

Circulating cat allergen and immune complexes in cat-allergic children

R. CASAS, P. DJERF, P. HÄGGSTRÖM, R. FERRÁNDIZ and B. BJÖRKSTÉN

Department of Health and Environment, Division of Paediatrics, Linköping University, Sweden

Summary

Background The first encounters with allergens seem to influence the development of allergy. Food antigens have been detected in sera as free antigens and in complexes with IgG but less is known about the presence of inhalant allergens.

Objective To investigate the presence of the major cat allergen Fel d 1, either as free allergen and/or in complexes with IgG and IgE antibodies in sera from atopic children.

Methods Serum samples from 33 cat allergic asthmatic children, 7–17 years old, and 15 non-allergic controls were investigated for the presence of Fel d 1 by ELISA (detection limit 0.13 µg/L). To detect immune complexes (IC), the IgG fraction from Fel d 1 positive sera was purified by affinity chromatography. Purified and non-absorbed material was then analysed for allergen content and specific IgG antibody levels. Immune complexes with Fel d 1 IgE were detected by coupling anti-Fel d 1 MoAb to paramagnetic particles.

Results Fel d 1 was detected (0.15–1.8 µg/L) in 23 of the 33 patients (70%) but not from any of the controls. Eighteen samples contained IgE-Fel d 1 IC and two of four tested samples contained Fel d 1 in the IgG fraction. Electrophoresis and Western blotting of IgG purified material using anti-Fel d 1 MoAb corroborated the presence of IgG-Fel d 1 IC.

Conclusion Free-circulating inhalant allergen and IC with allergens may contribute to maintaining immune responsiveness and sensitivity.

Keywords: allergen, Fel d 1, immunoglobulin-E, immunoglobulin-G, immune complex

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Introduction

The conditions under which an allergen is encountered in infancy seem to influence the development of allergy late in life [1–5]. It is known that very low doses of allergen may induce and maintain sensitization and that immune responses to inhaled allergens are detectable at sites distant from the respiratory tract [6,7].

It has been reported that dietary antigen may be present in the blood of healthy adults, as demonstrated by the presence of ovalbumin (OVA) and OVA in complex with IgG [8,9].

Less is known about the possible presence of circulating inhalant allergens, however, microparticles as pollens have also been detected in body fluids [10]. Inhalant antigens,

IgG antibodies against food antigens, or anti-idiotypic antibodies resembling the antigen, also appear to cross the 'placental barrier' and elicit a specific IgE response in the fetus [11]. Some researchers have claimed to detect house dust mite Der p 1 allergen up to 20 ng/mL in 35% of cord blood and maternal serum samples. Experimental studies adding Der p 1 to a serum sample suggest that the allergen may be coupled to antibodies, since 75% of the added allergen cannot be recovered. In a study comprising 43 mite-sensitive asthmatic patients, Der f 1 and Der f 1 IgE IC were detected in nine (21%) of the serum samples [12].

Cat and dog allergens are the major indoor allergens in Sweden [13,14], and about 70% of Scandinavian children with perennial asthma are sensitized to cat and/or dog dander [15]. These allergens are ubiquitous in the environment even in places where cats and dogs have never been kept [13,16–18].

The clinical significance of free-circulating allergens and

Correspondence: R. Casas, Department of Health and Environment, Division of Paediatrics, Linköping University Hospital, S-581 85 Linköping, Sweden.

IC with allergens is not fully understood. It is possible that these allergenic molecules, as well as anti-idiotypic antibodies in serum may play a role in sensitization and maintenance of sensitivity.

The aim of this work was to analyse serum samples from cat-allergic children for the presence of Fel d 1, either as free allergen and/or in complexes with IgG and IgE antibodies.

Materials and methods

Patients and sera

Serum samples were obtained from 33 cat-allergic asthmatic children, 13 girls and 20 boys, 7–17 years old, who participated in a prospective study with a corticosteroid drug. They all had a family history of atopy, positive skin prick test (SPT) to cat allergen and in addition to asthma they had other manifestations of atopy, e.g. eczema and/or rhinoconjunctivitis. Sera from 15 healthy children with undetectable IgE antibodies to cat allergen were included as controls. Three of the asthmatic children and two of the control children had a cat at home and all were frequently exposed to cats in houses of friends and relatives.

Antibody determination

The levels of IgE antibodies to cat allergens and total IgE were determined by a chemiluminescent immunoassay (Magic Lite[®], ALK-Abelló, Denmark). Levels higher than 1.43 SU/mL (class 1) were considered as positive for IgE antibodies against cat.

Circulating IgG to cat allergens were measured by a conventional ELISA as previously described for the birch major allergen Bet v 1 [19]. Briefly, microtitre plates were coated with 200 µL/well of cat extract (ALK-Abelló) at 10 mg/L in phosphate buffered saline (PBS). After blocking the protein binding sites with 200 µL/well of human albumin (HSA) 0.5% in PBS-Tween, 200 µL of serum samples were added. Alkaline phosphatase conjugated rabbit-anti mouse IgG was then added and the reaction was developed by FAST-pNPP (p-nitrophenyl phosphate) substrate (Sigma, St Louis, USA).

Determination of Fel d 1 allergen

The concentration of Fel d 1 allergen was determined by a sandwich ELISA (detection limit 0.14 µg/L) (ALK-Abelló). Costar 3590 microtitre plates (Life Technologies, Cambridge, USA) were coated with KAT 1 anti-Fel d 1 monoclonal antibody at 1 µg/well in 50 mmol/L carbonate-bicarbonate buffer, pH 9.6. After incubation with 100 µL of sample per well, a horse radish peroxidase-labelled

immunoabsorbed rabbit anti Fel d was used as detecting antibody. The reaction was developed with 1,2-phenylene-diamine (OPD).

Detection of immune complexes

Affinity chromatography purification of IgG complexes was performed by GammaBind G-Sepharose (Pharmacia, Uppsala, Sweden). After binding of serum samples, diluted 1:10 in 0.01 mol/L sodium phosphate buffer containing 0.15 mol/L NaCl and 0.001 mol/L EDTA pH 7.0, the IgG-containing fractions were eluted by 0.5 mol/L acetic acid, pH 3.0. Adsorbed and non-adsorbed (NA) materials were collected on 1 mol/L TRIS pH 9.0 and desalted against NH₄HCO₃ 0.005 mol/L by Sephadex G-25. The desalted samples were dried by speed vacuum overnight, and then kept frozen and reconstituted before used in half the volume of the total sample before purification. The IgG-purified and NA fractions were analysed for Fel d 1 content and specific IgG by ELISA as described above. To check the efficacy of this method, IgG–Fel d 1 IC complexes were induced. A serum, with no detectable Fel d 1 in the IgG fraction, was incubated with cat extract at a concentration of 100 ng/L Fel d 1 major allergen (ALK-Abelló) for 1 h at 37 °C and then subject to the purification steps.

To detect Fel d 1-IgE IC, a modification of the Magic Lite[®] chemiluminescent immunoassay (ALK-Abelló) was performed by coupling anti Fel d 1 KAT 1 monoclonal antibody (kindly supplied by Carsten Schou, ALK-Abelló) at 2.2 g/L to paramagnetic particles, according to the procedure recommended by the manufacturer. These particles were incubated overnight in duplicate with each of the 33 individual patient sera and the 15 controls, diluted 1:5 in PBS-Tween. Immune complexes with IgE and Fel d 1 were induced as controls by incubating overnight a serum sample containing specific IgE to cat, ≥20.0 standardized units/mL (SU/mL), but not detectable Fel d 1 and IgE–Fel d 1 complexes, with serial dilutions (12.5–0.35 mg/L) of cat extract. The particles were then washed and the serum was analysed as recommended by the manufacturer. The results were measured in relative light units (RLU). Levels above 5400 RLU/mL were regarded as positive. The results were also expressed as the ratio of the RLU of each sample to the mean RLU of the background controls (negative controls). Ratios higher than two were considered to be positive.

SDS-gPAGE and immunoblot analysis

Dried affinity purified IC were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions on a Mini Protean system (Bio-Rad, USA). After electrophoresis, the gels were transferred to 0.2 µm Hybond nitro-cellulose membranes

(Amersham, UK). Membranes were blocked by incubation with 5% defatted milk in PBS containing 0.1% Tween for 1 h at room temperature, and then washed and incubated overnight at 4°C with KAT 1 anti-Fel d 1 MoAb 1:10 000 in PBS-Tween 0.5%, or HP6064 anti-human IgG MoAb (BioZac, Sweden) diluted 1:50 000. The membranes were then extensively washed and incubated for 1 h at room temperature with Streptavidin-horseradish peroxidase (HRP) conjugate. After washing thoroughly, the membranes were exposed to the enhanced chemiluminescent system (ECL) detection solution during 1 min and autoradiographed on HyperfilmTM ECL (Amersham, UK) using a Kodak X-Omatic cassette with intensifying screen (Eastman Kodak, USA). One strip containing molecular weight markers (Kaleidoscope pre-stained standards, 6.9–202 kDa; Bio-Rad, USA) was cut after blotting, and used as a reference to estimate the molecular weights.

Statistical analysis

Relationship between the levels of Fel d 1, specific IgG and IgE, SPT and IgE-Fel d 1 IC were analysed by the non-parametric Spearman correlation test using the StatView software (Abacus, Concepts Inc., Berkeley, CA, USA).

Results

The cat allergen Fel d 1 was detected in 23 of 33 (70%) serum samples at concentrations varying from 0.15 to 1.8 µg/L (mean concentration 0.55 µg/L) (Table 1). Cat allergen was not detected in any of the samples from the 15 non-allergic children, not even in the serum of a cat owner who had a cat for several years.

To evaluate the presence of IgG-Fel d 1 IC, four of the samples with detectable levels of Fel d 1 were selected and further purified by affinity chromatography. In one of the samples Fel d 1 was detected in the IgG-bounded and non-adsorbed (NA) fractions at 0.59 µg/L and 6.45 µg/L, respectively (Table 2). The concentration of Fel d 1 in this sample was 1.88 µg/L before purification. In two other samples, Fel d 1 was detected only in the NA material after IgG purification and one sample contained Fel d 1 only in the IgG fraction. The Fel d 1 concentration in the purified IgG fraction from the control serum with induced Fel d 1-IgG IC was 25.3 µg/L, confirming the capacity of the method employed to catch Fel d 1-IgG IC.

Four distinctive bands were identified in the IgG fractions which were positive to cat by Western blotting and autoradiography using anti Fel d 1 MoAb (Fig. 1), i.e. one around 40 kDa, a second band approximately of 94 kDa, an intensive and broad band between 133 and 170 kDa, and a high molecular weight band around 300 kDa. The analysis of several negative samples to Fel d 1 showed only one

Table 1. Levels of Fel d 1 allergen, IgG and IgE antibodies and IgE–Fel d 1 immune complexes in serum samples from atopic children and in the non-allergic controls

Sample	Fel d 1 (µg/L)	IgG (µg/L)	IgE (SU/mL)	IgE–Fel d 1 (RLU)
<i>Atopic children</i>				
1	1.88	78	10	1.1
2	Neg	69	475	4.9
3	Neg	46	0.9	2.1
4	0.17	121	222	26
5	0.18	206	323	25
6	0.15	48	16	2.3
7	Neg	109	49	2.7
8	0.68	567	125	111
9	0.17	51	367	0.2
10	0.84	780	240	11
11	Neg	114	232	1.1
12	0.19	110	290	1.5
13	0.60	51	319	2.1
14	0.75	55	4.2	0.4
15	0.78	26	16	2.1
16	0.63	165	190	114
17	0.82	35	0.3	0.0
18	Neg	162	88	1.6
19	Neg	500	25	0.5
20	Neg	54	3.0	0.1
21	0.13	287	800	374
22	0.54	158	68	2.4
23	Neg	77	11	0.6
24	0.30	83	30	8.8
25	0.57	183	1.1	1.6
26	Neg	80	2.2	2.3
27	0.33	118	3.6	0.0
28	0.07	220	266	1457
29	0.34	269	96	7.2
30	0.86	114	3.7	0.0
31	0.59	785	76	0.9
32	0.35	876	249	2.2
33	0.29	131	22	0.9
<i>Non-allergic controls</i>				
1	Neg	22	0.1	0.0
2	Neg	62	0.0	0.0
3	Neg	210	0.0	0.4
4	Neg	27	0.0	0.5
5	Neg	0	0.1	0.4
6	Neg	88	0.0	0.1
7	Neg	0	0.2	0.3
8	Neg	0	0.0	0.0
9	Neg	204	0.0	0.5
10	Neg	0	0.0	0.0
11	Neg	0	0.0	0.0
12	Neg	48	0.0	0.9
13	Neg	28	0.0	0.0
14	Neg	22	0.0	0.0
15	Neg	26	0.0	0.5

Table 2. Fel d 1 concentration after affinity chromatography purification of four serum samples with detectable levels of Fel d 1 allergen

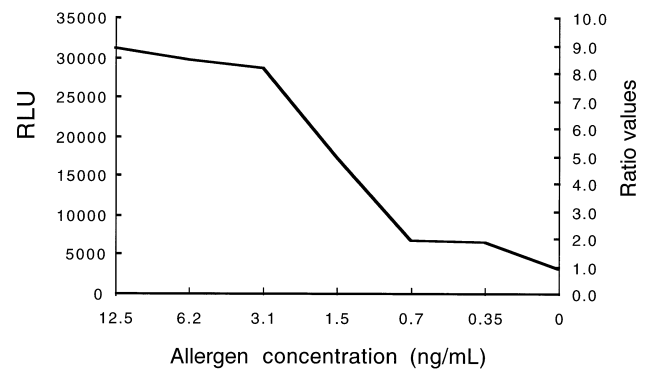
Sample	Fel d 1 ($\mu\text{g/L}$)	
	NA fraction	IgG fraction
1	6.45	0.59
8	Neg	0.34
10	0.32	Neg
15	0.52	Neg
Positive control	18.8	25.3
Negative control	Neg	Neg

NA and IgG fractions indicate the non-adsorbed fraction and the fraction bound to GammaBind Sepharose, respectively.

diffuse band. In contrast, when the IgG-purified fractions were incubated with anti-IgG MoAb showed numerous bands which were similarly distributed in Fel d 1 positive and negative samples.

Eighteen of 33 samples (55%) contained IgE–Fel d 1 complexes (Table 1), while all the 15 sera from the non-asthmatic children were negative in this assay. Immune complexes induced artificially were also detected in the sample incubated with varying concentrations of Fel d 1 (Fig. 2).

One of the samples with detectable Fel d 1 in the IgG fraction after purification, was negative for IgE–Fel d 1 by

**Fig. 2.** Induction of IgE–Fel d 1 allergen immune complexes by increasing concentrations of cat extract. Values are expressed both as relative light units (RLU) and as the RLU ratio in the sample over the background.

Magic Lite, suggesting that in this serum, Fel d 1 was present only as IgG complex. Another sample was positive for IgE–Fel d 1 IC, but negative in the IgG fraction after purification, indicating that in this serum Fel d 1 was in complex with IgE.

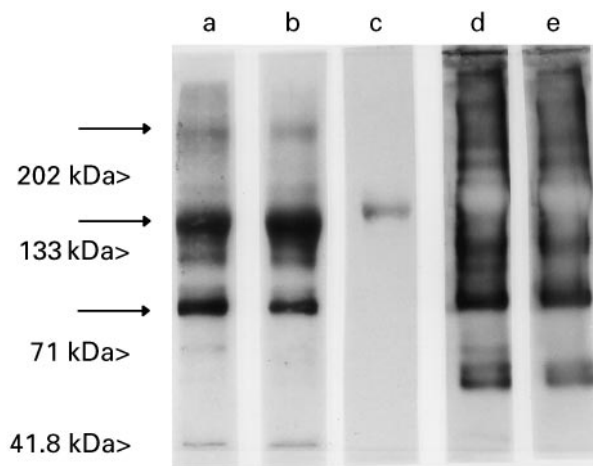
There was no significant correlation between the levels of Fel d 1 and either specific IgG ($r = 0.14$) or IgE ($r = 0.08$), nor with the size of the skin prick test results ($r = 0.14$). There was however, a relationship between the levels of IgE–Fel d 1 complexes and the levels of specific IgE antibodies to cat ($r = 0.6$; $P < 0.01$).

Discussion

The major cat allergen Fel d 1 was detected in 70% of the serum samples from asthmatic children and in none of the 15 healthy controls independently of the exposure to cat, indicating that high level of exposure was not by itself associated with the presence of Fel d 1 in serum. Some of the allergen was present as immune complexes with IgE and/or IgG.

The cat dander allergen Ag 1 which was later given the name Fel d 1 has been shown to bind to sera from cat-allergic patients [20]. The results were supported in a study showing that most patients reacted to a 14 kDa protein, which would correspond to the molecular weight of Fel d 1 [21]. Then, it is reasonable to suggest that most cat-specific IgE and IgG detected in our samples were directed against Fel d 1.

A possible explanation for the relatively low yield of Fel d 1 from the purified IgG-IC could be that when Fel d 1 is attached to the immunoglobulin molecules as complexes, the epitopes recognized by the monoclonal antibody were hidden by conformational changes in the IC. After purification by IgG-binding Fel d 1 was detected in complexes with IgG. However, no immune complexes were detected in the

**Fig. 1.** IgG–Fel d 1 immune complexes (indicated by arrows) in purified serum samples after SDS-PAGE and Western blotting. Lanes a and b: samples positive for Fel d 1 incubated with anti-Fel d 1 MoAb; lane c: sample negative to Fel d 1 incubated with anti-Fel d 1 MoAb; lanes d and e: samples negative and positive for Fel d 1, respectively, incubated with anti-IgG.

control samples, nor were any IgE–Fel d 1 complexes detected.

There was no correlation in the individual serum samples between the concentration of Fel d 1 and the presence of Fel d 1–IgE or Fel d 1–IgG, nor between the two types of IC.

Immune complexes of Der f 1–IgE have been previously detected in nine of 43 (21%) sera from house dust mite allergic patients at a concentration $\geq 2 \mu\text{g/L}$ [12]. In four of the nine patients with IC, free circulating Der f 1 was also detected, while all the 19 sera from non-atopic patients were negative. Our results confirm with another allergen that inhalant allergens may be present in the serum, both as free allergen and as IC.

The Fel d 1 allergen was detected in bands corresponding to molecular weights of ≈ 40 , 94, 133–170 and 300 kDa, indicating that some antigen was bound to IgG as Fel d 1–IgG complex (133–170 kDa band), and as aggregates with more than one immunoglobulin molecule (300 kDa band). The 40 kDa band, corresponding to the molecular weight of Fel d 1 [22–24], could represent free cat allergen dissociated from IC during the electrophoresis. The 94 kDa band could represent fragments of IgG after the electrophoresis. The diffused band found in the negative samples can be explained by some non-specific binding of anti-Fel d 1 MoAb, since no allergen was detected by ELISA in these samples before or after purification. The specificity of the recognition to Fel d 1 allergen in the positive sera was demonstrated by the bands identified when the samples were incubated with anti-IgG MoAb.

It is generally difficult to induce specific IgE and IgG responses in laboratory animals through inhalation of antigens. It has been suggested that the immune system in the respiratory tract of normal animals acts as an effective shield to most inhaled antigens [25]. Our findings would support this notion, as we could detect cat allergen only in cat-allergic individuals, whereas all the controls were negative, even those permanently exposed to cat. Possibly, the mucosal barrier in non-atopic individuals prevents the passage of allergens while increased mucosal permeability is present in atopics. No other inhalant allergens against which the patients were sensitized were studied, but our data could provide some indirect information for the same reasoning with other inhalant allergens.

Anti-idiotypic antibodies directed against idiotypes carried by allergen-specific IgG and IgE in atopic and non-atopic individuals have been reported [26–28]. The presence of IgG–Fel d 1 only after the induction of IC with cat allergen suggests that at least in that sample the Fel d 1 antigenic activity did not correspond to an anti-idiotypic IgG antibody.

The biological function of IC with IgG has been studied in experimental animals, and both enhancement and suppression of immune response have been described [29,30]. Immune complexes with allergens have also been used in

the treatment of patients with atopic dermatitis in an effort to suppress IgE antibody production, and a significant therapeutic improvement was reported [31]. In another study, the injection of allergen–antibody complexes resulted in a rapid and significant improvement of bronchial asthma in patients sensitive to *Dermatophagoides pteronyssinus* at 100-fold less amount of allergen as compared to conventional immunotherapy [32].

The biological significance of IgE–IC is unknown. Possibly, they are involved in the pathogenesis of allergic diseases, e.g. by enhancing the function of antigen presenting cells as IgE-mediated antigen presentation which may potentially provide an activation of the immune system [33]. Experimentally, Der p 2–IC stimulates the proliferation of specific T cells at 1000-fold lower Der p 2 concentration than that required for T-cell activation by native antigen [34]. This facilitated antigen presentation (FAP) activity may have a role in the pathogenesis of atopic dermatitis [35].

Although no other allergens were studied, the data obtained so far provide some evidence for a possible continuous presence of inhalant allergens in the circulation of allergic individuals which could maintain the sensitivity in mucosal membranes. It is also possible that such compounds passing over the placenta or via breast milk to fetus and offspring may influence sensitization and future development of allergy.

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