

Assessment of recombinant dog allergens Can f 1 and Can f 2 for the diagnosis of dog allergy

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Summary

Background The use of recombinant allergens for the diagnosis and immunotherapy of allergy may offer several advantages over allergen extracts.

Objective To produce recombinant dog allergens Can f 1 and Can f 2 in *Pichia pastoris* yeast and to assess their suitability for the diagnosis of dog allergy.

Methods Clinically diagnosed dog-allergic patients' and healthy non-atopic dog owners' reactivities against recombinant Can f 1 and Can f 2 and commercial dog epithelial extract were studied by a panel of methods including skin prick test (SPT), ELISA and IgE immunoblotting.

Results Recombinant Can f 1 and Can f 2 were found immunologically functional: they bound dog-allergic patients' IgE in immunoblotting and inhibited specifically the binding of IgE to their natural counterparts in the dog allergen extract. Moreover, patients' IgE reactivity in immunoblotting to natural Can f 1 and their SPT with the recombinant allergen were perfectly concordant (ϕ coefficient 1.0, $P < 0.001$). The concordance was slightly lower with recombinant Can f 2 (ϕ coefficient 0.92, $P < 0.001$). A lower number of dog-allergic patients, 52%, reacted against Can f 1 than previously reported. About one-third of the patients reacted to Can f 2. In immunoblotting, the highest prevalence of reactivity, 60%, was directed to an 18 kDa component. Aminoterminal sequencing showed this to be a previously unidentified allergenic protein.

Conclusions The recombinant allergens can be used reliably to identify Can f 1 and Can f 2-sensitized individuals. However, the two allergens are insufficient as reagents for diagnosing dog allergy.

Keywords Can f 1, Can f 2, diagnosis, dog allergy, recombinant allergens

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Introduction

The use of recombinant allergens for the diagnosis and immunotherapy of animal allergies can overcome several of the problems associated with natural allergen extracts. These include, for example, standardization, which will be straightforward in mass units once the immunoreactivity of the recombinant product has been verified [1]. In contrast, the amount of important allergens in extracts can vary extensively, as was shown with Can f 1, the major allergen of dog [2]. The same study also showed that natural preparations might contain mite allergens potentially causing false-positive skin prick test (SPT) results. Another problem associated with allergen extracts is their specificity. For the moment, the data on the cross-reactivity between major animal allergens are limited, but at the level of epithelial extracts cross-reactivity appears to exist [3, 4]. This is not necessarily a surprise since extracts contain potentially cross-reactive proteins, for

example, serum components, such as albumin [4]. It can be assumed that the specificity of a test for animal allergy increases by using purified major allergens.

Pure recombinant allergens, possibly genetically modified, or in conjunction with appropriate adjuvants, are expected to open new prospects for allergen immunotherapy [1]. Immunotherapy with pure preparations most probably also helps to avoid sensitization against new proteins, a phenomenon sometimes seen in immunotherapy based on allergen extracts [5, 6]. Moreover, if immunotherapy is carried out by administering pure recombinant allergens or their modifications, it is advantageous that the follow-up of the therapy can be based on measuring allergen-specific immunoparameters. In a study of grass pollen immunotherapy, measuring IgE response against purified major allergens offered a reliable way to monitor the outcome [7].

The purpose of this study was to produce recombinant (r) dog allergens Can f 1 and Can f 2 in *Pichia pastoris* and to evaluate their usability in the diagnostics of dog allergy. The allergens proved to be immunologically functional but insufficient as exclusive reagents in SPTs and ELISA assays for detecting allergy to dog.

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Materials and methods

Subjects

Atopic subjects with dog allergy diagnosed at the Department of Pulmonary Diseases of Kuopio University Hospital and healthy non-atopic dog owners as controls were recruited in the study (Table 1). For a person to be classified as allergic to dog, a positive result was required in the dog UniCAP fluoroenzyme-immunometric assay (FEIA, Pharmacia, Uppsala, Sweden) and in the SPT with dog allergen (epithelial preparation from ALK Abelló, Hørsholm, Denmark). Values considered positive in the two tests were >0.7 kU/L and ≥ 3 mm, respectively. The SPTs were performed according to European recommendations [8] as described previously [9]. To test the reactivity against rCan f 1 and rCan f 2, the allergens were applied on the back of the allergic patients and control subjects in duplicate at five 10-fold dilutions starting from 250 µg/mL. After 15 min, the weals were marked and documented by direct tracing onto strips of tape. The total serum IgE of patients and control subjects was measured by Immulite 2000 total IgE immuno-enzymometric assay (IEMA) using chemiluminescence substrate (Diagnostic Products Corporation, Los Angeles, CA, USA). The study was approved by the Ethics Committee of Kuopio University Hospital, and a written informed consent was given by all patients.

Allergen preparations

For SDS-PAGE and immunoblotting analyses, the same commercial dog epithelial extract was used as for SPTs. rCan f 1 and rCan f 2 were expressed in *P. pastoris* yeast (Invitrogen, San Diego, CA, USA). For rCan f 1, total RNA was isolated from the tongue tissue and for rCan f 2 from the parotid gland of a male dog by the RNeasy[®] total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. First-strand cDNAs were synthesized using random hexanucleotides. To generate the DNA fragments encoding Can f 1 and Can f 2, specific primers were designed based on the published nucleotide sequences of the allergens [10]. In both 3' primers, polyhistidine tag sequences were included. The generated Can f 1 and Can f 2 constructs were cloned into the pPIC9 and pHILS1 expression vectors, respectively. These constructs were transformed in *Escherichia coli*. Their nucleotide sequences were confirmed by DNA sequencing using Thermo Sequenase CY5 Dye Terminator Kit and ALF Express DNA Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). *P. pastoris* yeast was transformed with the constructs pPIC9Can f 1 and pHILS1Can f 2 and the allergens were

produced following the manufacturer's instructions (Invitrogen). The culture supernatants were harvested by centrifugation and concentrated for affinity purification. The histidine-tagged recombinant allergens were purified with HisTrap Kit (Amersham Pharmacia AB, Uppsala, Sweden) according to the manufacturer's instructions. The subsequent gel filtration was performed with Superdex 75 media (Amersham Pharmacia AB) with 0.15 M sodium chloride (Natrosteril 0.9, Orion OY, Espoo, Finland) as the eluant. The purity of the preparations was verified by SDS-PAGE. The recombinant allergens were then subjected to in-gel digestion, as described previously [11], with slight modifications. The sequences of the tryptic digests were confirmed by high-pressure liquid chromatography (HPLC)-electrospray ionization mass spectrometry as described previously [12]. The N-terminal sequencing of the natural 18 kDa allergen isolated from gel after the SDS-PAGE of the commercial dog epithelial extract was performed as described previously [13] using the Applied Biosystems Procise 494A (Foster City, CA, USA) protein sequencer. Protein concentrations were determined with the method of Bradford using the BioRad (Hercules, CA, USA) protein assay. Sterile-filtered preparations were stored at -70°C .

Antibody measurement by enzyme-linked immunosorbent assay

Can f 1- and Can f 2-specific IgE antibodies were measured with indirect ELISAs similarly as described previously [14, 15] with a few modifications. In brief, the plates were coated with 5 µg/mL of the recombinant dog allergens. Sera were diluted 1:10. The bound IgE was detected by rabbit anti-human IgE (1:500; Dako A/S, Glostrup, Denmark) combined with biotinylated goat anti-rabbit immunoglobulins (1:2000; Dako A/S). The results were expressed as optical density (OD) values.

Immunoblotting and immunoblot inhibition

Patients' and control subjects' IgE reactivity against the commercial dog SPT preparation, rCan f 1 and rCan f 2, was detected by SDS-PAGE immunoblotting as described previously [15, 16], with minor modifications. In brief, membrane strips were incubated overnight with patient and control sera (1:10), washed and then incubated with monoclonal horseradish peroxidase-labelled mouse anti-human IgE (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) at 1:2000. For detecting monoclonal mouse antibody (mAb) against Can f 1 (1:1000; Indoor Biotechnologies, Inc., Charlottesville, VA, USA), horseradish peroxidase-labelled

Table 1. Characteristics of dog-allergic patients and control subjects

	Sex		Age (years)*	Total IgE (kU/L)*	Dog SPT (mm)*	Dog RAST (kU/L)*
	Female	Male				
Patients $n = 25$	13	12	39 ± 9	442 ± 533	6.3 ± 3.3	15.6 ± 20.8
Controls $n = 11$	8	3	43 ± 13	29 ± 19	Negative	Negative

*Mean $\pm$ SD.

SPT, skin prick test.

rabbit anti-mouse immunoglobulins (Dako A/S) at 1:1000 were used. After washing, the reactive bands were visualized with ECL detection reagents (Amersham International, Buckinghamshire, UK). The inhibition of immunoblotting was performed essentially as described previously [16]. A human serum reactive to Can f 1 and Can f 2, diluted at 1:12.5, was mixed with an equal volume of rCan f 1, rCan f 2 or tetanus toxoid as a control so that the final protein concentration was 200 µg/mL. After an incubation of 6 h at room temperature, IgE reactivity against the dog epithelial preparation was visualized as described above.

Statistical analyses

Statistical differences between groups were determined with the Kruskal–Wallis test and *post hoc* comparisons with the Mann–Whitney *U*-test. For determining correlations, the Spearman rank correlation test was used. Concordances for results classified as positive or negative were determined by calculating the ϕ coefficient and the Fisher's exact *P*-value.

Results

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoglobulin E immunoblotting

SDS-PAGE revealed *P. pastoris*-produced rCan f 1 as a single band (21 kDa) and rCan f 2 as two bands (23 and 25 kDa). The sequences of the tryptic digests of rCan f 1 and rCan f 2 obtained by HPLC-electrospray ionization mass spectrometry matched with those reported by Konieczny et al. [10] (data not shown). Peptides from both bands of rCan f 2 were verified to contain the same amino acid sequences. The peptide WHSVALASNK of the higher molecular weight rCan f 2 was observed to be glycosylated with two *N*-acetyl glucosamines and 8–14 hexoses, whereas the same peptide of the smaller molecule was found to be unglycosylated.

The IgE binding capacities of rCan f 1 and rCan f 2 preparations were verified with patient sera in SDS-PAGE immunoblotting (Fig. 1 and data not shown). The same technique was used to assess the capacity of the recombinant allergens to inhibit the binding of a patient's IgE on Can f 1 and Can f 2 in the commercial dog allergen extract. Figure 1 shows that the prior incubation of a serum with rCan f 1 or rCan f 2 resulted in the elimination of IgE binding to the 22 or 25 kDa components in the extract, respectively. These molecular masses are close to those reported for Can f 1 and Can f 2 [10, 17]. The 22 kDa component in the extract was further verified as Can f 1 by its reactivity with Can f 1-specific mAb. This mAb also recognized rCan f 1 in immunoblotting (data not shown).

Patients' IgE reactivities against Can f 1 and Can f 2 are summarized in Table 2. According to the immunoblotting analyses, 18 out of 25 dog-allergic patients exhibited IgE reactivity against dog allergens (Fig. 2). Thirteen (13/25, 52%) of these had IgE against Can f 1. While six of the 13 patients (6/25, 24%) recognized Can f 1 only, seven of them (7/25, 28%) also recognized Can f 2. IgE reactivity against Can f 2 without reactivity against Can f 1 was not observed. IgE reactivity to the components other than Can f 1 and Can f 2 was mostly seen against those of 18 kDa (15/25, 60%),

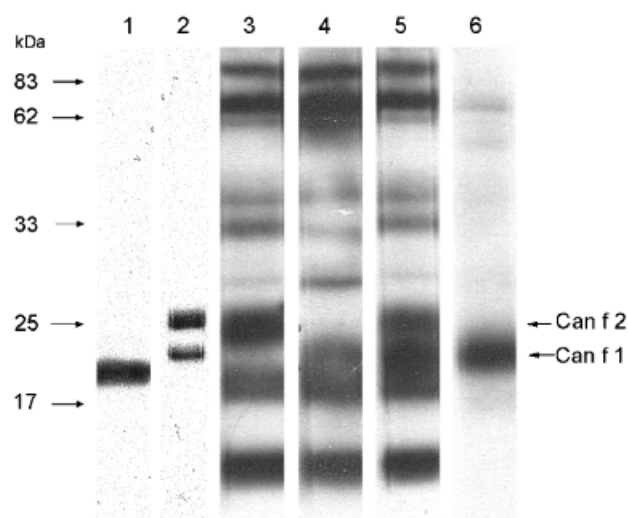


Fig. 1. IgE reactivity of a dog-allergic patient (#4) to rCan f 1 (lane 1) and rCan f 2 (lane 2), analysed by SDS-PAGE immunoblotting. Inhibition of IgE binding to natural Can f 1 and Can f 2 in the dog epithelial extract: before allowing serum of a patient (#21) to react against the extract, it was incubated with rCan f 1 (lane 3), rCan f 2 (lane 4) or with the control antigen tetanus toxoid (lane 5). Can f 1 was identified in the extract by a specific mAb (lane 6).

Table 2. Frequency of 25 dog-allergic patients' IgE reactivity against Can f 1 and Can f 2 analysed by IgE immunoblotting with commercial dog epithelial extract, and by SPTs and ELISA with recombinant allergens

Group	Frequency (n, (%)) of patients positive in		
	Immunoblotting	SPT	ELISA
Can f 1&2-positive	7 (28)	8 (32)	5 (20)
Can f 1-single positive	6 (24)	5 (20)	6 (24)
Can f 1&2-negative	12 (48)*	12 (48)	14 (56)

*Five patients exhibited IgE reactivity to allergens other than Can f 1 or Can f 2. SPT, skin prick test.

40 kDa (11/25, 44%) and 70 kDa (12/25, 48%). Five patients showing IgE reactivity in immunoblotting but being Can f 1- and Can f 2-negative had IgE against either the 70 kDa component (probably serum albumin [18]) and/or the 18 kDa component. Seven of the patients (Fig. 2) and the control subjects (data not shown) had no detectable IgE antibodies in immunoblotting.

Since dog-allergic patients' IgE reactivity was most frequently directed to the 18 kDa component, it was characterized further. The aminoterminal sequencing of the allergen suggested that it is a new member in the lipocalin family of proteins. The sequence (LPNVLTVQVSGPWK) that exhibits no homology with a known dog or other allergens (the SIB BLAST Network Service, October 20, 2003 [19]) contains the triplet glycine-x-tryptophan, which is characteristic for the first structurally conserved region of lipocalins [20].

Skin prick test reactivity to rCan f 1 and rCan f 2

Out of 25 dog-allergic patients 13 (52%) had a positive (≥ 3 mm) SPT result with rCan f 1 at 2.5 µg/mL (Table 2). Among these 13 patients, five (5/25, 20%) were positive with

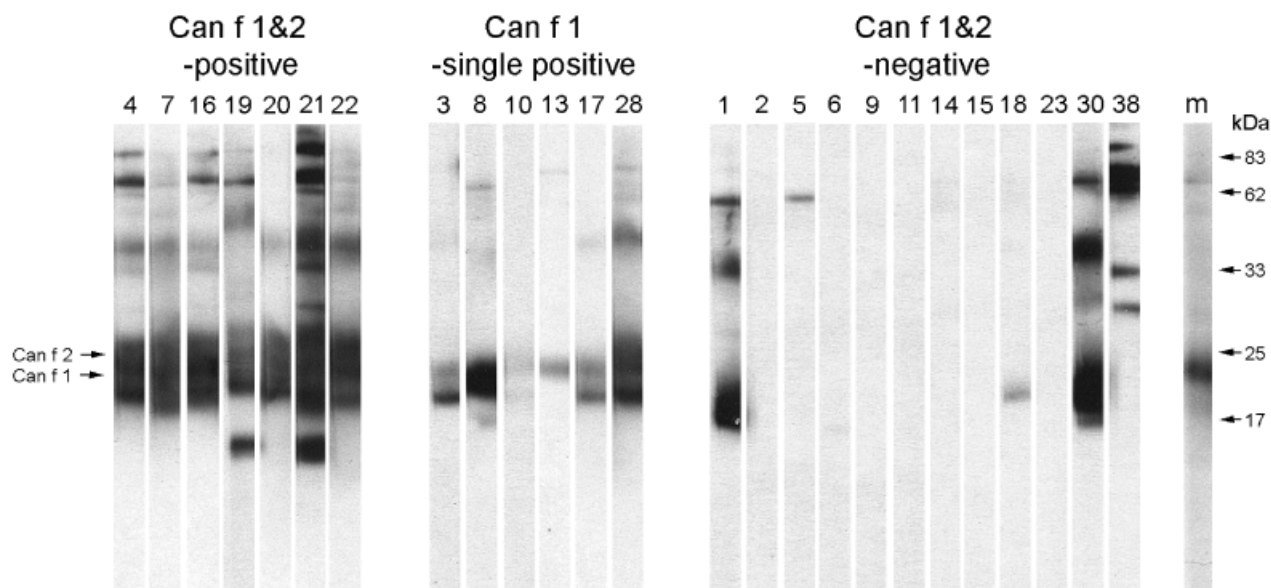


Fig. 2. Dog-allergic patients' IgE reactivity against the components of dog allergen extract analysed by SDS-PAGE immunoblotting. Patients are arranged in three groups: Can f 1&2-positive, Can f 1-positive only and Can f 1&2-negative. m – mAb to Can f 1.

rCan f 1 only (classified as SPT Can f 1-single positive) whereas eight (8/25, 32%) also had a positive SPT reaction with rCan f 2 (SPT Can f 1&2-positive). For one of these, 25 µg/mL of rCan f 2 was required for a positive reaction whereas for the others the concentration of 2.5 µg/mL was sufficient. No patient exhibited reactivity against rCan f 2 only. The SPT results of control subjects were negative.

The magnitudes of SPT reactions are shown in Fig. 3. With rCan f 1, they did not differ significantly ($P > 0.05$) between SPT Can f 1&2-positive patients and SPT Can f 1-single positive patients. The results of both groups differed significantly from those of control subjects and patients classified as SPT Can f 1&2-negative (Fig. 3). SPT Can f 1&2-positive patients' SPT reactions to rCan f 2 were significantly stronger than those of other groups (Fig. 3).

Specific immunoglobulin E levels to rCan f 1 and rCan f 2

When sensitization to the dog allergens was evaluated by measuring specific IgE by ELISA (Table 2), 11 (44%) of the 25 patients had the rCan f 1-specific IgE level above the arbitrary cut-off value (mean of the control subjects + 3 SD, i.e. OD 0.31). All control subjects were negative with this cut-off value. Among the 11 positive patients, six (6/25, 24%) patients exhibited IgE reactivity against rCan f 1 only whereas five (5/25, 20%) patients also had IgE against rCan f 2 above the cut-off value of OD 0.0 (control subjects had no specific IgE). No patient showed IgE reactivity against rCan f 2 only.

Figure 4 shows the magnitude of specific IgE levels of different groups classified according to their SPT results. While the rCan f 1-specific IgE levels did not differ between the SPT Can f 1&2-positive and SPT Can f 1-single positive patients, the results of both patient groups differed significantly from those of control subjects and SPT Can f 1&2-negative patients (Fig. 4). The difference of rCan f 2-specific IgE levels between SPT Can f 1&2-positive and SPT Can f 1-

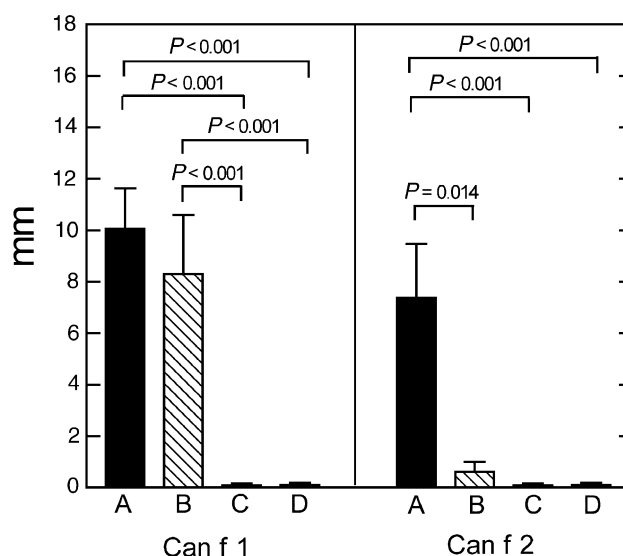


Fig. 3. Magnitude of dog-allergic patients' and control subjects' skin prick test (SPT) reactions (mean diameter $\pm$ SEM, mm) with rCan f 1 and rCan f 2 at 2.5 µg/mL. Statistically significant differences ($P < 0.05$) between groups are shown. Subjects were classified as SPT Can f 1&2-positive (patients with positive SPT reactions with both rCan f 1 and rCan f 2, $n = 8$, A (■)), SPT Can f 1-single positive (positive SPT reaction with rCan f 1 only, $n = 5$, B (▨)), SPT Can f 1&2-negative (negative SPT reactions with both allergens, $n = 12$, C (▧)), and control subjects ($n = 11$, D (□)).

single positive patients was not significant ($P = 0.08$) and most probably this was because of the small number of SPT Can f 1-single positive patients ($n = 5$) as well as the fact that one of these patients had a marginally positive rCan f 2 IgE result (OD 0.01). If this result was interpreted as negative, the difference was significant ($P = 0.04$). SPT Can f 1&2-positive patients' rCan f 2-specific IgE level differed significantly from those of the control subjects and the SPT Can f 1&2-negative patients (Fig. 4).

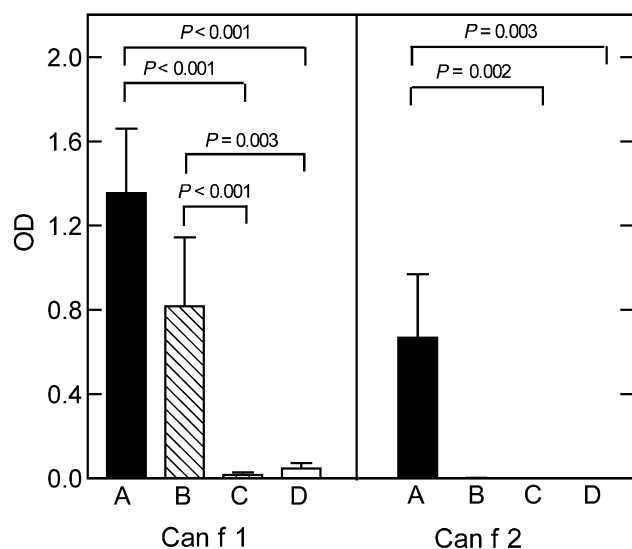


Fig. 4. Dog-allergic patients' and control subjects' rCan f 1 and rCan f 2-specific IgE levels (mean OD $\pm$ SEM). Statistically significant differences ($P < 0.05$) between groups are shown. Subjects were classified according to their skin prick test (SPT) results: SPT Can f 1&2-positive (patients with positive SPT reactions with both rCan f 1 and rCan f 2, $n = 8$, A (■)), SPT Can f 1-single positive (positive SPT reaction with rCan f 1 only, $n = 5$, B (▨)), SPT Can f 1&2-negative (negative SPT reactions with both allergens $n = 12$, C (▤)) and control subjects ($n = 11$, D (□)).

Relationship of results obtained with immunoglobulin E immunoblotting, skin prick tests and immunoglobulin E enzyme-linked immunosorbent assay

The concordance for positive and negative results (all subjects, $n = 36$) between the SPT results with rCan f 1 and the detection of Can f 1 in immunoblotting was perfect (ϕ coefficient 1.0, $P < 0.001$). However, two patients (#3 and #19) had rCan f 1-specific IgE below the cut-off level and therefore the SPT results and the results of rCan f 1-specific IgE measurements were less concordant (ϕ coefficient 0.88, $P < 0.001$). Because of the same two persons, the concordance of the results between rCan f 1-specific IgE measurements and immunoblotting showed a lower value (ϕ coefficient 0.88, $P < 0.001$). As for the reactivity against Can f 2, the highest concordance was obtained between SPT and immunoblotting results (ϕ coefficient 0.92, $P < 0.001$). In this case, one patient (#17) positive in SPT was negative in immunoblotting. Out of eight patients positive in SPTs with rCan f 2, three patients (#17, #19 and #22) turned out to be negative in rCan f 2-specific IgE measurement (ϕ coefficient 0.75, $P < 0.001$). When the results of immunoblotting and specific IgE measurements were analysed, two patients (#19 and #22) with negative results in rCan f 2-specific IgE measurement had positive reactions in immunoblotting against Can f 2 (ϕ coefficient 0.82, $P < 0.001$).

The weal sizes in SPTs at 2.5 $\mu\text{g/mL}$ and the levels of rCan f 1-specific IgE (all subjects, $n = 36$) exhibited a high correlation ($r = 0.82$, $P < 0.001$). The correlation was lower for rCan f 2 ($r = 0.66$, $P < 0.001$).

The specificities of SPTs and specific IgE measurements with the recombinant allergens for detecting dog allergy were 100%. The sensitivity was the highest in SPT with rCan f 1, 52%, while that of rCan f 1-specific IgE measurement was

44%. The sensitivities in tests with rCan f 2 remained lower; it was 32% in SPT and 20% in the IgE measurement.

Discussion

Instead of using a bacterial expression system [10], we chose to use *P. pastoris* for producing rCan f 1 and rCan f 2 since in our and other researchers' experience it has proved to yield a fully immunoreactive allergen in high amounts [21, 22]. The mass spectrometric analyses of the recombinant allergens confirmed that they were sequentially correct. Interestingly, rCan f 2 was expressed in two forms, one of which was glycosylated. The differential glycosylation of the two forms of rCan f 2, sometimes observed with proteins produced in *P. pastoris* [22], most probably accounts for the small difference in their molecular masses since the sequences of the allergens did not differ. Our tests with dog-allergic patients' sera in IgE immunoblotting (Fig. 1 and data not shown), inhibition experiments (Fig. 1) and the binding of a specific mAb to rCan f 1 all showed that the recombinant Can f 1 and Can f 2 produced in *P. pastoris* exhibit antibody-binding capacities comparable with those of the natural allergens. Moreover, the perfect concordance of SPT and immunoblotting results with Can f 1 and the very high concordance with Can f 2 support this view.

An unexpected finding in our study was that the prevalence of IgE reactivity of dog-allergic patients against Can f 1 was considerably lower than that reported by Konieczny et al. [10]. While Konieczny et al. found rCan f 1-specific IgE in 75% of dog-allergic patients' sera, our ELISA with the recombinant allergen indicated that 44% of dog-allergic patients recognized Can f 1. Only a slightly higher prevalence of sensitization, 52%, was revealed both by the immunoblotting analyses with the commercial dog dander extract and by the SPTs with the recombinant Can f 1. One possibility for the discrepancy between the studies is the selection of patients. Although our patients were clinically verified as being allergic to dog, it is possible that reactivity against Can f 1 could be higher among patients with more severe forms of allergy. The criteria adapted for a positive reaction in ELISAs in the two studies may also account for the difference. Interestingly, there was not a great difference in reactivity against Can f 2 between the studies although we found by ELISA slightly fewer patients sensitized to the allergen than Konieczny et al. In any case, our results with rCan f 1 and 2 emphasize the need for information on the reactivity to the allergens in other populations, although Finns are known not to differ significantly, for example, in the prevalence of allergic diseases from other West Europeans [23] or in the human leucocyte antigen allele distribution from most other North Europeans [24]. If, however, local environmental or demographic factors accounted for our findings it could have important implications. For example, it may turn out to be necessary to use different repertoires of recombinant preparations for the diagnosis and immunotherapy of allergy in different populations.

Our results also highlight the significance of other dog allergens than Can f 1 and Can f 2 as sensitizers. Especially the 18 kDa component could be useful for the diagnosis and

therapy of dog allergy since it was recognized by allergic subjects more frequently than Can f 1. Moreover, the 70 kDa component, the probable serum albumin according to the molecular mass [18], was recognized by a considerable number of allergic patients. The molecular mass of the 18 kDa allergen and the fact that it contains a characteristic sequence motif of lipocalins at the amino terminus of the molecule [20] suggest that it is a previously unidentified allergenic lipocalin protein. For reasons to be determined, most of the important animal-derived respiratory allergens belong to this protein family [25].

Our results show that the recombinant allergens can be reliably used to identify dog-allergic individuals sensitized to Can f 1 and Can f 2. However, diagnosing dog allergy by using the two recombinant allergens is not satisfactory since the prevalence of dog-allergic patients' IgE reactivity to Can f 1 and Can f 2 is relatively low. Using rCan f 2 in addition to rCan f 1 does not increase sensitivity since all individuals reactive to rCan f 2 are also reactive to rCan f 1. In this regard, our results are similar to those of Konieczny et al. [10]. The situation with the dog allergens is, however, quite different from that of bovine Bos d 2 or cat Fel d 1. Over 90% of cow dust-allergic patients had IgE against Bos d 2, analysed by ELISA [14] or by immunoblotting [26]. The percentage of cat-allergic subjects' sera reactive to Fel d 1 in RAST was equally high [27]. The allergen component-specific diagnosis of dog allergy is further hampered by the fact that additional candidate allergens are few according to the immunoblotting analyses (Fig. 2). If we assume that the five patients who were negative in SPTs with rCan f 1, but exhibited IgE reactivity against the 70 and the 18 kDa components in immunoblotting, were positive to these allergens in SPTs, the sensitivity for detecting dog allergy by using the three allergens in SPTs would increase up to 72% (while the specificity would be 100%). As for the seven dog-allergic patients negative in IgE immunoblotting (with the commercial SPT extract), our results do not indicate which are the allergens in the commercial RAST and SPT test preparations that they reacted to. It can be speculated that the patients exhibit low-level reactivity to more than one dog allergen, which results in a positive reaction in the commercial tests. Most probably the low sensitivity of SPT with rCan f 1 cannot be ascribed to a low protein concentration since the number of positive cases did not increase even at the concentration of 250 µg/mL of the allergen. It cannot be ruled out, however, that the sensitivity of rCan f 1-specific IgE ELISA could be increased by careful optimization of the assay (without losing specificity).

Taken together, our results indicate that the dog allergens Can f 1 and Can f 2 can be produced in a fully functional form in *P. pastoris*. Their use for diagnosing dog allergy is, however, limited by the fact that lower numbers of dog-allergic subjects react against them than reported previously. Consequently, allergy diagnosis and immunotherapy with recombinant Can f 1 and Can f 2 may turn out to be more difficult than with allergens such as cat Fel d 1 or bovine Bos d 2 which are recognized by the majority of allergic individuals. The clinical trials of immunotherapy with the dog allergens will ultimately show whether their use provides a sufficient therapeutic efficacy among individuals sensitized to them. Including the previously unidentified 18 kDa allergen

in the panel of recombinant dog allergens most probably improves the diagnostics and allergen immunotherapy of dog allergy in the future.

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References

- 1 Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000; 106:409–18.
- 2 van der Veen MJ, Mulder M, Witteman AM et al. False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. *J Allergy Clin Immunol* 1996; 98:1028–34.
- 3 Spitzauer S, Pandjaitan B, Mühl S et al. Major cat and dog allergens share IgE epitopes. *J Allergy Clin Immunol* 1997; 99: 100–6.
- 4 Cabanas R, Lopez-Serrano MC, Carreira J et al. Importance of albumin in cross-reactivity among cat, dog and horse allergens. *J Invest Allergol Clin Immunol* 2000; 10:71–7.
- 5 Ball T, Sperr WR, Valent P et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 1999; 29:2026–36.
- 6 Moverare R, Elfman L, Vesterinen E, Metso T, Haahtela T. Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP System. *Allergy* 2002; 57:423–30.
- 7 van Ree R, van Leeuwen WA, Dieges PH et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. *Clin Exp Allergy* 1997; 27:68–74.
- 8 Dreborg S, Frew A, eds. Position paper: allergen standardization and skin tests. *Allergy* 1993; 48:49–82.
- 9 Zeiler T, Mäntyjärvi R, Rautiainen J et al. T cell epitopes of a lipocalin allergen colocalize with the conserved regions of the molecule. *J Immunol* 1999; 162:1415–22.
- 10 Konieczny A, Morgenstern JP, Bizinkauskas CB et al. The major dog allergens, Can f 1 and Can f 2, are salivary lipocalin proteins: cloning and immunological characterization of the recombinant forms. *Immunology* 1997; 92:577–86.
- 11 Rautiainen J, Rytkönen M, Parkkinen S et al. cDNA cloning and protein analysis of a bovine dermal allergen with homology to psoriasin. *J Invest Dermatol* 1995; 105:660–3.
- 12 Rautiainen J, Auriola S, Rouvinen J et al. Molecular and crystal properties of Bos d 2, an allergenic protein of the lipocalin family. *Biochem Biophys Res Commun* 1998; 247:746–50.
- 13 Rautiainen J, Pentikäinen J, Rytkönen M et al. Molecular analysis of allergenic proteins in bovine dander. *Allergy* 1996; 51:378–82.
- 14 Virtanen T, Zeiler T, Rautiainen J et al. Immune reactivity of cow-asthmatic dairy farmers to the major allergen of cow (BDA20) and to other cow-derived proteins. The use of purified BDA20 increases the performance of diagnostic tests in respiratory cow allergy. *Clin Exp Allergy* 1996; 26:188–96.

- 15 Zeiler T, Taivainen A, Rytönen M et al. Recombinant allergen fragments as candidate preparations for allergen immunotherapy. *J Allergy Clin Immunol* 1997; 100:721–7.
- 16 Rautiainen J, Rytönen M, Virtanen T, Pentikäinen J, Zeiler T, Mäntyjärvi R. BDA20, a major bovine dander allergen characterized at the sequence level, is Bos d 2. *J Allergy Clin Immunol* 1997; 100:251–2.
- 17 de Groot H, Goei KG, van Swieten P, Aalberse RC. Affinity purification of a major and a minor allergen from dog extract: serologic activity of affinity-purified Can f I and of Can f I-depleted extract. *J Allergy Clin Immunol* 1991; 87:1056–65.
- 18 Pandjaitan B, Swoboda I, Brandesky-Pichler F, Rumpold H, Valenta R, Spitzauer S. *Escherichia coli* expression and purification of recombinant dog albumin, a cross-reactive animal allergen. *J Allergy Clin Immunol* 2000; 105:279–85.
- 19 Altschul SF, Madden TL, Schaffer AA et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; 25:3389–402.
- 20 Flower DR, North AC, Sansom CE. The lipocalin protein family: structural and sequence overview. *Biochim Biophys Acta* 2000; 1482:9–24.
- 21 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.
- 22 Vailes LD, Kinter MT, Arruda LK, Chapman MD. High-level expression of cockroach allergen, Bla g 4, in *Pichia pastoris*. *J Allergy Clin Immunol* 1998; 101:274–80.
- 23 The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema. *Lancet* 1998; 351:1225–32.
- 24 Partanen J, Westman P. Caucasian Finnish normal. In: Terasaki PI, Gjertson DW, eds. *HLA* 1997. Los Angeles: UCLA Tissue Typing Laboratory, 1997; 214–5.
- 25 Mäntyjärvi R, Rautiainen J, Virtanen T. Lipocalins as allergens. *Biochim Biophys Acta* 2000; 1482:308–17.
- 26 Ylönen J, Mäntyjärvi R, Taivainen A, Virtanen T. Comparison of the antigenic and allergenic properties of three types of bovine epithelial material. *Int Arch Allergy Immunol* 1992; 99:112–7.
- 27 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.