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The major dog allergens, *Can f* 1 and *Can f* 2, are salivary lipocalin proteins: cloning and immunological characterization of the recombinant forms

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SUMMARY

Canis familiaris allergen 1 (*Can f* 1) and *Canis familiaris* allergen 2 (*Can f* 2) are the two major allergens present in dog dander extracts. We now report the isolation of cDNAs encoding both proteins and present their nucleotide and deduced amino acid sequences. *Can f* 1, produced by tongue epithelial tissue, has homology with the von Ebner's gland (VEG) protein, a salivary protein not previously thought to have allergenic properties. *Can f* 2, produced by tongue and parotid gland, has homology with mouse urinary protein (MUP), a known allergen. Both VEG protein and MUP are members of the lipocalin family of small ligand-binding proteins. Recombinant forms of *Can f* 1 and *Can f* 2 were produced and tested for immunoglobulin E (IgE) reactivity. Among dog-allergic subjects, 45% had IgE directed exclusively to r*Can f* 1, and 25% had IgE to both r*Can f* 1 and r*Can f* 2. In addition, both recombinant proteins were able to cross-link IgE and elicit histamine release from peripheral blood leucocytes *in vitro*. These findings confirm that *Can f* 1 and *Can f* 2 are major and minor dog allergens, respectively, and demonstrate that recombinant forms of dog allergens retain at least some IgE-binding epitopes.

INTRODUCTION

Allergy to the domestic dog (*Canis familiaris*) is prominent worldwide.[1] Dog allergens can be detected not only in houses where dogs are kept as pets but also in other places such as schools and day care centres where dogs are not present on a regular basis.[2–5] Chronic exposure to dog and other indoor allergens, including mite and cat, may lead to allergic sensitization and contribute to the development of bronchial hyperreactivity and asthma in children.[3–5]

Dog hair and dander extracts are complex mixtures containing a number of allergenic proteins.[6–8] Two major allergens present in dog hair and dander have been purified using immunoaffinity chromatography and further characterized.[9,10] *Canis familiaris* allergen 1 (*Can f* 1), a protein with molecular weight reported to range from 21 to 25 kD, binds immunoglobulin E (IgE) from the majority of dog-allergic subjects.[6–10] Immunoaffinity–purified *Can f* 1 was found to elicit a high frequency of positive skin prick test reactions[9,10] and to account for, on average, 70% of IgE binding to dog dander extracts.[10]

Canis familiaris allergen 2 (*Can f* 2; originally named Dog 2), with molecular weight reported at 19 kD[7] or 27 kD,[8] was detected by several groups through human IgE binding studies with dog hair and dander extracts.[7,8,10] This allergen was affinity purified using specific monoclonal antibodies and found to react with IgE of only 66% of dog allergic patients, and to bind 23% of the IgE directed against dog dander extract, supporting its role as a minor allergen.[10]

In the present study, the cloning and sequencing of cDNAs encoding *Can f* 1 and *Can f* 2 are reported. The differential expression of *Can f* 1 and *Can f* 2 in various dog tissues has also been determined. In addition, the isolation of recombinant forms of *Can f* 1 and *Can f* 2 using an *Escherichia coli* expression and purification system is described. Recombinant *Can f* 1 and, to a lesser extent, recombinant *Can f* 2, bound not only to monoclonal antibodies raised against the native proteins, but also to the IgE of dog-allergic subjects. In addition, the recombinant proteins were able to elicit histamine release from the blood leucocytes of dog-allergic patients *in vitro*, further confirming their allergenicity.

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MATERIALS AND METHODS

Protein sequence analysis of purified Can f *1 and* Can f *2*

Affinity–purified *Can f* 1 and *Can f* 2 proteins obtained as described[10] were subjected to N-terminal sequence analysis,[11]

using an Applied Biosystems Model 477A (Applied Biosystems, Foster City, CA) gas phase sequencer with online phenylthiohydantion (HTH) amino acid analysis (Model 120A).

Isolation and Characterization of Can f *1 cDNA*

RNA was extracted from fresh parotid glands of a single outbred dog with guanidine thiocyanate and centrifugation through a CsCl cushion. mRNA was isolated by chromatography on olido(dT) cellulose, and total dog parotid gland cDNA was obtained using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (cDNA Synthesis System Plus, Amersham Corp., Arlington Heights, IL) and an oligo(dT)$_{12-18}$ primer. *Can f* 1 cDNA was amplified by polymerase chain reaction (PCR; GeneAmp kit, Perkin Elmer Cetus, Norwalk, CT) in a Minicycler (MJ Research, Inc., Watertown, MA) using primers (Table 1) based on partial protein sequence analysis (data not shown). Initially, cDNA encoding residues 15 to 29 was amplified using the 5′ primer C1S1B, along with the degenerate 3′ primers, C1AS2A and C1AS2B, in 40 cycles (denaturation: 92°, 30 s; annealing: 55°, 1 min; extension: 75°, 1 min). cDNA encoding residues 28 to 87 were amplified using the sense C1SA in conjunction with the degenerate antisense primers, C1AS3A and C1AS3B, followed by secondary amplification using the combination of C1SB and C1AS3A/C1AS3B primers.

To obtain the 3′ portion of the *Can f* 1 cDNA, the rapid amplification of cDNA ends (RACE) PCR protocol was employed.[12] First-strand cDNA was synthesized from total dog parotid gland RNA using the JM3 antisense primer, which contains an arbitrary adaptor sequence with restriction enzyme sites located 5′ to an oligo(dT)$_{17}$ tract. First, JM3-primed cDNA was amplified using the XSD sense primer in conjunction with the JM3-3XB antisense primer. The first round product was amplified with a nested sense primer (XSE), along with JM3-3XB. The product was subcloned, sequenced, and found to encode the 3′ portion (residues 87 to 142) of *Can f* 1.

The 5′ end of *Can f* 1 cDNA was cloned using an anchored PCR technique.[13] Double-stranded cDNA (ds cDNA) was synthesized from total dog parotid gland RNA using the BRL Superscript cDNA Synthesis System (Life Technologies Inc., Gaithersburg, MD), made blunt using DNA polymerase I Klenow fragment (New England BioLabs, Beverly, MA), ligated to anchor adaptor AT (Clontech, Palo Alto, CA) and used as a template to amplify the 5′ end of *Can f* 1 cDNA. A

Table 1. Primer sequences used in cloning *Can f* 1

Primer	Sequence (5′–3′)	Orientation	Corresponding residues*
C1S1B:	GGGAATTCTGGTAYCTXAARGCXATGAC *Eco*RI	Sense	9–14
C1AS2A:	GGGGATCCYTTYTGXGCYTTYAADATCAT *Bam*HI	Antisense	30–36
C1AS2B:	GGGGATCCYTTYTGXGCYTTYAGDATCAT *Bam*HI	Antisense	30–36
C1SA:	ATGACAGCAGACCAGGAG	Sense	14–19
C1SB:	CCTGAGAAGCCTGACTCAGTG	Sense	21–27
C1AS3A:	GGGGATCCYTCACARTAYAADATRTA *Bam*HI	Antisense	88–93
C1AS3B:	GGGGATCCYTCACARTAXAGDATRTA *Bam*HI	Antisense	88–93
JM3:	CGAATACGACTCACTATAGGAAGCTGCGGCCGCTGCAGTAC (A)$_{17}$ *Xma*III *Pst*I	Antisense	Adaptor
JM3-3XB:	GGGGAGATCTCGAGAGGAAGCTGCGGCCGCTGCA *Bgl*II *Xho*I *Xma*III *Pst*I	Antisense	Adaptor
XSD:	GGGCTCGAGGCCAGCGTGTCGTGTTCATC *Xho*I	Sense	73–79
XSE:	GGGCTCGAGCAGCCGTCCCCGGTGAGGGAC *Xho*I	Sense	80–86
ASA:	GGGGATCCAGGCTTCTCAGGCACCTCCTG *Bam*HI	Antisense	18–24
ASB:	GGGGATCCATGGGAGTCACTGAGTC *Bam*HI	Antisense	25–30
D1Start:	CCTCGAGATGAAGACCCTGCTCCTCACCAT *Xho*I	Sense	$^{-}$26–$^{-}$20
D1Stop:	GGGAGATCTCAGAGGGTCATGGAGCTGCTGCCC *Bgl*II	Antisense	Downstream†
D1Exp:	GAATTCGACACTGTGGCTGTGTCAGGGAA *Eco*RI	Sense	1–8
D1ExpAS:	GGGAGATCTACTGTCCTCCTGGAGAGCAGG *Bgl*II	Antisense	143–148

*Based on amino acid sequence of the mature protein.

†Corresponds to 24 bp of sequence located 40 bp downstream of the stop codon.

sense primer (AP1; Clontech) based on the anchor sequence was used in conjunction with antisense primer ASB. The first round product was amplified with the nested sense primer AP2 (Clontech) and the nested antisense primer ASA, using 40 cycles (denaturation: 92°, 30 s; annealing: 60°, 1 min; extension: 75°, 1 min). The amplified fragment was subcloned, sequenced, and found to encode the N-terminal 13 residues of mature *Can f* 1 preceded by a 5′ leader sequence.

A contiguous *Can f* 1 cDNA was then amplified from dog parotid gland cDNA using the sense D1Start and antisense D1Stop primers in 40 cycles (denaturation: 95°, 30 s; annealing: 60°, 45 s; extension: 75°, 45 s), using *Pfu*I DNA polymerase (Stratagene, La Jolla, CA). The sequence of the amplified product was confirmed using the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus, Norwalk, CT).

Isolation and characterization of Can f *2 cDNA*

The sequences of primers used in cloning *Can f* 2 (Table 2) were deduced from the partial amino acid sequence (data not shown). An internal fragment encoding residues 7 to 32 of *Can f* 2 was obtained by PCR amplification of dog parotid gland cDNA using the D2S1A sense primer and degenerate antisense primers (ASP2A and ASP2B) in 30 cycles (denaturation: 1 min, 94°; annealing: 1 min, 42°; extension: 1 min, 72°).

This sequence was then utilized to construct primers for amplification of the 5′ and 3′ ends of *Can f* 2 cDNA using anchored PCR techniques similar to those used in cloning *Can f* 1. To clone the 5′ end of *Can f* 2, blunt-ended ds cDNA ligated to anchor adaptor AT (Clontech) was amplified in three sequential PCR reactions using a sense primer based on the anchor sequence (AP2; Clontech) in conjunction with a group of nested antisense primers based on the *Can f* 2 cDNA sequence. In the primary reaction, anchored ds cDNA was amplified using the 5′ AP2 and 3′ D2-1 primers. In the secondary reaction, the primary reaction mixture was amplified using the 5′ AP2 and 3′ D2-2 primers. In the tertiary reaction, the secondary reaction mixture was amplified using the 5′ AP2 and 3′ D2-3 primers. A final product was sequenced and found to encode residues 1–15 of *Can f* 2. The 3′ portion of *Can f* 2 was cloned in an analogous manner, using JM3-primed single-stranded cDNA as a template. DNA amplified in three sequential rounds using the 3′ APA primer and a group of nested 5′ primers, D2–4, D2–5, and D2–6, was sequenced and found to encode residues 29 to 36 of *Can f* 2.

In order to clone the full-length *Can f* 2 cDNA, a λgt10 library containing cDNA inserts from poly(A)$^+$RNA was screened with a probe obtained by PCR amplification of dog parotid gland cDNA using the D2–9 sense and D2–13 antisense primers. Phage DNA extracted from each of 20 positive clones was subcloned into pUC18 (Clontech). Clones containing the largest inserts (1a, 1c, and 1j) were used to obtain the full-length nucleotide sequence encoding *Can f* 2.

Northern blots

Poly(A)$^+$ RNA or total RNA from various tissues was fractionated by electrophoresis through a 1·5% agarose gel containing 2·2 M formaldehyde, and transferred onto a GeneScreen membrane (DuPont-NEN, Boston, MA). Transfer, hybridization and washing of the filter were performed according to the manufacturer's instructions. The probes used for Northern blot analyses were generated by

Table 2. Primer sequences used in cloning *Can f* 2

Primer	Sequence (5′–3′)	Orientation	Corresponding residues*
D2S1A:	GGGAATTCAAYCAYGARGAR *Eco*RI	Sense	3–6
ASP2A:	XCGRAARTGXCCCCAXGG	Antisense	33–38
ASP2B:	XCTRAARTGXCCCCAXGG	Antisense	33–38
D2-1:	GGGGGATCCCGGATCGGACTTGTTGGAGGC *Bam*HI	Antisense	24–30
D2-2:	GGGGGATCCGGAGGCCAGGGCAACGGA *Bam*HI	Antisense	20–25
D2-3:	GGGGGATCCAACGGAGTGCCACCTCCC *Bam*HI	Antisense	16–21
APA:	GGGCTCGAGGTCGAGTTTTTTTTTTTTTTTTV *Xho*I	Antisense	Adaptor
D2-4:	GGGGGAATTCGAGGAGCTGTCTGGGAGGTGG *Eco*RI	Sense	12–18
D2-5:	GGGGGAATTCAGGTGGCACTCCGTTGCCCTG *Eco*RI	Sense	17–23
D2-6:	GGGGGAATTCGCCCTGGCCTCCAACAAGTCC *Eco*RI	Sense	22–28
D2-9:	GGGGGAATTCGAGGGAAACCATGAGGAGCC *Eco*RI	Sense	1–6
D2-13:	GGGGGATCCAAGTGCCCCCAGGGTTTGAT *Bam*HI	Antisense	31–36
D2ExpAS:	GGGGGATCCCTAGTCTCTGGAACCCTG *Bam*HI	Antisense	157–161

*Based on amino acid sequence of the mature protein.

PCR using internal primers and plasmid templates containing *Can f* 1 or *Can f* 2 inserts.

Bacterial expression of recombinant proteins

Escheriania coli expression of recombinant *Can f* 1 and *Can f* 2 proteins was carried out as described.[14] The full-length sequences encoding mature *Can f* 1 and *Can f* 2 were obtained from dog parotid gland cDNA by PCR amplification utilizing the primers D1Exp and D1ExpAS for *Can f* 1 (Table 1), and D2–9 and D2ExpAS for *Can f* 2 (Table 2). These primers were designed to introduce *Eco*RI and *Bam*HI restriction sites at the 5′ and 3′ ends respectively of the amplified cDNA, to allow subcloning into the pET11d His_6 vector (Novagen Inc., Madison, WI). Prior to subcloning, *Can f* 1 was modified by removal of the internal *Eco*RI site at residues $E_{123}F_{124}$, by introducing a silent A to G point mutation at position 447 of the cDNA. BL21(DE3)-transformed cells were grown overnight and recombinant proteins were purified using NTA Ni^{++} chelating resin (Qiagen Inc., Chatsworth, CA). Recombinant His_6–*Can f* 1 or His_6–*Can f* 2 proteins were eluted from the resin with 8 M urea, 100 mM NaOAc, 10 mM Tris, pH 4·5, as previously described.[15] The purity of the recombinant proteins as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining was approximately 80%.

Immunoblot analysis of native and recombinant proteins (Western)

Samples of r*Can f* 1, r*Can f* 2, or dog skin test extract (Miles Inc. Elkhart IN, lot #B63D4563) were denatured by boiling with guanidine hydrochloride, dithiothreitol (DTT) and SDS, then applied to a 15% SDS–PAGE gel and transferred onto nitrocellulose. The membrane was blocked with 1% non-fat dry milk in 0·5% fetal calf serum, and incubated with plasma from dog allergic subjects overnight at room temperature or with monoclonal antibodies against *Can f* 1 and *Can f* 2[10] for 2 hr at room temperature. The blots were treated with biotinylated goat anti-human IgE (Kirkegaard and Perry Labs, Gaithersburg, MA) or biotinylated goat anti-mouse IgG (H+L, Kirkegaard and Perry Labs), followed by peroxidase-linked strepavidin (Southern Biotechnology Associates, Inc., Birmingham, AL). Bands were visualized using the ECL system (Amersham Corp., Arlington Heights, IL).

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Costar, Cambridge, MA) were coated with 5 μg/ml r*Can f* 1 or r*Can f* 2, or a 1:50 dilution of dog skin test extract in phosphate-buffered saline (PBS). Plasma samples from dog-allergic subjects (skin test 4^+) were added (1:2 to 1:512 dilution) in PBS containing 0·05% Tween–20 (PBS–Tween). Bound IgE was detected using biotinylated goat-anti-human IgE (Kirkegaard and Perry Labs), peroxidase-linked strepavidin (Southern Biotechnology Associates, Inc.), and TMB substrate (Kirkegaard and Perry Labs). As a control for non-specific binding, serum samples were run on uncoated ELISA plates. Positive sera were defined as those that gave an absorbance reading of at least double the uncoated plate background at a dilution of 1:4.

Histamine release assays

Peripheral blood was drawn into CAST® venipuncture tubes (ALPCO Inc., Windham, NH). Leucocytes were isolated following dextran sedimentation of platelets and erythrocytes, and challenged with allergen as described.[16] Total histamine release was determined following cell disruption, in a bath of boiling water for 3 min. Cell supernatants were harvested and analysed for histamine content using a glass fibre assay.[17]

RESULTS

Protein sequence analysis of purified *Can f* 1 and *Can f* 2

Partial amino acid sequences were obtained for immunoaffinity–purified *Can f* 1 and *Can f* 2 proteins. The N-terminus of *Can f* 1 was not blocked, as had been previously reported,[9] and the 65 N-terminal amino acids were identified through multiple rounds of N-terminal sequence analysis. Subsequent analysis of high-performance liquid chromatography (HPLC)-isolated CNBr cleavage fragments further extended the N-terminal sequence to 94 amino acid residues. *In situ* CNBr cleavage in conjunction with OPA blocking[11] identified residues 104–142. By protein sequence analysis, the residue at position 54 could not be identified, but was subsequently deduced to be Asn from the sequence of the cloned cDNA. Similarly, the 38 N-terminal residues of *Can f* 2 were identified by protein sequence analysis. No residues could be definitively assigned to positions 26 and 32, but subsequent analysis of the sequence deduced from cDNA identified them as Asn and Lys, respectively.

Cloning of *Can f* 1

The full-length cDNA encoding *Can f* 1 was amplified from total parotid gland cDNA, using primers designed based on limited amino acid analysis of the native protein (Table 1). First, the internal portion of the gene encoding *Can f* 1 was obtained by two discrete PCR amplifications as described in the Materials and Methods. The 5′ and 3′ portions of the *Can f* 1 gene were then isolated using anchored PCR methods. Finally, a contiguous *Can f* 1 cDNA was generated by PCR, directly sequenced, and found to encode a 26 amino acid signal peptide and a 148 residue mature protein (Fig. 1), with predicted MW of 16·5 kD. A single potential N-linked glycosylation site was found, corresponding to the Asn residue at position 54 (Fig. 1).

Cloning of *Can f* 2

Full-length *Can f* 2 cDNA was cloned following screening of a dog parotid gland cDNA library with a specific probe, generated using primers based on limited amino acid and nucleotide analyses (see the Materials and Methods). Twenty positive clones were plaque purified and those containing the three longest inserts (designated 1a, 1c, and 1j) were subcloned into pUC18 and sequenced. The sequences of all three clones contained an open reading frame encoding the 38 N-terminal amino acid residues of *Can f* 2 previously identified by protein sequence analysis (amino acids 1–38). The clones displayed limited sequence polymorphisms. Clone 1j contained two substitutions compared with clones 1a and 1c (T to C at position 347, resulting in a Ile to Thr replacement at position 347; and G to T at position 401, resulting in a Gly to Val replacement at position 49). Clone 1c (791 bp), which spanned the full-length *Can f* 2 precursor protein, including signal

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  1   ATG AAG ACC CTG CTC CTC ACC ATC GGC TTC AGC CTC ATT GCG ATC CTG CAG GCC CAG GAT    60
-26   M   K   T   L   L   L   T   I   G   F   S   L   I   A   I   L   Q   A   Q   D    -7

 61   ACC CCA GCC TTG GGA AAG GAC ACT GTG GCT GTG TCA GGG AAA TGG TAT CTG AAG GCC ATG   120
 -6   T   P   A   L   G   K   D   T   V   A   V   S   G   K   W   Y   L   K   A   M    14

121   ACA GCA GAC CAG GAG GTG CCT GAG AAG CCT GAC TCA GTG ACT CCC ATG ATC CTC AAA GCC   180
 15   T   A   D   Q   E   V   P   E   K   P   D   S   V   T   P   M   I   L   K   A    34

181   CAG AAG GGG GGC AAC CTG GAA GCC AAG ATC ACC ATG CTG ACA AAT GGT CAG TGC CAG AAC   240
 35   Q   K   G   G   N   L   E   A   K   I   T   M   L   T   N   G   Q   C   Q   N    54

241   ATC ACG GTG GTC CTG CAC AAA ACC TCT GAG CCT GGC AAA TAC ACG GCA TAC GAG GGC CAG   300
 55   I   T   V   V   L   H   K   T   S   E   P   G   K   Y   T   A   Y   E   G   Q    74

301   CGT GTC GTG TTC ATC CAG CCG TCC CCG GTG AGG GAC CAC TAC ATT CTC TAC TGC GAG GGC   360
 75   R   V   V   F   I   Q   P   S   P   V   R   D   H   Y   I   L   Y   C   E   G    94

361   GAG CTC CAT GGG AGG CAG ATC CGA ATG GCC AAG CTT CTG GGA AGG GAT CCT GAG CAG AGC   420
 95   E   L   H   G   R   Q   I   R   M   A   K   L   L   G   R   D   P   E   Q   S   114

421   CAA GAG GCC TTG GAG GAT TTT CGG GAA TTC TCA AGA GCC AAA GGA TTG AAC CAG GAG ATT   480
115   Q   E   A   L   E   D   F   R   E   F   S   R   A   K   G   L   N   Q   E   I   134

481   TTG GAA CTC GCG CAG AGC GAA ACC TGC TCT CCA GGA GGA CAG TAG                       525
135   L   E   L   A   Q   S   E   T   C   S   P   G   G   Q   Stop                    148
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Figure 1. Nucleotide sequence and deduced amino acid sequence of *Can f* 1 (Genbank accession no. AFO27177). The sequence encodes a 26 amino acid signal peptide followed by a 148 amino acid protein. The arrow indicates the putative cleavage site of the signal peptide. A potential N-glycosylation site is boxed.

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  1    AG AGC TGG ACC CGT GTG TGT GCT GGC CAA TGA GCC CTG GAG GGT CCG GCT CCA GAG TAC    59

 60   CCT CTT GGC ACA GGG CCG AGT CCA TCG GGA CAG ATG AAC CTA GAG GAC TCC ACT GCC CTC   119

120   CCA TCC ACG GGG CCG GGT CAC CAG ACT CTG CAA GTC TCC AGC TGT CGC CAA ACC CAG ACA   179

180   GAA GGT GCT GTG GAC ATG CAG CTC CTA CTG CTG ACC GTG GGC CTG GCA CTG ATC TGT GGC   239
-20                       M   Q   L   L   L   L   T   V   G   L   A   L   I   C   G    -5

240   CTC CAG GCT CAG GAG GGA AAC CAT GAG GAG CCC CAG GGA GGC CTA GAG GAG CTG TCT GGG   299
 -4   L   Q   A   Q   E   G   N   H   E   E   P   Q   G   G   L   E   E   L   S   G    16

300   AGG TGG CAC TCC GTT GCC CTG GCC TCC AAC AAG TCC GAT CTG ATC AAA CCC TGG GGG CAC   359
 17   R   W   H   S   V   A   L   A   S   N   K   S   D   L   I   K   P   W   G   H    36

360   TTC AGG GTT TTC ATC CAC AGC ATG AGC GCA AAG GAC GGC AAC CTG CAC GGG GAT ATC CTT   419
 37   F   R   V   F   I   H   S   M   S   A   K   D   G   N   L   H   G   D   I   L    56

420   ATA CCG CAG GAC GGC CAG TGC GAG AAA GTC TCC CTC ACT GCG TTC AAG ACT GCC ACC AGC   479
 57   I   P   Q   D   G   Q   C   E   K   V   S   L   T   A   F   K   T   A   T   S    76

480   AAC AAA TTT GAC CTG GAG TAC TGG GGA CAC AAT GAC CTG TAC CTG GCA GAG GTA GAC CCC   539
 77   N   K   F   D   L   E   Y   W   G   H   N   D   L   Y   L   A   E   V   D   P    96

540   AAG AGC TAC CTG ATT CTC TAC ATG ATC AAC CAG TAC AAC GAT GAC ACC AGC CTG GTG GCT   599
 97   K   S   Y   L   I   L   Y   M   I   N   Q   Y   N   D   D   T   S   L   V   A   116

600   CAC TTG ATG GTC CGG GAC CTC AGC AGG CAG CAG GAC TTC CTG CCG GCA TTC GAA TCT GTA   659
117   H   L   M   V   R   D   L   S   R   Q   Q   D   F   L   P   A   F   E   S   V   136

660   TGT GAA GAC ATC GGT CTG CAC AAG GAC CAG ATT GTG GTT CTG AGC GAT GAC GAT CGC TGC   719
137   C   E   D   I   G   L   H   K   D   Q   I   V   V   L   S   D   D   D   R   C   156

720   CAG GGT TCC AGA GAC TAG GGC CTC AGC CAC GCA GAG AGC CAA GCA GCA GGA TCT CAC CTG   779
157   Q   G   S   R   D   Stop                                                       161

780   CCT GAG TAC GGT                                                                  791
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Figure 2. Nucleotide sequence and deduced amino acid sequence of *Can f* 2 (Genbank accession no. AFO27178). The sequence encodes a 19 amino acid signal peptide followed by a 161 amino acid protein. The arrow indicates the putative cleavage site of the signal peptide. A potential N-glycosylation site is boxed.

peptide and 5′ and 3′ untranslated regions (Fig. 2), encoded a 19 amino acid signal peptide followed by a 161 residue mature protein with a predicted MW of 18·2 kD and a single potential N-linked glycosylation site at position 26 (Fig. 2).

Homology between dog allergens and related proteins

Amino acid sequence comparisons revealed that *Can f* 1 showed a high degree of homology to the human[18] and rat[19]

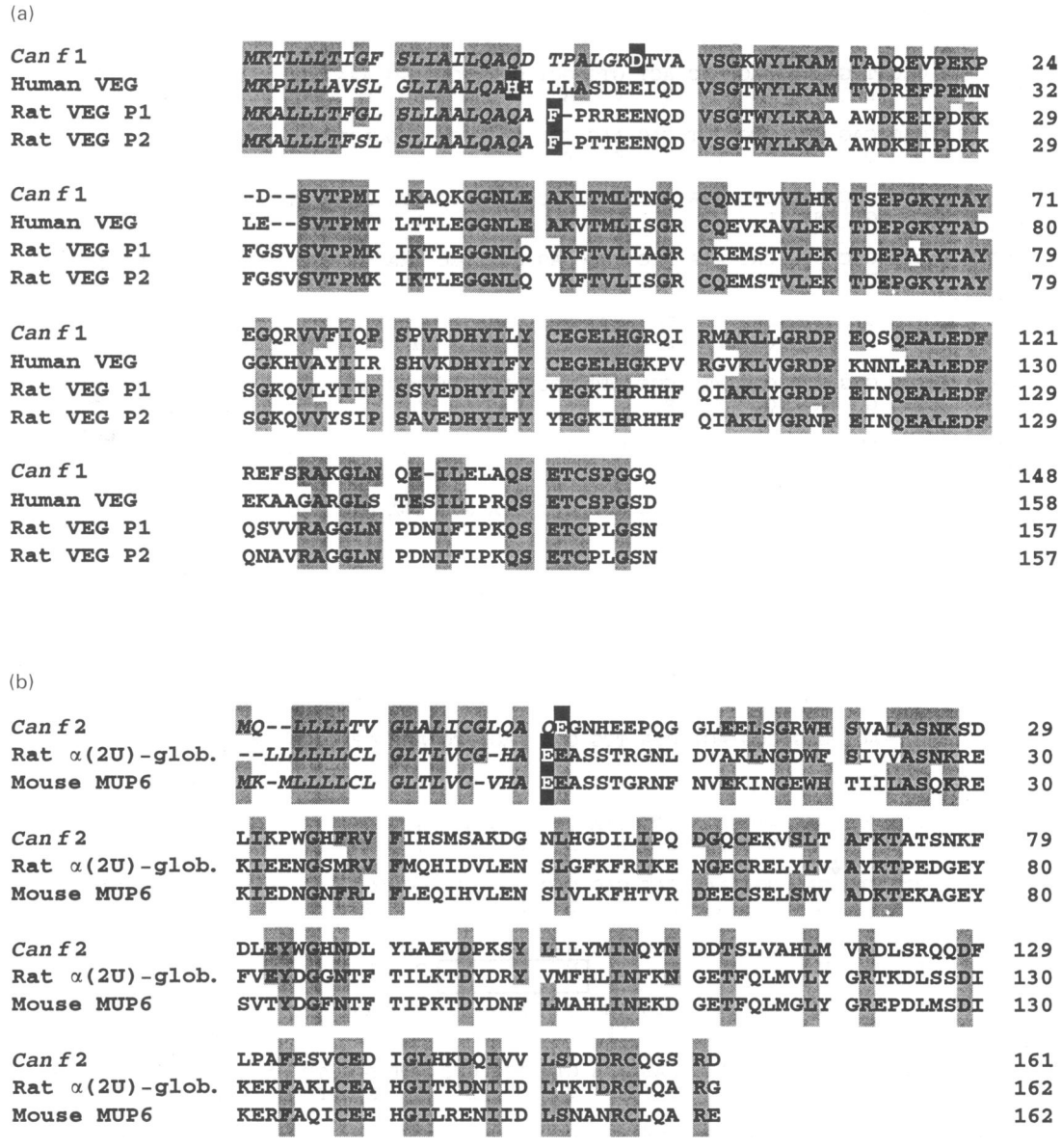

Figure 3. (a) Homology of *Can f* 1 to human and rat von Ebner's gland protein. Amino acids 1 to 148 of *Can f* 1 are aligned with the corresponding residues of human VEG[18] and rat[19] VEG p1 and p2 proteins. (b) Homology of *Can f* 2 to rat and mouse urinary proteins. Amino acids 1 to 161 of *Can f* 2 are aligned with the corresponding residues of rat α(2u)-globulin[21] and mouse MUP6[20] proteins. Dashes indicate gaps introduced to aid alignment. Shaded boxes indicate conserved residues. Leader sequences are shown in italics. The first residue of the mature protein is highlighted.

von Ebner's gland (VEG) proteins (57%, 54% and 54% identity to the human VEG, rat VEG P1, and rat VEG P2 proteins, respectively; Fig. 3a). *Can f* 2 was most similar to mouse urinary protein (MUP;[20]) and its rat homologue, urinary α(2U)-globulin[21] (29% and 32% identity, respectively; Fig. 3b).

Tissue distribution of *Can f* 1 and *Can f* 2

The expression pattern of *Can f* 1 and *Can f* 2 mRNA in various dog tissues was examined by Northern blot analysis. *Can f* 1 mRNA was found in tongue epithelial tissue (von Ebner's gland; Fig. 4a lanes 2 and 4) but not in parotid gland (Fig. 4a, lanes 1 and 5) or skin (Fig. 4a, lanes 3 and 6), whereas *Can f* 2 mRNA was found predominantly in parotid gland (Fig. 4b, lanes 1 and 4) and, to a lesser extent in tongue tissue (Fig. 4b, lanes 2 and 5) but was absent from skin (Fig. 4b, lanes 3 and 6). Neither *Can f* 1 nor *Can f* 2 mRNA was expressed in the liver (not shown).

Binding of human IgE to native and recombinant allergens

cDNAs encoding both *Can f* 1 and *Can f* 2 were subcloned into the *E. coli* expression vector pET11d, recombinant protein expression was induced, and the resultant protein purified by metal chelate chromatography, as described in the Materials and Methods. The antigenicity of r*Can f* 1 and r*Can f* 2

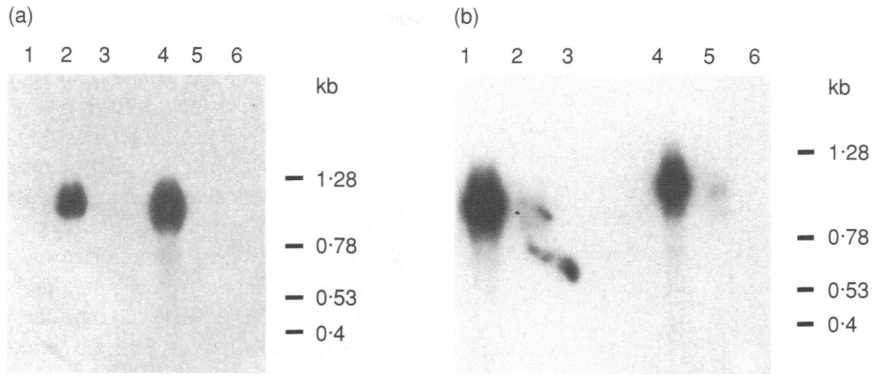

Figure 4. Northern blot analysis of *Can f* 1 and *Can f* 2 mRNA expression in different tissues. Total cellular RNA [25 μg; panel (a) lanes 1–3, and panel (b) lanes 4–6] or polyA+RNA [5 μg; panel A lanes 4–6, and panel B lanes 1–3] from dog parotid salivary gland (lanes 1 and 5), tongue epithelial tissue (von Ebner's gland, lanes 2 and 4), or dog skin (lanes 3 and 6) were subjected to Northern blot analysis as described in the Materials and Methods and probed using *Can f* 1 (a) or *Can f* 2 (b) cDNA. The positions of RNA markers are indicated in kb.

proteins was confirmed by Western blot analysis using monoclonal antibodies raised against the native allergens, indicating that the recombinantly expressed forms of *Can f* 1 and *Can f* 2 retained at least some antigenic epitopes of the native proteins (data not shown). In order to test whether recombinant *Can f* 1 and *Can f* 2 are able to bind to human IgE, plasma from 20 dog-allergic subjects was tested by ELISA for the presence of IgE to recombinant *Can f* 1, recombinant *Can f* 2, and dog dander skin test extracts (Table 3). Plasma from 15 (75%) and five (25%) of the subjects contained IgE to recombinant *Can f* 1 and recombinant *Can f* 2, respectively. Of the 20 plasma samples tested with both allergens, five (25%) showed IgE reactivity to both recombinant dog allergens, and nine (45%) bound to *Can f* 1 exclusively. None of the plasma samples bound exclusively to *Can f* 2, and five (25%) lacked detectable IgE to either allergen. Each sample that contained IgE to one of the recombinant allergens also bound to the commercial dog skin test extract (Table 3).

Table 3. Plasma IgE reactivity to recombinant dog allergens and dog skin test extract*

Patient	rCan f 1	rCan f 2	Extract†
226	++†	−	+
227	+++	++	++
267	++	+	+
281	++	NT	++
358	+	−	+
406	++	+	++
1056	+	−	+
1057	−	−	−
1092	++	−	++
1114	+	−	+
901	+++	−	+++
1018	−	−	+
1070	−	−	+
237	NT	−	+
383	+	+	++
330	+	−	++
234	+	−	++
257	++	+	+++
1194	−	−	+
1918	+	−	+
1383	−	−	−
Total	20	20	21
No. positive	15	5	19
% positive	75	25	90·4

*Plasma IgE reactivity was tested by ELISA as described in the Materials and Methods.

†Dog skin test extract (Miles, Inc., lot #B63D4563).

‡Positive samples were defined as those that gave an absorbance reading of at least double background at a serum dilution of 1:4.

NT, not tested.

Plasma from three subjects (patients nos. 1018, 1070 and 1194) did not contain detectable IgE to either recombinant allergen, but did bind to the skin test extract (Table 3). These subjects may have recognized epitopes of the native allergens that are not retained in the recombinant forms. Alternatively, IgE to these allergens may have been present at titres too low to be detected by ELISA. A third possibility is that some patients respond primarily to dog allergens other than *Can f* 1 or *Can f* 2, such as dog serum albumin.[22]

The presence of IgE to dog allergens was confirmed in some cases by Western blot. A representative example is shown in Fig. 5, in which plasma IgE from patient no. 1918 bound to recombinant *Can f* 1 but not recombinant *Can f* 2, in agreement with ELISA results (Table 3). Plasma from this patient also contained IgE binding to a 22 000 mw protein present in skin test extract (Fig. 5), which likely corresponds to *Can f* 1. Monoclonal antibodies raised against native *Can f* 1 and *Can f* 2 each recognized components of the skin test extract with molecular weights of approximately 22 kD (data not shown).

In vitro histamine release from dog allergic patient blood using recombinant *Can f* 1, *Can f* 2 and dog skin test extracts

In order to test the biological activity of recombinant dog allergens, we performed histamine release assays using peripheral blood leucocytes of dog-allergic subject no. 1918. Dose-dependent histamine release was found in response to the dog skin test extract (Fig. 6a) and to recombinant *Can f* 1 (Fig. 6b). In addition, cells of patient no. 1918 showed low levels of histamine release in response to recombinant *Can f* 2 (Fig. 6b),

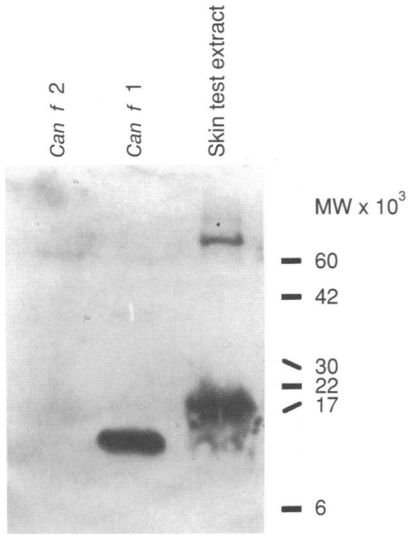

Figure 5. IgE reactivity to recombinant dog allergens analysed by Western blot. Approximately 4 μg of r*Can f* 2, r*Can f* 1, or dog dander skin test extract (Miles) protein was loaded in each lane, electrophoresed and analysed by Western blot using plasma from dog-allergic subject no. 1918. Bands show the location of proteins binding to human IgE.

an allergen to which no plasma IgE could be detected by ELISA (Table 3) or Western blot (Fig. 5). These findings underscore the greater sensitivity of the histamine release assay as opposed to direct measures of IgE titres in determining the allergen sensitivity of patients and the allergenicity of antigens.

DISCUSSION

Cloning and expression of recombinant forms of allergens has proved to be valuable not only for understanding the common sequence and structural motifs that underlie allergenicity, but also as a basis for the design of sequence-specific immunotherapeutics.[23] In this paper, we describe cloning of the major dog allergen, *Can f* 1, and the minor dog allergen, *Can f* 2, and report their complete amino acid sequences as well as their expression patterns in various dog tissues. Recombinant forms of one or both of these allergens bound serum IgE from the vast majority (75%) of dog-allergic subjects, and both r*Can f* 1- and r*Can f* 2-induced histamine release from blood basophils of dog-allergic but not non-allergic subjects. These findings indicate that at least some allergenic epitopes are conserved on the recombinant proteins, making them useful reagents for the study of allergen-specific immune responses, and support the characterization of *Can f* 1 and *Can f* 2 as important allergenic components of dog hair/dander.

The genes encoding *Can f* 1 and *Can f* 2 were cloned from dog parotid gland cDNA. The *Can f* 1 gene was identified by direct PCR amplification using primers based on partial protein sequence analysis, and found to encode a 26 amino acid signal sequence followed by a 148 residue mature protein. The predicted molecular weight of 16·5 kD differs appreciably from the approximately 22-kD size of the native protein, indicating that the native protein undergoes post-translational modification. Consistent with this, protein sequence analysis of native *Can f* 1 could not resolve the residue at position 54, and the deduced amino acid sequence of the cDNA revealed Asn at this residue within a consensus sequence for N-linked glycosylation ($N_{54}I_{55}T_{56}$).

The nucleotide sequence encoding *Can f* 2 was determined from three clones isolated from a dog parotid gland cDNA library. These clones displayed limited sequence polymorphisms, including two replacement mutations in the coding region. This variation may represent allelic polymorphism, or could be due to cloning artefacts related to the high GC content of the *Can f* 2 mRNA template. As for *Can f* 1, the predicted size of the mature protein encoded by the *Can f* 2 gene (18·2 kD) is smaller than the approximately 22-kD size of the form present in skin test extract, suggesting post-translational modification. The residue at position 26 of native

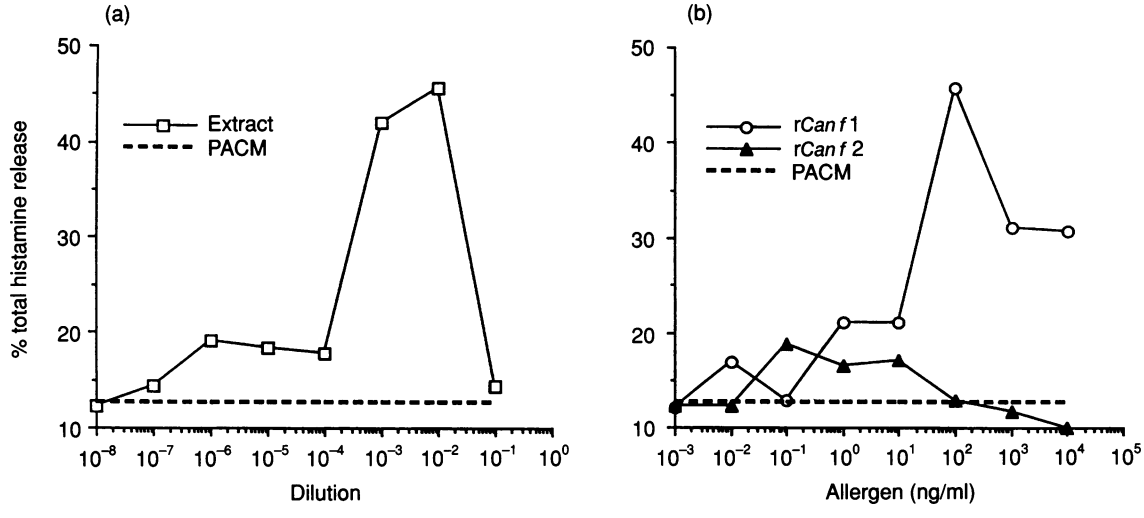

Figure 6. Histamine release from human basophils in response to recombinant *Can f* 1, *Can f* 2 and dog skin test extract. Freshly isolated peripheral blood leucocytes from patient no. 1918 were incubated with the indicated levels of recombinant *Can f* 1, recombinant *Can f* 2, dog dander skin extract (Miles) or a PACM buffer control for 30 min at 37°. Histamine released into the supernatant was quantitated as described in the Materials and Methods.

Can f 2 could not be resolved by protein sequence analysis, and the deduced amino acid sequence of the cDNA revealed Asn at this residue within a consensus sequence for N-linked glycosylation ($N_{26}K_{27}S_{28}$).

The amino acid sequence of *Can f* 1 displayed over 50% amino acid identity to the human[18] and rat[19] VEG proteins. The extent of homology suggests that *Can f* 1 is the canine homologue of VEG protein, rather than a highly related protein. This conclusion is supported by the localization of mRNA encoding *Can f* 1 to VEG tissue. Consistent with the known localization of dog allergenic proteins to saliva,[7,8,10] VEG protein is produced exclusively by the lingual salivary VEGs, and is their major secretory product.[24] Recently, this protein has been shown to have cysteine protease inhibitor activity.[25]

Can f 2, localized to parotid gland and tongue tissue, had approximately 30% amino acid identity to mouse urinary protein (MUP;[20]) and its rat homologue, urinary α(2U)-globulin.[21] MUPs and MUP-related proteins correspond to fatty acid binding proteins found in the kidneys,[26] and to sex-specific proteins found in the livers,[20,21] of male rodents. These proteins are thought to function in pheromone transport,[27] and immunological characterization has shown them to be important allergens.[28,29] The major allergen of horse dander and urine, *Equ c* 1, also displays amino acid sequence homology with proteins of the α(2U)-globulin family.[30] Interestingly, a dog urinary component has been reported to elicit a positive skin prick reaction in dog-allergic subjects.[31] Based on the homology to MUP, it would be of interest to determine if *Can f* 2 is detectable in urine and could account for this activity.

The rodent urinary proteins[20,29] and VEG[18,19] are members of the lipocalin protein family which forms part of the calycin protein superfamily.[32] The lipocalins are extracellular proteins that bind small hydrophobic molecules with high affinity and selectivity, and have been implicated in a wide variety of biological functions, ranging from transport of serum proteins to modulation of the immune response.[32] A major cockroach allergen, *Bla g* 4, has recently been assigned to this protein family,[33] and the milk allergen, β-lactoglobulin[34] and bovine dander allergen, BDA20,[35] are lipocalin proteins. Analysis of lipocalin crystal structures has revealed that the single protein chain adapts a conserved β-barrel configuration comprised of eight contiguous hydrogen-bonded anti-parallel β-sheets, which encompass the internal ligand binding site.[32] Because the majority of lipocalins have no allergenic activity, further examination of the structural similarities and ligand binding properties of the allergenic lipocalins is necessary to define the major determinants responsible for their allergenicity.

The molecular analysis of dog allergens allows us to definitively address their homology to cat allergens. There has long been widespread speculation that dog and cat allergens are related proteins, and there are several descriptions of immunological cross-reactivity between crude preparations of these allergens.[31,36–38] The hair and dander-specific IgE of dog-allergic subjects has been reported to cross-react with allergenic components of cat fur[31,37] and with epithelial allergens of cat dander.[38] The major cat allergen, *Fel d* 1, was found to inhibit binding of IgE from dog-allergic patients to a dog allergen extract, and this effect was reduced by pre-incubation of patient sera with cat hair/dander extract.[38] Furthermore, like *Can f* 1 and *Can f* 2,[7,8,10] *Fel d* 1 can be found in saliva[39] and in salivary glands.[40] With the present report, the major allergens of both dog and cat[41] have been cloned, and sequence comparisons reveal that neither *Can f* 1 nor *Can f* 2 shares appreciable nucleotide or amino acid sequence homology with *Fel d* 1. Although we cannot rule out that minor cross-reactive allergenic components, such as dog and cat serum albumin, would be present in crude preparations of these allergens, the lack of sequence homology makes it unlikely that there is true immunological cross-reactivity between the major allergens of dog and cat.

The cloning of two important dog allergens will allow the identification of major T-cell epitope sequences, which is essential for the design of a specific immunotherapeutic for desensitization treatment of dog allergy.[23] Furthermore, definition of the expression patterns of the major and minor allergens in dog tissues may permit the preparation of higher potency allergen extracts derived from sources enriched in these allergens. Immunotherapy with dog dander extracts has shown only partial success in ameliorating symptoms,[42–44] underscoring the need for development of more effective immunotherapeutics for this important indoor allergen. Finally, the demonstration that recombinant forms of dog allergens can bind to human IgE will allow these molecules to be utilized in future studies of allergic reactivity.

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REFERENCES

1. KJELLMAN B. & PETTERSSON R. (1983) The problem of furred pets in childhood atopic disease. *Allergy* **38,** 65.
2. SAKAGUCHI M.S., INOUYE S., IRIE T. *et al.* (1993) Airborne cat (*Fel d* I), dog (*Can f* I), and mite (*Der* I and *Der* II) allergen levels in the homes of Japan. *J Allergy Clin Immunol* **92,** 797.
3. MUNIR A.K.M., EINARSSON R., SCHOU C. & DREBORG S.K.G. (1993) Allergens in school dust. I. The amount of the major cat (*Fel d* I) and dog (*Can f* I) allergens in dust from Swedish schools is high enough to probably cause perennial symptoms in most children with asthma who are sensitized to cat and dog. *J Allergy Clin Immunol* **91,** 1067.
4. MUNIR A.K.M., EINARSSON R. & DREBORG S.K.G. (1995) Mite (*Der p* I, *Der f* I), cat (*Fel d* I) and dog (*Can f* I) allergens in dust from Swedish day-care centres. *Clin Exp Allergy* **25,** 119.
5. DEANDRADE A., CHARPIN D., BIRNBAUM J., LANTEAUME A., CHAPMAN M. & VERVLOET D. (1995) Indoor allergen levels in day nurseries. *J Allergy Clin Immunol* **95,** 1158.
6. FORD A.W., ALTERMAN L. & KEMENY D.M. (1989) The allergens of dog. I. Identification using crossed radio-immunoelectrophoresis. *Clin Exp Allergy* **19,** 183.
7. SPITZAUER S., SCHWEIGE C., ANRATHE J. *et al.* (1993) Characterization of dog allergens by means of immunoblotting. *Int Arch Allergy Immunol* **100,** 60.
8. FORD A.W. & KEMENY D.M. (1992) The allergens of dog. II. Identification and partial purification of a major dander allergen. *Clin Exp Allergy* **22,** 793.
9. SCHOU C., SVENDSEN U.G. & LOWENSTEIN H. (1991) Purification and characterization of the major dog allergen *Can f* I. *Clin Exp Allergy* **21,** 321.

10. DE GROOT H., GOEI K.G.H., VAN SWIETEN P. & AALBERSE R.C. (1991) Affinity purification of a major and a minor allergen from dog extract: serologic activity of affinity-purified *Can f* I-depleted extract. *J Allergy Clin Immunol* **87,** 1056.
11. BRAUER A.W., OMAN C.L. & MARGOLIES M.N. (1984) Use of o-phthalaldehyde to reduce background during automated Edman degradation. *Anal Biochem* **137,** 134.
12. FROHMAN M.A., DUSH M.K. & MARTIN G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* **85,** 8998.
13. ROUX K.H. & DHANARAJAN P. (1990) A strategy for single site PCR amplification of dsDNA: Priming digested cloned or genomic DNA from an anchor-modified restriction site and a short internal sequence. *Biotechniques* **8,** 48.
14. STUDIER F.W., ROSENBERG A.H., DUNN J.J. & DUBENDORF J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185,** 60.
15. ROGERS B.L., MORGENSTERN J.P., GARMAN R.D., BOND J.F. & KUO M.-C. (1993) Recombinant *Fel d* I: expression, purification, IgE binding and reaction with cat allergic human T cells. *Mol Immunol* **30,** 559.
16. BLACK K.M., LUSSIER A.M., GION W.R. & KASAIAN M.T. (1996) Cytokine priming of human basophils: Description of allergen 'nonreleasers'. *Int Arch Allergy Immunol* **111,** 141.
17. NOLTE H., SCHIOTZ O. & STAHL SKOV P. (1987) A new glass microfibre-based histamine analysis for allergy testing in children. *Allergy* **42,** 366.
18. BLAKER M., KOCK K., AHLERS C., BUCK F. & SCHMALE H. (1993) Molecular cloning of human von Ebner's gland protein, a member of the lipocalin superfamily highly expressed in lingual salivary glands. *Biochim Biophys Acta* **1172,** 131.
19. KOCK K., AHLERS C. & SCHMALE H. (1994) Structural organization of the genes for rat von Ebner's gland proteins 1 and 2 reveals their close relationship to lipocalins. *Eur J Biochem* **221,** 905.
20. DERMAN E. (1981) Isolation of a cDNA clone for mouse urinary proteins: Age- and sex-related expression of mouse urinary protein genes is transcriptionally controlled. *Proc Natl Acad Sci USA* **78,** 5425.
21. UNTERMAN R.D., LYNCH K.R., NAKHASI H.L. *et al.* (1981) Cloning and sequence of several α_{2u}-globulin cDNAs. *Proc Natl Acad Sci USA* **78,** 3478.
22. SPITZAUER S., SCHWEIGER C., SPERR W.R. *et al.* (1994) Molecular characterization of dog albumin as a cross-reactive allergen. *J Allergy Clin Immunol* **93,** 614.
23. WALLNER B.P. & GEFTER M.L. (1996) Peptide therapy for treatment of allergic diseases. *Clin Immunol Immunopathol* **80,** 105.
24. KOCK M., BLAKER M. & SCHMALE H. (1992) Postnatal development of von Ebner's glands: accumulation of a protein of the lipocalin superfamily in taste papillae of rat tongue. *Cell Tissue Res* **267,** 313.
25. VAN'T HOF W., BLANKENVOORDE M.F.J., VEERMAN E.C.I. & AMERONGEN A.V.N. (1997) The salivary lipocalin von Ebner's gland protein is a cystein proteinase inhibitor. *J Biol Chem* **272,** 1837.
26. KIMURA H., ODANI S., NISHI S., SATO H., ARAKAWA M. & ONO T. (1991) Primary structure and cellular distribution of two fatty acid-binding proteins in adult rat kidneys. *J Biol Chem* **266,** 5963.
27. BOCSKEI Z., GROOM C.R., FLOWER D.R. *et al.* (1992) Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. *Nature* **360,** 186.
28. LORUSSO J.R., MOFFAT S. & OHMAN J.L., JR. (1986) Immunologic and biochemical properties of the major mouse urinary allergen (Mus m 1). *J Allergy Clin Immunol* **78,** 928.
29. BAYARD C., HOLMQUIST L. & VESTERBERG O. (1996) Purification and identification of allergenic alpha(2u)-globulin species of rat urine. *Biochim Biophys Acta* **1290,** 129.
30. GREGOIRE C., ROSINSKI-CHUPIN I., RABILLON J., ALZARI P.M., DAVID B. & DANDEU J.-P. (1996) cDNA cloning and sequencing reveal the major horse allergen Equ c1 to be a glycoprotein member of the lipocalin superfamily. *J Biol Chem* **271,** 32951.
31. VIANDER M., VALOVIRTA E., VANTO T. & KOIVIKKO A. (1983) Cross-reactivity of cat and dog allergen extracts. RAST inhibition studies with special reference to the allergenic activity in saliva and urine. *Int Arch Allergy Appl Immunol* **71,** 252.
32. FLOWER D.R. (1996) The lipocalin protein family: structure and function. *Biochem J* **318,** 1.
33. ARRUDA L.K., VAILES L.D., HAYDEN M.L., BENJAMIN D.C. & CHAPMAN M.D. (1995) Cloning of cockroach allergen, *Bla g* 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J Biol Chem* **270,** 31196.
34. MONACO H.L. & ZANOTTI G. (1992) Three-dimensional structure and active site of three hydrophobic molecule-binding proteins with significant amino acid sequence similarity. *Biopolymers* **32,** 457.
35. MANTYJARVI R.A., RYTKONEN M., PENTIKAINEN J. *et al.* (1996) Bovine dander allergen BDA20 is a lipocalin identical or closely related to *Bos d* 2. *J Allergy Clin Immunol* **97,** 212 (Abstr. 117).
36. WUTHRICH B., GUERIN B. & HEWITT B.E. (1985) Cross-allergenicity between extracts of hair from different dog breeds and cat fur. *Clin Allergy* **15,** 87.
37. BOUTIN Y., HEBERT H., VRANCKEN E.R. & MOURAD W. (1988) Allergenicity and cross-reactivity of cat and dog allergenic extracts. *Clin Allergy* **18,** 287.
38. SPITZAUER S., PANDJAITAN B., MUHL S. *et al.* (1997) Major cat and dog allergens share IgE epitopes. *J Allergy Clin Immunol* **99,** 100.
39. BROWN P.R., LETTERMANN K. & OHMAN J.L., JR. (1984) Distribution of cat allergen 1 in cat tissues and fluids. *Int Arch Allergy Appl Immunol* **74,** 67.
40. VAN MILLIGEN F.J., VROOM T.M. & AALBERSE R.C. (1990) Presence of *Felis domesticus* allergen I in the cat's salivary and lacrimal glands. *Int Arch Allergy Appl Immunol* **92,** 375.
41. MORGENSTERN J.P., GRIFFITH I.J., BRAUER A.W. *et al.* (1991) Amino acid sequence of *Fel d* I, the major allergen of the domestic cat: Protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci USA* **88,** 9690.
42. HEDLIN G., GRAFF-LONNEVIG V., HEILBORN G. *et al.* (1991) Immunotherapy with cat- and dog-dander extracts. V. Effects of 3 years of treatment. *J Allergy Clin Immunol* **87,** 955.
43. HAUGAARD L. & DAHL R. (1992) Immunotherapy in patients allergic to cat and dog dander. *Allergy* **47,** 249.
44. HEDLIN G., HEILBORN H., LILJA G. *et al.* (1995) Immunotherapy with cat- and dog-dander extracts. II. *In vivo* and *in vitro* immunologic effects observed in a 1 year double-blind placebo study. *J Allergy Clin Immunol* **96,** 879.