 1, nFel d 2, and rFel d 4), and horse allergens (rEqu c 1 and nEqu c 3).[E3] Quality controls were performed with IgE and IgG standards with established IgE and IgG reactivity to allergen components on the chip. For IgE detection, 30 μL of serum, or for IgG detection 30 μL of a 1:50 dilution of serum, was added to the microarray and incubated for 120 minutes, followed by washing and incubation with fluorescence-labeled anti-IgE or anti-IgG antibodies (Thermo Fisher), respectively, for 30 minutes. Then, chips were washed, dried, and analyzed with a Laser Scan Confocal microarray reader (LuxScan 10K/A; Capital-Bio, Beijing, China) and evaluated with Phadia Microarray Image Analysis software. Antibody levels are given as semiquantitative ISAC Standardized Units (ISUs) with a cutoff of 0.1 ISU. The allergen-specific antibody levels were classified as low (0.1-1 ISUs), moderate to high (>1-15 ISUs), or very high (>15 ISUs).

Statistical analysis

For all statistical analyses, the statistical Program SPSS (2008, version 16.0; SPSS, Inc, Chicago, Ill) was used. *P* values of less than .05 were considered significant. Correlations between variables were analyzed by using the Spearman-Rho test.

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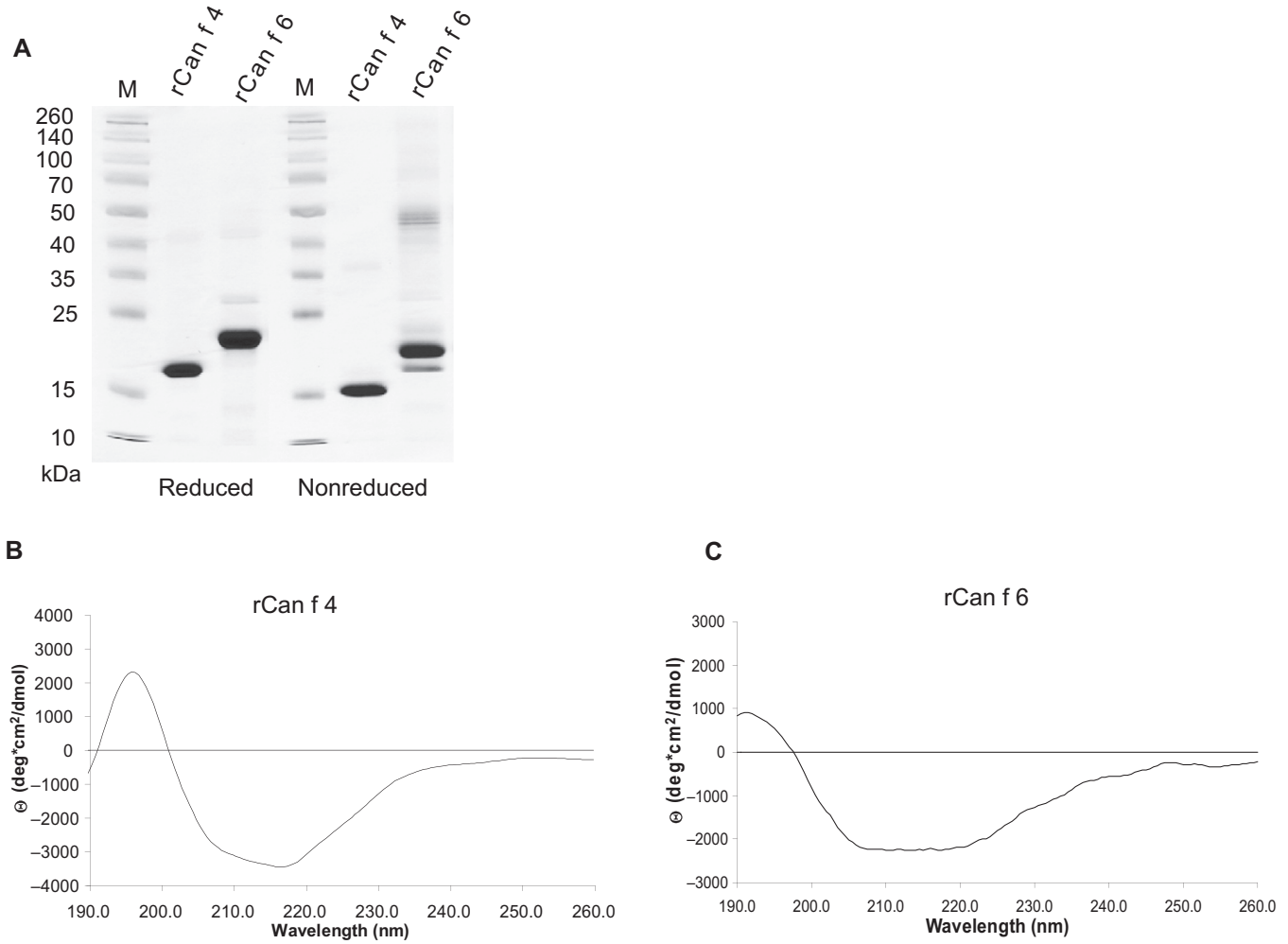

FIG E1. Biochemical and structural characterization of rCan f 4 and rCan f 6. **A,** Coomassie brilliant blue–stained SDS-PAGE containing rCan f 4 and rCan f 6 under reducing and nonreducing conditions. Molecular weights *(M)* are indicated at the left margins. **B,** Circular dichroism spectra of rCan f 4 and rCan f 6. The mean residue ellipticities (Θ) *(y-axes)* are shown at given wavelengths *(x-axes)*.

		Der p 1		Der p 2		Der p 4		Der p 5		Der p 7		Der p 10		Der p 11		Der p 14		Der p 15		Der p 18		Der p 21		Der p 23	
group	patient	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE	IgG
	1	2.30	6.11	8.20	9.66	0.00	1.88	1.03	2.18	0.62	1.65	0.00	0.90	0.00	6.31	0.00	6.40	0.00	1.96	0.00	4.92	1.42	4.64	8.92	6.76
	2	0.00	3.02	3.43	1.02	0.00	3.81	0.00	4.19	0.00	1.09	0.00	0.00	0.00	2.63	0.00	2.34	0.00	1.71	0.00	1.93	0.00	3.23	0.00	6.42
	3	0.85	2.62	0.69	0.70	0.00	0.66	0.00	0.86	0.00	1.01	0.00	0.78	0.00	2.22	0.00	2.28	0.00	1.89	0.00	2.53	0.00	4.75	0.90	1.07
	4	0.00	1.97	0.00	0.00	0.00	8.00	0.00	2.22	0.00	7.47	0.00	0.00	0.00	1.82	0.00	5.96	0.00	2.76	0.00	9.26	0.00	0.00	0.00	0.00
DCA	5	0.00	0.71	8.70	8.80	0.00	1.26	0.00	0.72	0.00	1.24	0.00	0.71	0.00	1.93	0.00	1.92	0.00	1.98	0.00	2.94	0.00	1.10	13.24	3.71
	6	1.05	4.35	10.20	4.57	0.00	4.76	0.00	1.50	0.17	6.35	0.93	0.00	0.25	3.62	0.00	4.60	0.00	1.75	0.00	5.39	0.00	1.52	7.52	5.08
	7	2.23	1.55	5.65	2.71	0.00	6.94	0.00	1.85	0.00	1.09	0.00	0.70	0.12	2.07	0.00	1.88	0.00	1.86	0.00	5.24	0.00	0.00	5.36	4.53
	8	0.00	4.35	0.90	2.72	0.00	6.46	0.00	3.59	0.00	20.40	0.00	0.65	0.00	2.99	0.00	3.29	0.00	3.13	0.00	9.80	0.00	3.02	1.64	2.85
	9	0.00	5.54	0.00	1.09	0.00	0.00	0.00	1.33	0.00	1.50	0.00	0.00	0.00	4.04	0.00	7.23	0.00	10.67	0.00	7.60	0.00	3.61	0.00	2.50
	10	0.00	0.00	0.00	1.73	0.00	0.00	0.00	0.00	0.00	0.81	0.00	0.00	0.00	1.29	0.00	1.12	0.00	2.18	0.00	1.64	0.00	0.00	0.00	2.04
	11	0.00	1.22	0.00	0.56	0.00	0.76	0.00	0.84	0.00	1.14	0.00	0.47	0.00	2.24	0.00	2.05	0.00	2.52	0.00	3.22	0.00	1.91	0.00	2.38
	12	0.00	2.55	1.63	0.00	0.00	0.00	0.00	0.98	0.25	8.53	0.00	0.00	0.00	1.63	0.00	3.26	0.00	2.99	0.00	7.13	0.00	1.78	0.25	3.26
	13	0.00	2.27	0.55	1.03	0.00	1.34	0.00	3.44	0.00	1.25	0.00	0.83	0.00	2.21	0.00	1.83	0.00	1.94	0.00	4.17	0.14	0.00	0.54	2.94
	14	0.00	4.15	5.38	0.00	0.00	0.00	0.00	2.30	0.00	1.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.12	0.00	3.36	0.00	0.00	0.00	1.16
	15	0.00	3.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.02	0.00	14.40	0.00	0.00	0.00	12.23	0.00	0.00	0.00	0.00
	16	0.00	1.33	0.00	2.43	0.00	1.10	0.00	0.39	0.00	0.65	0.00	0.00	0.00	0.88	0.00	0.85	0.00	1.81	0.00	1.69	0.00	0.00	0.00	2.07
	17	0.00	2.72	0.00	0.65	0.00	1.24	0.00	4.84	0.11	1.23	0.00	1.00	0.00	5.54	0.00	6.65	0.00	2.69	0.00	8.97	0.00	1.63	5.58	5.32
	18	0.00	0.00	0.00	0.00	0.00	0.98	0.00	0.00	0.00	0.93	0.00	0.00	0.00	1.44	0.00	1.76	0.00	0.00	0.00	3.42	0.00	0.92	0.00	2.12
	19	21.65	0.00	52.60	0.00	0.71	0.00	0.86	0.00	0.31	0.00	0.00	0.00	0.62	0.00	0.00	0.00	0.41	0.00	0.31	0.00	0.17	0.00	0.00	0.00
	20	0.00	5.71	0.00	2.15	0.00	8.94	0.00	2.93	0.00	3.18	0.00	1.52	0.00	8.19	0.00	8.03	0.00	4.50	0.00	16.19	0.00	7.14	0.00	7.82
CA	21	0.00	2.06	0.00	0.84	0.00	1.02	0.00	5.77	0.00	3.13	0.00	0.99	0.00	4.04	0.00	3.31	0.00	1.71	0.00	4.52	0.00	0.00	0.00	2.39
	22	0.00	5.90	19.74	10.92	0.00	4.00	0.27	3.19	0.14	1.90	0.59	0.91	0.00	2.17	0.00	2.28	0.00	2.79	0.00	6.14	0.00	1.98	9.27	3.33
	23	12.61	4.09	17.59	2.99	0.35	3.33	7.08	1.05	0.76	1.67	0.00	0.00	0.00	2.30	0.00	3.58	0.00	1.65	0.00	2.78	7.68	3.52	33.95	3.73
	24	3.39	13.46	12.22	11.71	0.15	2.01	1.14	2.41	0.15	4.35	1.13	0.00	0.20	3.07	0.00	3.11	0.00	2.92	0.00	7.40	0.00	3.15	7.30	5.04
	25	0.00	2.91	0.00	1.14	0.00	1.35	0.00	0.59	0.00	0.82	0.00	0.86	0.00	1.61	0.00	2.96	0.00	2.46	0.00	6.67	0.00	0.00	0.82	0.00
OA	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	27	0.00	2.77	0.00	1.76	0.00	4.54	0.00	1.88	0.00	1.17	0.00	0.68	0.00	3.67	0.00	5.26	0.00	1.65	0.00	8.57	0.00	2.05	0.00	3.24
	28	0.00	1.46	0.00	0.00	0.00	1.68	0.00	0.33	0.00	0.80	0.00	0.85	0.00	1.46	0.00	3.06	0.00	2.26	0.00	1.31	0.00	0.81	0.00	0.48
	29	0.00	3.64	0.00	1.22	0.00	0.00	0.00	1.01	0.00	0.76	0.00	1.34	0.00	3.40	0.00	1.80	0.00	1.32	0.00	2.36	0.00	1.26	0.00	0.81
	30	0.00	0.90	0.00	0.28	0.00	0.00	0.00	0.31	0.00	0.43	0.00	0.00	0.00	1.32	0.00	1.09	0.00	1.59	0.00	2.30	0.00	0.95	0.00	0.49
NA	31	0.00	1.97	0.00	0.61	0.00	5.70	0.00	5.31	0.00	1.07	0.00	0.84	0.00	2.31	0.00	1.43	0.00	2.31	0.00	7.85	0.00	0.65	0.00	2.02
	32	0.00	3.37	0.00	0.45	0.00	2.89	0.00	0.91	0.00	3.03	0.00	0.00	0.00	2.36	0.00	1.02	0.00	0.00	0.00	7.44	0.00	0.99	0.00	1.50
	33	0.00	1.55	0.00	0.84	0.00	0.00	0.00	5.05	0.00	0.00	0.00	0.00	0.00	2.56	0.00	2.66	0.00	0.79	0.00	6.77	0.00	0.73	0.00	1.38
	34	0.00	0.47	0.00	0.58	0.00	0.47	0.00	0.53	0.00	2.12	0.00	0.27	0.00	1.28	0.00	1.26	0.00	1.03	0.00	3.05	0.00	0.86	0.00	1.87
	35	0.00	1.63	0.00	0.00	0.00	0.00	0.00	0.37	0.00	0.59	0.00	0.00	0.00	0.40	0.00	1.35	0.00	1.29	0.00	1.92	0.00	0.00	0.00	0.39
	36	0.00	2.21	0.00	2.62	0.00	7.92	0.00	11.83	0.00	6.09	0.00	4.64	0.00	10.34	0.00	7.86	0.00	3.58	0.00	17.40	0.00	9.79	0.00	4.00

IgE reactivity
- negative
- 0.1-0.9
- 1-14.9
- >15

IgG reactivity
- negative
- 0.1-0.9
- 1-14.9
- >15

FIG E2. Heat map of patients' IgE and IgG reactivity. IgE and IgG levels (inserts show color codes for the levels) specific for microarrayed house dust mite allergens (nDer p 1, rDer p 2, rDer p 4, rDer p 5, rDer p 7, rDer p 10, rDer p 11, rDer p 14, rDer p 15, rDer p 18, rDer p 21, and rDer p 23) are displayed for 4 groups of subjects (*DCA*, patients with symptoms to dog and/or cat; *CA*, cat-allergic patients; *OA*, allergic patients without allergy to animals; *NA*, nonallergic subjects). *n*, Natural; *r*, recombinant.

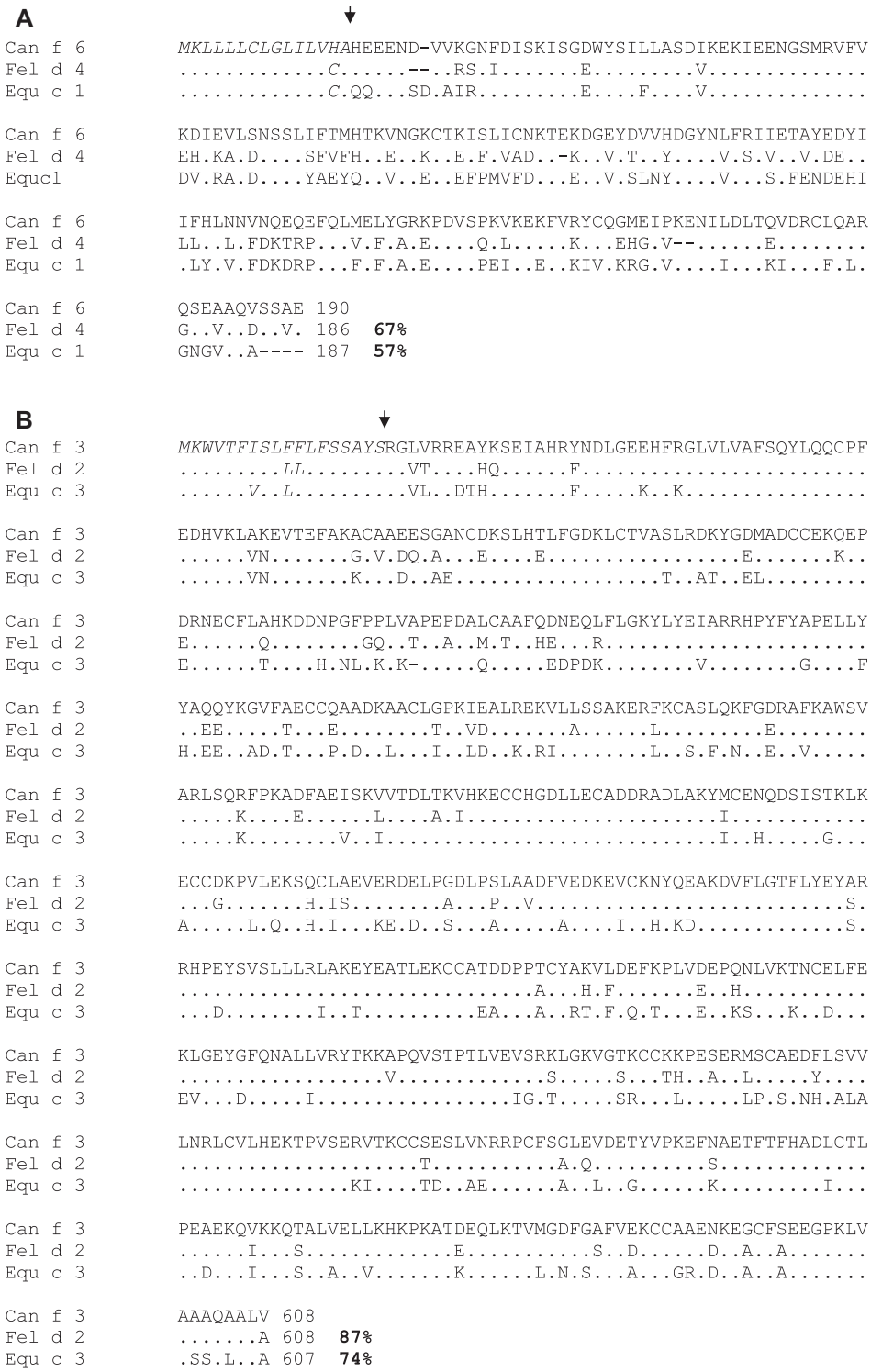

FIG E3. Alignment of protein sequences of Can f 6, Fel d 4, and Equ c 1 **(A)** and Can f 3, Fel d 2, and Equ c 3 **(B)**. Predicted signal sequences are marked in *italics*, and *arrows* mark the first amino acid of the mature form of protein. *Points* indicate identical residues, and *dashes* indicate gaps. Sequence identities of allergens to the mature Can f 6 and Can f 3 allergens are shown on the right side of the last lines of the alignments.

TABLE E1. Demographic, clinical, and serological characteristics of patients and control subjects

Allergy	Subject	Age (y)	Sex	Symptoms on contact with the following animals	Symptoms to animals	Other allergies	Symptoms to other allergen sources	Specific IgE level (kU_A/L)		Total IgE level (kU/L)
								Cat (e1)	Dog (e5)	
	1	23	F	Dog, cat, horse, rabbit	AS, RH,	tp, gp, hdm, nu	AS, RH, CO, OAS	4.31	4.96	140
	2	42	F	Dog, cat, horse	AS, RH, CO	tp, gp, hdm, ve, he, cm	AS, RH, CO, AD	>100	66.70	>2000
	3	26	F	Dog, cat, horse, rodents	AS, RH, CO, U	tp, gp, hdm, fi, nu	AS, RH, CO	>100	14.60	570
	4	36	F	Dog, cat, horse	AS, U	gp, hdm	RH	15.70	6.27	218
DCA	5	29	M	Dog, cat	AS, RH, CO	tp, gp, hdm	AS, RH, CO, AD	19.70	63.00	>2000
	6	34	F	Dog, cat	AS, CO, AD	tp, gp, hdm	AS, RH, CO, AD	75.60	78.10	>2000
	7	26	M	Dog, cat, guinea pig, sheep	CO, AD	tp, gp, hdm, he, nu	AS, RH, CO, AD, OAS	19.30	4.47	>2000
	8	23	M	Dog, cat	AS, RH, CO	tp, gp, hdm, fr	AS, RH, CO, OAS	10.00	16.40	316
	9	27	M	Dog, cat, horse, rabbit, guinea pig	RH, CO	tp, me	RH	31.30	4.44	129
	10	26	M	Dog, cat, horse, rabbit, guinea pig	AS, AD	tp, fr, nu	RH, CO, AD, OAS	52.70	12.70	>5000
	11	37	M	Dog, cat, horse	AS, RH, CO, AD	tp, gp	RH, CO	3.60	1.46	141
	12	47	F	Dog, cat	AS, RH, AD	tp, gp, hdm, fi	AS, RH, AD, U	>100	>100	1224
	13	41	M	Dog, cat	RH	tp, gp, ve	RH, CO, OAS	0.55	1.81	44
	14	30	F	Dog, cat, guinea pig	AS, RH, CO	tp, gp, hdm, mo, me, fi, nu	AS, RH, CO, OAS	3.55	5.20	997
	15	47	M	Dog, cat, horse	AS, RH, CO, U	tp, gp	AS, RH, CO	13.20	20.10	420
	16	26	M	Dog, cat, horse, guinea pig	RH, CO	tp, gp	RH, CO	62.20	6.00	798
	17	27	M	Dog, cat, guinea pig	AS, RH	tp, gp, hdm, fr	AS, RH, OAS	61.00	20.80	938
	18	27	M	Cat	AS	gp, me	AS	>100	73.20	2706
	19	19	M	Cat	AS	gp, hdm	AS	4.97	9.97	1771
	20	36	F	Cat	AS, RH, CO	wf	RH	>100	17.10	1035
CA	21	29	F	Cat, horse	AS, RH, CO	me	U	11.30	2.46	101
	22	29	F	Cat	RH, CO	tp, gp, hdm, fr	AS, RH, CO, OAS	3.51	0.75	490
	23	27	F	Cat, horse	RH, CO	tp, gp, hdm, fr	RH, CO, OAS	3.47	2.89	>5000
	24	26	M	Cat	RH, CO	gp, hdm	AS, RH, CO	3.20	0.48	296
	25	23	M	0	0	tp, gp, fr, ve	RH, CO, OAS	0	0.78	410
OA	26	25	F	0	0	tp, gp, hdm, fr, nu	RH, CO, OAS	0.41	0.68	130
	27	34	M	0	0	gp	RH, CO	0	0	45
	28	29	F	0	0	0	0	0	0	31
	29	29	M	0	0	0	0	0	0	44
	30	27	F	0	0	0	0	0	0	8
NA	31	49	M	0	0	0	0	0	0	26
	32	44	F	0	0	0	0	0	0	52
	33	22	F	0	0	0	0	0	0	19
	34	36	M	0	0	0	0	0	0	19
	35	39	M	0	0	0	0	0	0	20
	36	54	F	0	0	0	0	0	0	8

Demographic data and symptoms and sensitization to animal allergens are displayed for 24 dog/cat-allergic patients, 3 controls with allergy to pollen and/or house dust mite, and 9 nonallergic individuals. Total and allergen-specific IgE levels were measured by using ImmunoCAP and are displayed in kilo units/liter (kU/L) and kilo units of antigen/liter (kU_A/L), respectively. The cutoff value is 0.35 kU_A/L.

AD, Atopic dermatitis; *AS*, asthma; *CA*, cat allergy; *cm*, cow's milk; *CO*, conjunctivitis; *DCA*, dog or cat allergic; *e1*, cat dander extract; *e5*, dog dander extract; *F*, female; *fi*, fish; *fr*, fruits; *gp*, grass pollen; *hdm*, house dust mite; *he*, hens egg; *M*, male; *me*, medicaments; *mo*, moulds; *NA*, not allergic; *nu*, nuts; *OA*, other allergies than to animal dander; *OAS*, oral allergy syndrome; *RH*, rhinitis; *tp*, tree pollen; *U*, urticaria; *ve*, vegetables; *wf*, wheat flour.

TABLE E2. Correlations between specific IgE levels to homologous allergens for the group of patients with dog and/or cat allergy (n = 24)

	Can f 6	Fel d 4	Equ c 1	Can f 3	Fel d 2	Equ c 3
Can f 6	1	$r = 0.163$ $P > .05$	$r = 0.325$ $P > .05$			
Fel d 4		1	$r = 0.557$* $P < .003$			
Equ c 1			1			
Can f 3				1	$r = 0.788$* $P < .001$	$r = 0.690$* $P < .001$
Fel d 2					1	$r = 0.684$* $P < .001$
Equ c 3						1

*P values less than .005 were considered highly significant.