

Purification and characterization of the major dog allergen, *Can f I*

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Summary

An important dog-hair and dander-specific allergen Ag13 has been purified by means of immunoaffinity chromatography utilizing rabbit antibody specific for Ag13. Purity was judged to be very high as detected by crossed immunoelectrophoresis and SDS-PAGE. The purified allergen was subjected to amino acid analyses. Molecular weight was about 22 kD in HPLC–gel filtration and 25 kD in SDS-PAGE with an additional band at 18 kD. *In vitro* IgE binding of the allergen was investigated by luminescence immunoassay (LIA) inhibition. Removal of Ag13 from dog hair and dander extract (DHD) removed $50 \pm 1.5\%$ of the IgE binding capacity. The purified allergen inhibited up to 56.5% of the IgE activity to DHD as measured with a pool of serum from dog-allergic patients. Out of 26 dog-allergic patients, 24 had a positive skin-prick test to the allergen. Out of 23 dog-allergic patients, 16 reacted with the allergen in IgE immunoblotting. We suggest that Ag13 be termed *Can f I*. The allergen will be a marker allergen for environmental dog hair and dander exposure.

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Introduction

Dogs are kept as household pets worldwide. The presence of dog antigen has been shown in up to 63% of homes of the normal population in Baltimore, MD, as analysed by immunochemical means [1]. Dog hair is readily transported on clothes and is likely to be found in areas where dogs do not themselves necessarily live, e.g. in schools [2]. Allergy towards dog dander defined as the observation of a positive skin-prick test has been found in 14% of unselected adolescents in Finland [3]. Among children with asthma, 40% of the patients demonstrate a positive skin-prick test with dog dander, even though they had no direct contact with dogs [4].

Extracts of mammalian hair and dander are complex mixtures of components [5,6], some of which are potent allergens. This is acknowledged by the International Union of Immunological Societies (IUIS), who have issued an international standard for dog hair and dander extract [7].

Two groups of investigators have relied on crossed radioimmuno-electrophoresis (CRIE) for the identifica-

tion of dog hair and dander allergens: Blands *et al.* [8] pointed to dog serum albumin and the hair and dander-specific Ag13 as potent allergens. Recently, Ford *et al.* [9] pointed to the same molecule as an important allergen; they named it Ag8.

In order to identify unequivocally a major allergen specific for dog hair and dander, and to show its clinical significance, we have purified Ag13 and investigated its importance as a sensitizing agent by means of skin-prick testing. We suggest that Ag13 is termed *Can f I*, as it fulfils the criteria of IUIS [10].

Material and methods

Allergen extracts

Dog hair and dander extract (DHD) used for purification purposes was prepared from a mixture of dog breeds, including alsatian and poodle. The hair was cut close to the skin without damaging the epithelium. The material was not defatted before extraction at 1:10 (w/v) in 0.5% phenol, 0.9% NaCl, 0.1% NaHCO₃ for 60 hr at 4°C. The extract was concentrated by ultrafiltration and extensively dialysed against distilled water and finally freeze-dried.

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Antibodies

Rabbit antibody to dog hair and dander and to dog serum was prepared using an immunization schedule as described by Harboe and Ingild [11]. Monospecific rabbit antibody to *Can f* I was prepared by immunizing rabbits with partially purified *Can f* I. The ensuing serum was rendered monospecific by successive solid-phase absorption with fractions obtained from DEAE ion exchange and Sephacryl S-200 gel filtration (Pharmacia, Uppsala, Sweden) of DHD. Briefly, pools of fractions not containing *Can f* I, as determined by fused rocket immunoelectrophoresis (FRIE) [12] were coupled onto CNBr-Sephacryl-4B (Pharmacia) according to the manufacturer's instructions, and rabbit serum was applied in phosphate-buffered saline (PBS), pH 7.3. The effluent antibodies were rendered monospecific for *Can f* I by affinity chromatography on CNBr-Sephacryl-4B to which was coupled *Can f* I containing fractions from successive DEAE ion-exchange and S-200 gel filtration; the preparation coupled was judged to be more than 95% pure as determined by crossed immunoelectrophoresis (CIE). The affinity chromatography procedure used was as described by Lind *et al.* [13]. Monospecificity was verified by CIE. CIE and CRIE were performed as described by Weeke *et al.* [14].

Immuno affinity columns

Anti-Can f I column: 5 ml (13.25 mg/ml) of monospecific rabbit antibody to *Can f* I were coupled to 4.5 g CNBr-Sephacryl-4B according to the manufacturer's instructions.

Anti-dog serum column: 0.5 ml (60 mg/ml) of rabbit antibody to dog serum was coupled to 2 g of CNBr-Sephacryl-4B according to the manufacturer's instructions.

Application buffer for purification was PBS. After application the columns were washed with 10 volumes of PBS, 0.5 M NaCl. The bound material was eluted with 0.1 M glycine, 0.15 M NaCl, pH 11. The immunoaffinity-purified *Can f* I did not contain rabbit immunoglobulin as revealed by rocket immunoelectrophoresis (RIE) with swine anti-rabbit Ig (Z196; Dakopatts, Glostrup, Denmark).

SDS-PAGE and immunoblotting

Electrophoresis in polyacrylamide gels was performed according to the method of Schäfer-Nielsen and Rose [15]. Before electrophoresis, the samples were pretreated for 2 min at 100°C in application buffer (0.150 M Tris/HCl, 0.324 M dithiothreitol and 1% w/v SDS, adjusted to pH 7.25, with bromophenol blue as marker). Electroblotting of SDS-PAGE gels to nitrocellulose paper

(GSWP 0.22 µm, Millipore, Milford, MA) was performed semi-dry at approximately 2.4 mA/cm² for 90 min as described earlier by Kyhse-Andersen [16]. The nitrocellulose papers were immediately blocked for 2 min in Tween buffer (2% v/v Tween 20 in 0.05 M Tris and 0.15 M NaCl, adjusted to pH 10.3) and washed.

Incubation with rabbit antibody was performed overnight at room temperature at a final dilution of the antibody of 1/1000. After three washings the strips were incubated for 2 hr at room temperature with alkaline-phosphatase-labelled secondary antibody (P217 from Dakopatts). Staining was performed with nitroblue tetrazolium. To obtain IgE immunoblots, nitrocellulose strips were incubated overnight at room temperature with 40 µl/sample of human serum diluted 1/50 (v/v) in PBS, 0.005% Tween 20, 0.01% sodium azide. After three washings the strips were incubated overnight with ¹²⁵I-tagged anti-IgE (Pharmacia) with approximately 50 000 c.p.m. After four washings the bound IgE was revealed by 12 days' exposure at -80°C to X-ray film (Kodak XL1; Kodak, Heidelberg, Germany).

Amino acid composition

The amino acid composition was determined as described by Barkholt and Jensen [17].

HPLC gel filtration

Gel filtration of purified *Can f* I was performed on a Superose 12 column in 0.125 M NH₄ HCO₃ (flow rate 0.75 ml/min) on an HPLC system (Pharmacia LKB Biotechnology, Bromma, Sweden). About 10 µg of the purified fraction were applied on the column and the absorption spectrum (200–370 nm) was monitored using a diode array detector (Pharmacia LKB Biotechnology; rapid spectral detector, 2140). The mol. wt of the purified protein was determined from interpolation of mol. wt standards.

LIA inhibition experiment

Test of allergenicity of *Can f* I was performed by a luminescence immunoassay (LIA) for specific IgE (Ciba-Corning Diagnostics, Walpole, MA), essentially as described [18,19]. Briefly, DHD was covalently coupled to microscopic paramagnetic particles (PMP) at 10 mg/ml w/v and used as solid phase in an inhibition experiment. Serum pooled from dog allergic patients was inhibited with DHD (25 µl, two-fold dilution series, 10 steps, duplicate determination, highest concentration was 2 mg/ml w/v, lowest, 1 µg/ml) or with equal amounts of *Can f* I-depleted DHD (as measured in RIE using dog albumin as

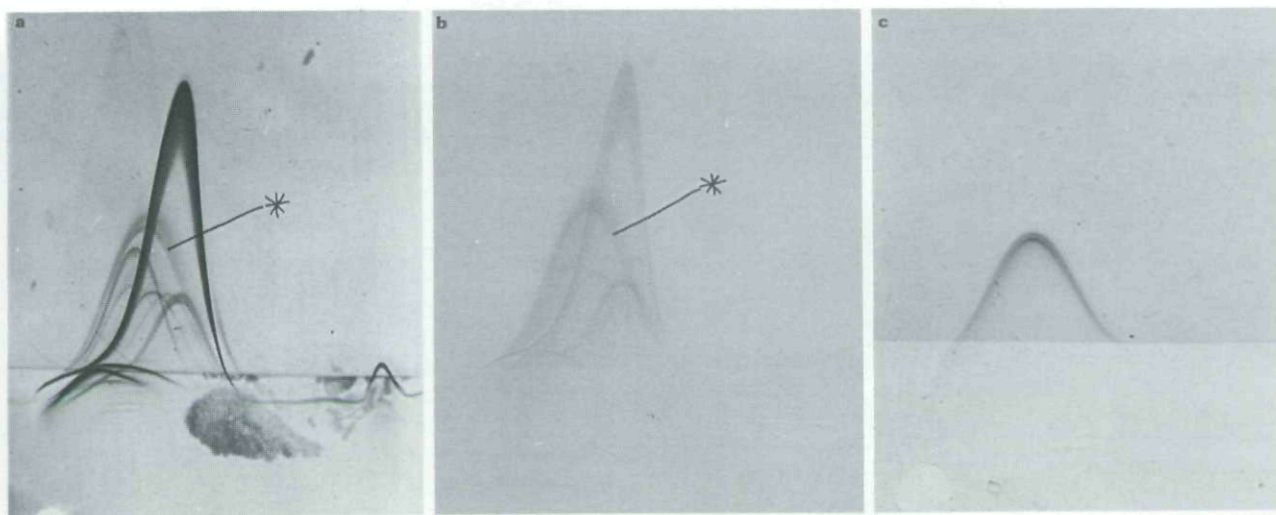


Fig. 1. Identification and purification of *Can f I*. (a) CIE of 10 µg of DHD precipitated in second dimension gel by rabbit antibody to DHD; (b) CIE overlaid with a pool of serum from dog-allergic patients; the IgE bound was revealed with iodinated anti-IgE and subsequent autoradiography; (c) CIE of 0.8 µg of purified *Can f I* precipitated by rabbit antibody to DHD in the upper second dimension gel. The intermediary second dimension gel contained rabbit antibody to dog serum. *Allergen *Can f I*.

a marker protein) or with purified *Can f I* at an identical concentration to that of DHD (as measured in RIE with rabbit anti-*Can f I*); this was followed by incubation with DHD-coated PMP. Free and bound IgE were magnetically separated and non-inhibited IgE was detected in a luminometer (Magic Lite Analyser, Ciba-Corning Diagnostics) using monoclonal anti-IgE (ALK, Hørsholm, Denmark) coupled to the chemiluminescent acridinium esther [20]. Three separate experiments were performed.

Skin-prick test

Twenty-six adult dog-allergic patients (criteria for admittance to the study were clinical symptoms and a positive skin test with Soluprick® DHD 10 HEP, from ALK) attending the Outpatients' Allergy Clinic were tested with 1.79 mg/ml w/v DHD (comparable to Soluprick DHD), *Can f I*-depleted DHD, 1.45 mg/ml w/v and with purified *Can f I*. DHD and *Can f I*-depleted DHD were identical respecting concentration of antigens other than *Can f I*. The concentration of the purified *Can f I* sample was identical to that of *Can f I* in DHD. The three samples were tested as undiluted, 1/5 and 1/25, diluted in Soluprick diluent (ALK). All tests were run in duplicate by the same experienced and specialized nurse. Positive control was histamine dihydrochloride, 10 mg/ml, negative control was Soluprick diluent. Weals were compared with the negative control and a diameter of ≥ 3 mm was considered positive [21].

Serum samples

Serum samples were obtained from 23 of the 26 of the subjects who were skin tested. The remaining three patients declined to give blood samples.

Statistical analysis

Significance of differences between LIA inhibition curves was evaluated by Student's *t*-test.

Results

Identification of *Can f I*

By means of CIE of DHD against rabbit serum to DHD (Fig. 1a) it was possible to separate in the order of 30 antigens of which several bound IgE from dog allergic patients (Fig. 1b). The important DHD-specific allergen Ag13 (*Can f I*) which is identified by an asterisk in Fig. 1a and b was selected for purification. Figure 1c shows the purified *Can f I*.

Purification of *Can f I*

DHD was applied to an affinity column of CNBr-Sepharose 4B coupled with monospecific rabbit antibody to *Can f I*. As shown by SDS-PAGE blotting immunostained with rabbit antibody to DHD and to dog serum, DHD contains antigens ranging from a mol. wt of less than 20 kD to more than 100 kD (Fig. 2, B and C, lane 6).

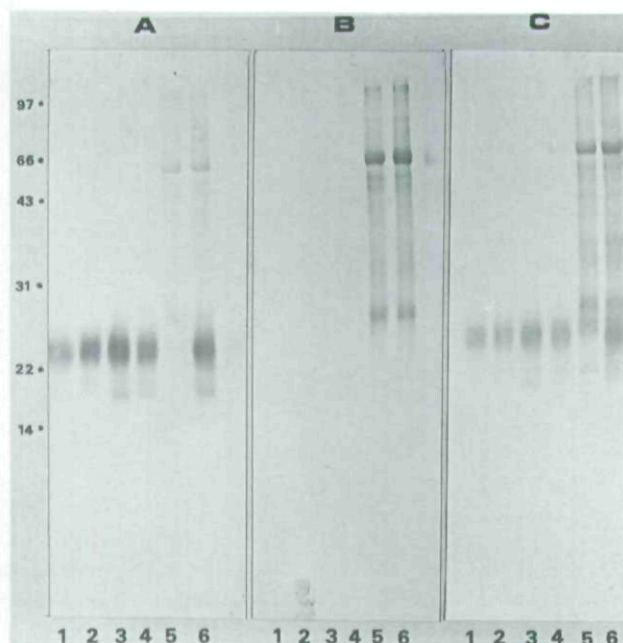


Fig. 2. Purification of the allergen *Can f I* from DHD. Blottings from SDS-PAGE. A, Blottings immunostained with rabbit antibody to *Can f I*; B, blottings immunostained with rabbit antibody to dog serum; C, blottings immunostained with rabbit antibody to DHD. Lanes 1, 2, 3, 4 are immunoaffinity-purified *Can f I*, four individual purifications, 0.3–0.5 μ g protein/lane. Lane 5 is effluent from anti-*Can f I* column, 25 μ g of protein was applied. Lane 6 is 25 μ g protein of DHD. Apparent molecular size in kD. The gel was run under reducing conditions. The main *Can f I* band is about 25 kD; a minor band appears at approximately 18 kD.

The effluent from the column (Fig. 2, B and C, lane 5) contained bands in the same mol. wt range. The eluate (Fig. 2A, lanes 1–4) showed a main antigen band at 25 kD. If the preparation contained residual amounts of dog albumin this was removed by passage through an anti-dog serum affinity column (data not shown). The purified material presented here did not contain dog albumin as detected by immunoblotting with anti-dog-serum (Fig. 2B). The *Can f I* thus purified retained its electrophoretic characteristics as demonstrated by CIE (Fig. 1c) and had an approximate mol. wt of 25 kD in SDS-PAGE. A minor band at about 18 kD reacted with the rabbit antibody to *Can f I* (Fig. 2A). Typically the final yield from the immunoaffinity columns was 2.4 mg when 100 mg of DHD were used as source material. The purified *Can f I* had a K_{av} corresponding to 22 kD in HPLC gel-filtration (data not shown).

Amino acid composition

The amino acid composition of the purified *Can f I* was analysed and is shown in Table 1. The amino acid

Table 1. Amino acid analysis of *Can f I*

Amino acid	Occurrence (%)
Asp	6.8
Thr	5.8
Ser	6.3
Glu	21.3
Pro	6.7
Gly	10.4
Ala	7.1
Val	6.0
Met	1.8
Ile	4.4
Leu	9.4
Tyr	2.9
Phe	2.5
His	1.6
Lys	4.1
Arg	2.8
Total	99.9

Means of two separate runs.

distribution did not show any remarkable features with the possible exception of a rather high concentration of glutamine, i.e. >1/5 amino acids. The amino terminal amino acid of *Can f I* was blocked and attempts at sequencing the allergen have therefore hitherto been unsuccessful.

Allergenic activity of *Can f I* in vitro

To determine the allergenic activity of the purified *Can f I* and its contribution to the total allergenic activity of DHD we used LIA inhibition. This analysis is akin to RAST inhibition but the bound IgE is revealed by chemiluminescence instead of radioactivity. DHD was coupled to the solid-phase particles and a pool of serum from dog-allergic patients was used as a source of specific IgE: 100% binding = 52 000 c.p.s. Background was 60 c.p.s. Maximum obtainable inhibition with DHD was 97.1% (Fig. 3). The *Can f I*-depleted DHD inhibited IgE binding with a potency of $50 \pm 1.5\%$ that of DHD in three separate set-ups as calculated by distance between regression lines at the 50% inhibition level (difference statistically significant: $P < 0.05$). The inhibition curves obtained with purified *Can f I* were not parallel to those obtained with the two other inhibitor preparations. The purified *Can f I* inhibited the IgE binding with 45%, 51.9% and 56.5%, respectively, in three separate experiments.

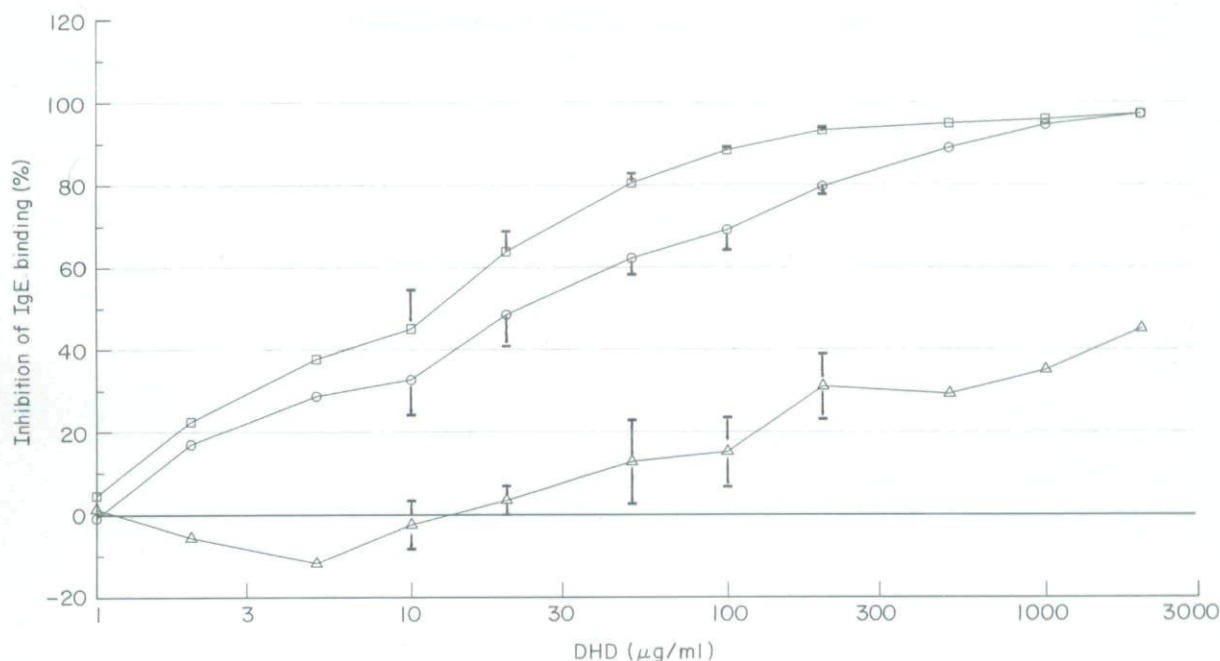


Fig. 3. LIA inhibition. In vitro allergenic activity of *Can f*I was investigated by LIA inhibition. Dog hair and dander extract (DHD) (equivalent concentration, $\mu\text{g/ml}$) was coupled to the solid phase. A pool of serum from dog allergic patients was used as source of IgE. □, Inhibition with DHD; △, inhibition with purified *Can f*I; ○, inhibition with *Can f*I-depleted DHD. Three individual experiments with duplicate determinations were performed. Vertical bars show s.d. Difference between DHD and *Can f*I-depleted DHD was statistically significant ($P < 0.05$); differences between purified *Can f*I and the two other samples were statistically significant ($P < 0.0001$).

Allergenic activity of *Can f*I in vivo

Three different preparations were used to evaluate the importance of *Can f*I as a sensitizing agent in dog-allergic patients, i.e. DHD, *Can f*I-depleted DHD and purified *Can f*I. Duplicated skin-prick testings were performed with undiluted samples and 1/5 and 1/25 dilutions of the material. As shown in Table 2, all 26 patients reacted to DHD in all three concentrations. Of the 26, 24 reacted to all three concentrations of *Can f*I-depleted DHD; the remaining two (patients no. 17 and 18) did not react with the most dilute sample. Purified *Can f*I elicited weals to all three concentrations in 23 out of 26 patients; one patient (no. 9) reacted only to the undiluted samples and the other two (nos. 3 and 17) did not react with *Can f*I in this test at all. All patients reacted to the positive histamine control and none reacted to the negative diluent control.

IgE immunoblotting

Serum samples were obtained from 23 of 26 dog-allergic patients that were skin-prick tested with purified *Can f*I. All serum samples were assayed for IgE-binding to *Can f*I in immunoblotting to DHD separated by SDS-PAGE (Fig. 4). A prominent IgE-binding band was revealed at about 25 kD, corresponding to that of *Can f*I. With the

conditions used for this assay 16 out of 23 patients reacted with an intensity high enough to be detected after exposure for 12 days. Several other bands bound IgE to varying degrees from the different patients. The most notable bands had an apparent mol. wt of approximately 18 kD, 42 kD and 66 kD. Seven patients did not stain any bands strongly enough to be revealed under the conditions used here.

Discussion

The comparison of allergens identified by different methods and different studies has been hampered by the fact that hitherto no dog-hair and dander-specific allergens had been purified and identified unequivocally. In the present study we aimed at purifying Ag13 that was identified as an important dander-related allergen by Blands *et al.* [8]. Other investigators have pointed to this allergen as an important component in allergy to dog hair and dander. Ford *et al.* [9] identified an allergen termed Ag8 that had been shown to be identical to our Ag13 as it reacts with our monospecific rabbit antibody to Ag13 (unpublished data). This molecule has also been recognized as an important allergen by Dr H. de Groot (Red Cross Blood Centre, Amsterdam, The Netherlands), who

Table 2. Allergenic activity of *Can f* I as measured by skin prick test

Patient no.	DHD			<i>Can f</i> I-depleted DHD			Purified <i>Can f</i> I		
	—	1/5	1/25	—	1/5	1/25	—	1/5	1/25
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	—	—	—
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	—	—
10	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	—	—	—	—
18	+	+	+	+	+	—	+	+	+
19	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+

All testings were run in duplicate by the same nurse; duplicate results were similar. Concentration of the purified *Can f* I was identical to that of *Can f* I in DHD. Only the lack of *Can f* I distinguishes DHD from *Can f* I-depleted DHD. —, undiluted; 1/5 and 1/25, diluted in Soluprick® diluent (ALK, Denmark). Diluent was negative control, 10 mg/ml of histamine was positive control. Weals ≥ 3 mm in diameter were considered positive.

kindly supplied us with two recently developed monoclonal antibodies that were shown to react with Ag13 by dot-blot analysis (unpublished data). We therefore selected Ag13 for purification and characterization and we tentatively termed it *Can f* I.

The purification of *Can f* I on the immunoaffinity column had a tendency to co-purify trace amounts of dog serum albumin as detected by CIE. Serum albumin has been shown to be an allergen [8,22] so it was necessary to remove the remaining albumin from the purified *Can f* I before we could assess the allergenic activity of *Can f* I. This was done by passing the material through an anti-dog serum column; the procedure completely removed the albumin, as shown in Fig. 1c, where the intermediary

gel containing anti-dog serum did not precipitate any antigens. As analysed in SDS-PAGE the purified *Can f* I had a major band at 25 kD and a minor band at 18 kD. The inter-relationship between the bands is not known at present, but some allergens, e.g. *Fel d* I have been found to be dimeric [23–25] so the molecule could be a heterodimer or the 18-kD band could be a breakdown product. The purified molecule looked homogenous in CIE and in HPLC gel filtration where a band was found at 22 kD.

LIA inhibition experiments demonstrate that the removal of *Can f* I from DHD removed about half of the allergenic activity of the extract. Even when the anti-*Can f* I column was far from overloaded, it was still possible to show small amounts of remaining *Can f* I in the effluent

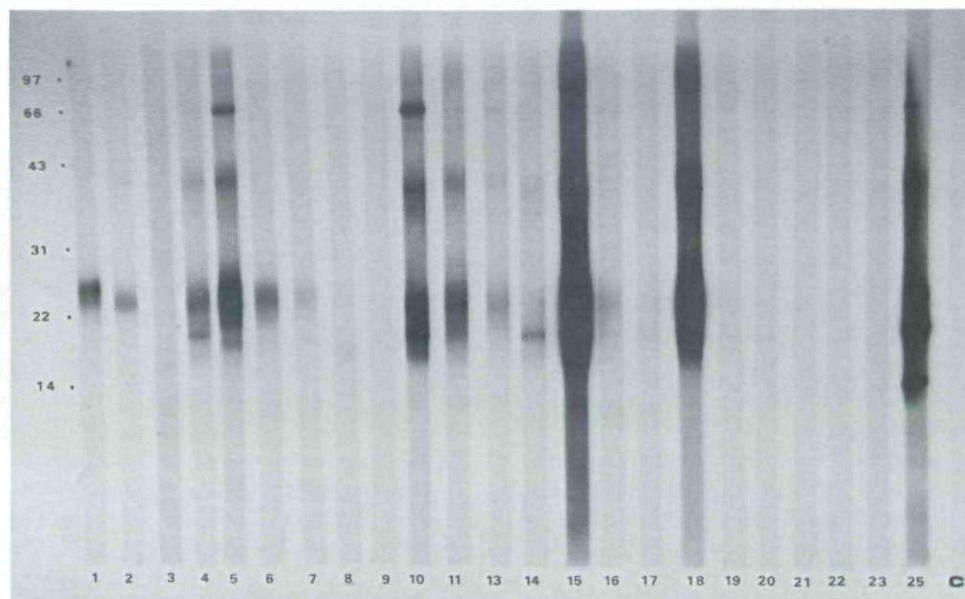


Fig. 4. IgE immunoblotting with patient serum. Serum from 23 dog-allergic patients (nos. 1–11; 13–23; 25) that were skin-prick-test positive to purified *Can f I* was tested for IgE reactivity to DHD. In each lane 10 μ g of DHD were separated by SDS-PAGE. Apparent mol. wt in kD. C, Pool of serum from non-allergic persons.

by means of immunoblotting with rabbit anti *Can f I* (Fig. 2A) and with human IgE (data not shown). This could explain why high concentrations of *Can f I*-depleted extract were able to inhibit IgE-binding to DHD almost completely. Theoretically we would have expected both lowered potency and a lowered maximum inhibitory ability of the *Can f I*-depleted extract. The purified *Can f I* inhibited the binding of IgE to DHD with up to more than 50% in our system. This value corresponds to the value expected from the diminished IgE binding obtained with *Can f I*-depleted DHD.

We investigated the sensitivity of individual dog-allergic patients by means of skin-prick testing with DHD, *Can f I*-depleted DHD and purified *Can f I*. The criterion for positive skin test was a weal diameter ≥ 3 mm. The purified *Can f I* elicited positive skin reactions in 92% of the patients tested and we therefore conclude that the allergen plays an important role in the development of allergy to dog hair and dander. As detected by CRIE analysis [8,9] the allergen was not as reactive with the IgE from individual patients and when we tested the serum from the skin-prick-positive subjects in immunoblotting (Fig. 4) we saw IgE binding to the *Can f I* in approximately 70% of the sera. Differences observed could be due to different sensitivities of the methods used and/or the patient populations. It has been shown that DHD contains several allergens of varying importance (Figs 1b, 3) [8, 9, 26, 27] so our finding that all dog-allergic patients

investigated were skin-test-positive to the *Can f I*-depleted DHD is compatible with the present knowledge.

The allergen *Can f I* is the first purified dog-hair and dander-specific allergen, and we suggest that it could be used as a marker allergen for future studies of the environmental exposure to dog hair and dander, and for the study of the relationship between exposure and sensitization. Furthermore, it is likely that the information obtained will benefit future DHD extracts used for specific immunotherapy.

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