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Role of the Major Allergen (*Fel d I*) in Patients Sensitized to Cat Allergens

Key Words

Fel d I
 Histamine release
 Skin prick test

Abstract

Forty patients with a positive case history and specific serum IgE to cat allergens were evaluated using titrated skin prick test (SPT), histamine release studies (HR) and immunoblotting studies. To determine the relevance of the major allergen, a monospecific antibody to *Fel d I* was preincubated in different concentrations with the cat allergen dose required for maximum response in the HR assay (10 SQ/ml). Dose-dependent inhibition of histamine release ($n = 30$, $x = 66\% \pm 5\%$ $IC_{30} = 0.36 \mu\text{g/ml}$) was found in all patients sensitized to cat allergens as shown by antigen-induced HR. The greater the HR response to the cat allergen extract the higher were the antibody concentrations necessary for inhibition ($p < 0.05$). Immunoblot experiments ($n = 22$) using SDS-PAGE demonstrated five different locations of IgE binding in 15 sera: 18, 35, 46, 54 and 66 kD. IgE binding at 18 kD was found in 14 patients. Anti-*Fel d I* possessed two strong bands at about 18 and 35 kD which are representative of the major allergen *Fel d I*. Our findings emphasize the major role of *Fel d I* in patients sensitized to cat allergens.

Introduction

In recent years, the role of pets as an important allergen source in Europe and North America became evident since 20–30% of individuals with IgE-mediated respiratory disorders were found to be allergic to pets and other animals [1, 2]. Continued exposure to these indoor allergens can cause allergic sensitization; this was shown by quantitative investigations of house dust by ELISA techniques which demonstrated the prevalence of allergen particles of cats [3, 4]. Several predominant cat allergens have been identified by crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) [5]. The appearance of major cat allergens in commercial

extracts is largely dependent on the origin of the raw materials used: *Fel d I*, suspected to be one of the most important allergens in cat allergen extracts [6], was isolated from cat pelt extracts [7], although it is known that its main source is saliva [8]. Recent data suggest that *Fel d I* is produced by sebaceous cells and stored mainly on the surface of the epidermis and fur [9]. The 39-kD molecule is very stable [10]; however, it has been demonstrated that *Fel d I* is a noncovalently linked dimer consisting of two monomers with molecular weights (MW) of 18 kD [11]. Monoclonal antibodies to *Fel d I* have been used to isolate and further purify *Fel d I* from different materials [12], and also to detect this major allergen in house dust samples and allergen extracts, and seem to be appropriate tools for the

standardization of cat allergen extracts [13]. Recently, a specific immunoassay has been developed to detect *Fel d I*, the major allergen of cat-derived allergens [14]. Little knowledge exists about the frequency of IgE-mediated sensitization to *Fel d I* in patients allergic to cat allergens. Therefore, monospecific antibodies to *Fel d I* might be a useful tool to study its clinical relevance in cases of allergic sensitization to cat allergens.

Subjects and Methods

Forty patients (age 10–65 years, average 35.8, 20 males and 20 females) with inhalent allergies, who had given informed consent were included in the study based on their specific IgE levels to cat allergen (class > 2) measured by Phadebas-RAST (Pharmacia, Uppsala, Sweden). After evaluation of the case history, skin prick tests (SPT), histamine release studies (HR), inhibition of HR and immunoblot techniques using SDS-PAGE were performed.

Allergen Extract

For all investigations (in vitro and in vivo), well-characterized and standardized extracts of cat allergens were used (ALK SQ Aquagen and Alk SQ Soluprick, ALK, Denmark). By quantitative immunoelectrophoresis and CRIE analysis, the *Fel d I* content in 100,000 SQ has been determined to be about 43 µg [15]. The lyophilized stock containing 500,000 SQ was dissolved in 5 ml of a Tris buffer (pH 7.4), containing 25 mM Tris (Serva, Heidelberg, FRG), 0.4% human serum albumin (HSA Armour Pharma, Eschwege, FRG) and 0.01 M CaCl₂, 0.01 M MgCl₂ for HR.

Monospecific Antibodies to *Fel d I*

Immunization of each of 2 rabbits with approximately 15 µg of partially purified *Fel d I* was repeated 5 times (1x/month) before the purification of the antibodies according to the method described by Harboe and Ingild [16]. Non-cat specificities were removed by solid phase absorption to cat extract, and the bound antibodies are eluted under mild conditions. After being concentrated, the monospecificity of the antibodies was assessed using CIE.

SPT

SPT were performed on the volar forearm using the following concentrations of the diluted cat extract: 10, 100, 1,000, 10,000, 100,000 SQ/ml. Histamine (10 mg/ml) and physiological saline were used as positive and negative controls. After 15 min, the wheals were marked and documented with a strip of tape. The diameters were calculated using the formula $(d_{\max} + d_{\min})/2 = d_{\text{mean}}$ and plotted against the allergen concentrations.

HR

After sedimentation of 20 ml peripheral blood in a solution containing 5 ml 6% dextran (MW: 60 kD, Knoll, Ludwigshafen, FRG), 2 ml 0.1 M EDTA and 0.6 ml 50% glucose (Braun, Melsungen, FRG) for 90 min, the basophil-containing supernatant was centrifuged (400 g, 4 °C). The pellet was gently resuspended and washed twice by centrifugation (400 g, 4 °C) with 50 ml of a buffer containing 25 mM Tris, 0.1 ml of 20% human serum albumin, either complemented with 0.01 M EDTA or without EDTA. The cells were resuspended in the same

buffer without EDTA, but with 0.01 M CaCl₂ and 0.01 M MgCl₂. For IgE-mediated HR, 250 µl of cells were incubated in duplicate with 250 µl of the allergen dilutions (final concentrations: 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1,000 SQ/ml) or anti-IgE (final concentrations: 1, 10, 100, 1,000 IU/ml) in a water bath (37 °C, 45 min). For the determination of total histamine, four portions of 250 µl cells were lysed by 250 µl 0.4 N HClO₄. Spontaneous HR was determined by incubation of 250 µl cells with 250 µl buffer. After removal of the cells by centrifugation (800 g, 4 °C, 15 min), the supernatants were assayed for histamine using an automated fluorometric method [17]. HR was expressed as a percentage of the total histamine content after subtracting the spontaneous release. All experiments were performed in duplicate. HR of $\geq 30\%$ was defined as a positive result. The dose-response curves obtained with the cat allergen extract were used to calculate the maximum of antigen-induced HR ($\text{HR}_{\max}$ = cell reactivity) and the allergen concentration for 30% HR (Ag_{30} = cell sensitivity) [17].

Inhibition of HR

For inhibition of HR, different concentrations of the monospecific anti-*Fel d I* antibody (0.001, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 µg/ml) were incubated (12 h, 20 °C, vortexing slowly) with 10 SQ/ml of the cat allergen extract. Then, the cells of so-called responders were triggered with 250 µl of the mixture of monospecific antibody plus allergen using the same HR protocol described above. The relative inhibition (Inh) was calculated using the following formula:

$$\text{Inh (\%)} = 100\% - [(\% \text{HR}^+ / \% \text{HR}^-) \times 100].$$

$\% \text{HR}^+ / \% \text{HR}^-$ means HR after incubation with/without the mixture of the monospecific antibody and the cat allergen (10 SQ/ml).

Three additional experiments were performed using the rabbit anti-*Fel d I* antibody and a rabbit anti-human IgG antibody at the same concentrations as a control to demonstrate that the inhibition of HR is due to the specific anti-*Fel d I* antibody rather than to a non-specific inhibition of release due to rabbit immunoglobulins.

SDS-PAGE/Western Blot

About 200 µl of the cat allergen extract (180,000 SQ/ml = 77.4 µg/ml *Fel d I*) were separated on a 7.5–20% sodium dodecylsulfate-polyacrylamide gel (Sigma, Deisenhofen, FRG) by electrophoresis (SDS-PAGE) modified from Neville [18] and transferred electrophoretically to a nitrocellulose membrane (Schleicher, Dassel, FRG). The nitrocellulose was blocked with 0.1% Tween 20 (Sigma) in 0.1 M Tris-HCl, pH 7.4, 0.1 M NaCl, 2.5 mM MgCl₂, cut in strips of 2-mm width and incubated with patient sera as described previously [19]. For detection of IgE, an alkaline-phosphatase-linked goat anti-human IgE antibody (Dianova, Hamburg, FRG) was used.

Statistical Calculation

Data, obtained by SPT, HR, RAST, were expressed as means $\pm$ SEM. Statistically significant differences in allergen-induced histamine levels within the group were analyzed using Wilcoxon's signed rank test for paired data. Values were considered significant at $p < 0.05$. Correlations between the in vitro and in vivo parameters were calculated using the Spearman rank test.

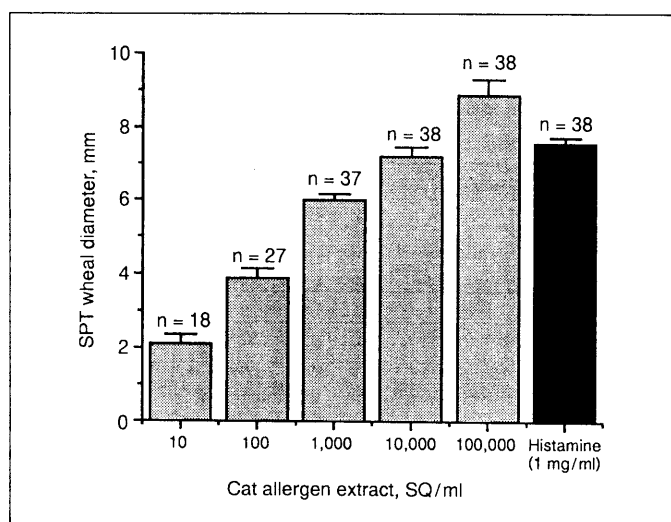


Fig. 1. SPT results (average of the wheal diameter $\pm$ SEM) in subjects sensitized to cat allergens (for number of positive results see above the columns). The individual wheal diameters were calculated according to the formula $d_{\text{mean}} = (d_{\text{max}} + d_{\text{min}})/2$.

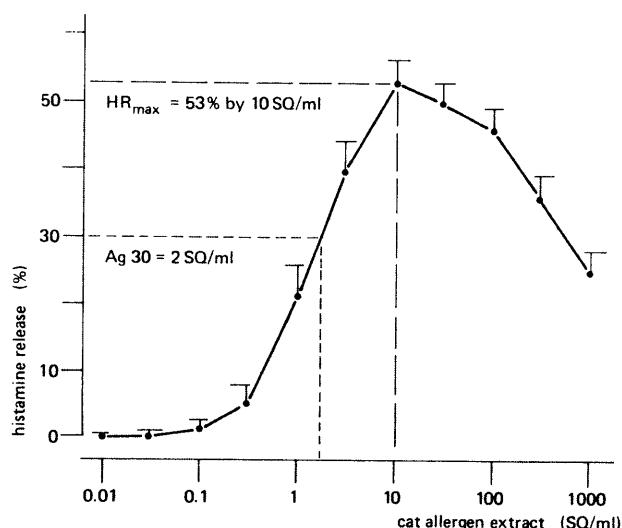


Fig. 2. HR (means $\pm$ SEM) from peripheral leukocytes in subjects sensitized to cat allergens ($n = 30$). Mediator release was triggered by a partially purified and standardized cat allergen extract and expressed as a percentage of the total cellular histamine content after subtraction of spontaneous release.

Results

SPT

The dose-dependent wheal responses with different concentrations of the cat extract are summarized in figure 1. Two patients were excluded from the calculation because a wheal induced by physiological saline indicated a dermographic urticaria. The mean allergen dose required for a wheal equivalent to histamine was found to be 18,000 SQ/ml.

Anti-IgE-Induced HR

Bell-shaped response curves were found after triggering HR by anti-IgE for all patients (HR_{max} : $42 \pm 4\%$, range: 14–80%). Thirty-seven of 40 released more than 30% of their total histamine, demonstrating a maximum release at 100 IU/ml anti-IgE (data not shown).

Cat-Induced HR

Positive HR ($>30\%$) induced by the cat allergen extract was obtained in 30 individuals. Spontaneous HR did not exceed 6% of the total cellular histamine content in any of the experiments and was found to be $2.0 \pm 0.2\%$ overall. The bell-shaped dose-response curves were summarized and are depicted in figure 2. Cell reactivity (HR_{max} : $59 \pm 4\%$, range: 33–94%) and cell sensitivity varied over a wide range. In 4 of 30 individuals, strong sensitization with an Ag_{30} of 0.01–0.1 SQ/ml were found, in 13 subjects Ag_{30} was detected between 0.1–1, in 10 cell donors between 1 and 10, and in 3 cases with relatively high doses of 10–100 SQ/ml. A close negative relationship was found between cell reactivity and cell sensitivity to the cat extract ($r_s = -0.53$, $p < 0.01$); thus, a high maximum of HR corresponded to small Ag_{30} concentrations, reflecting an increased cell sensitivity. A positive correlation was also found between the reactivity to anti-IgE and cat-induced HR ($r_s = 0.61$, $p < 0.01$).

Inhibition of HR

Anti-*Fel d* I induced a dose-dependent inhibition of HR in the range of 0.01–1 $\mu\text{g/ml}$ of anti-*Fel d* I in 29 of 30 cell donors (fig. 3); overall, the maximum inhibition was $66 \pm 5\%$ (range: 15–100%) at approximately 1 $\mu\text{g/ml}$ of the antibody. Spontaneous HR was $2.1 \pm 0.25\%$ and did not exceed 7% (data not shown). The mediator response of 3 individuals could only be inhibited by 25%, in 7 subjects inhibition between 25 and 50% was obtained, in 5 cell donors between 50 and 75%, and in at least 15 cat-sensitized individuals inhibition by anti-*Fel d* I was between 75 and 100% (data not shown). The IC_{30} was found to be 0.41 $\mu\text{g/ml}$ of

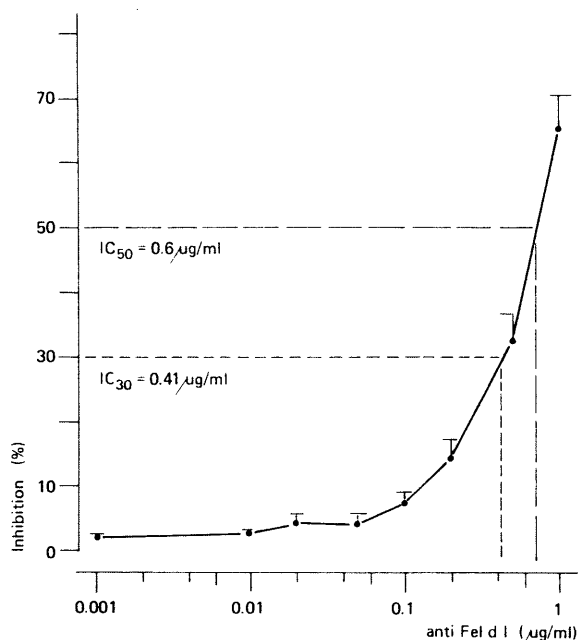


Fig. 3. Inhibition (means $\pm$ SEM, $n = 30$) of cat-induced leukocyte HR by anti-*Fel d I*. HR studies were performed after major allergen depletion of one cat extract dilution (10 SQ/ml) with different concentrations of the antibody (x-axis) binding to the major allergen of the extract (*Fel d I*).

anti-*Fel d I* (range: 0.05–0.9 $\mu\text{g/ml}$). A negative, close relationship was found between individual cellular sensitivity and the IC_{30} levels ($r_s = -0.53$, $p < 0.01$): in cases of weak cellular sensitivity (high Ag30 levels), only a small amount of anti-*Fel d I* was required for pronounced inhibition (fig. 4a). A similar relationship was reflected by a positive correlation between cell sensitivity and the individual maxima of inhibition ($r_s = 0.41$, $p < 0.05$). In cases in which large amounts of cat allergen were required for 30% HR, a more pronounced inhibition of HR by anti-*Fel d I* was detected (fig. 4b).

No inhibition occurred in three control experiments using a nonrelated rabbit anti-human IgG antibody at concentrations up to 1 $\mu\text{g/ml}$. At this concentration, anti-*Fel d I*-induced inhibition was found to be $74 \pm 23\%$, while the control antibody did not show inhibition ($-8 \pm 4.9\%$) of cat-allergen-induced histamine release (data not shown).

SDS PAGE/Western Blot

After separation from the allergen extract by SDS PAGE, 12 single bands were located and analyzed for their molecular weight using defined marker proteins: 10, 30, 35,

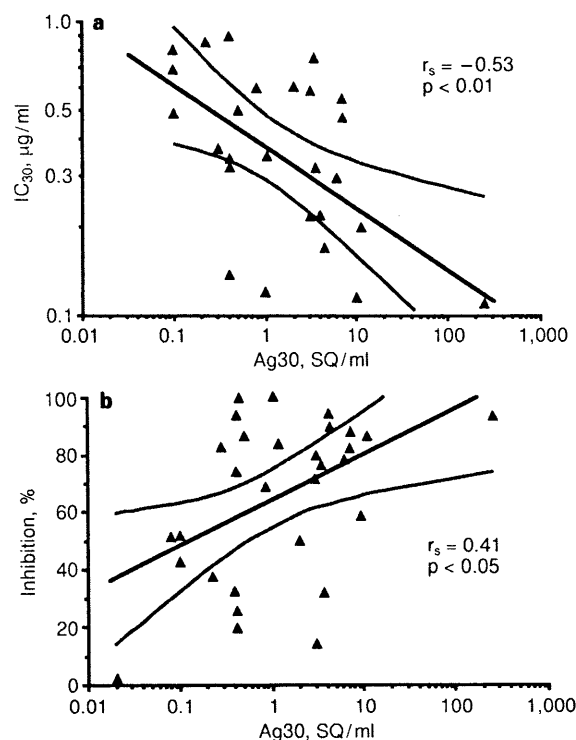
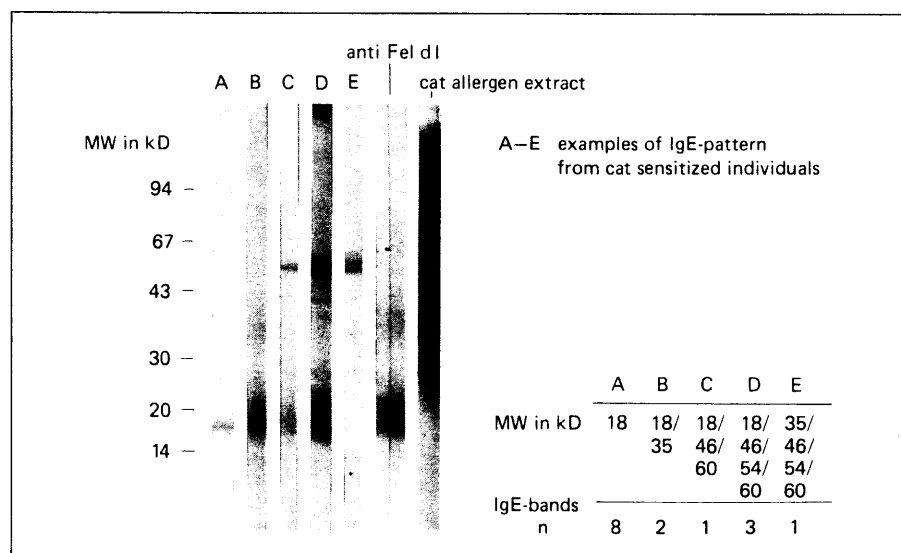


Fig. 4. Relationship between cellular sensitivity to mixed cat allergens (Ag30, x-axis) and the anti-*Fel d I* concentration to induce 30% inhibition of cat-allergen-induced HR (IC_{30} , y-axis; **a**) and the amount of inhibition at 1 $\mu\text{g/ml}$ of anti-*Fel d I* (maximum inhibition, y-axis; **b**). The regression lines and the 95% confidence bands for the true mean of y are shown. Correlations are given by Spearman's ρ (r_s).

40, 42, 46, 54, 58, 60, 65, 76, 98 kD (data not shown). Anti-*Fel d I* was found to bind at 18 ± 2 and 35 ± 2 kD (fig. 5). A negative control serum and 5 of 20 patient sera did not show any IgE binding (data not shown). The following IgE-binding bands of the cat allergen extract could be identified in 15 sera of patients with immediate type allergic reactions to cat allergens (fig. 5): 18 ($n = 8$), 18/35 ($n = 2$), 18/46/66 ($n = 1$), 18/46/54/66 ($n = 3$), 35, 46, 54 and 66 kD ($n = 1$). Thus, 14 of 15 patients exhibited IgE binding at 18 kD, while only 3 sera were found with IgE binding at 35 kD. A positive relation was found between the cat-allergen-specific serum IgE and the number of cat-allergen-specific IgE-binding bands ($r_s = 0.46$, $p < 0.05$, data not shown). Other attempts to correlate the individual IgE binding patterns with the level of sensitization (i.e. results in SPT, HR studies) or characteristics of inhibition by anti-*Fel d I* failed; no relationship between frequency or strength of IgE binding and the other diagnostic results could be determined.

Fig. 5. Immunoprint examples of cat-sensitized patients' sera after SDS-PAGE of a partially purified and standardized cat allergen extract. A–E represent examples of different IgE-binding patterns from 5 subjects. Additionally, the binding of anti-*Fel d I* and the individual proteins of the cat allergen extract are shown in the right lanes. The table in the lower right corner contains the approximated MW (kD) of the IgE-binding band(s) and their frequency of binding by sera of cat-sensitized subjects.



Discussion

SPT

An immediate type allergic sensitization could be demonstrated in 38 of 40 patients with cat-specific serum IgE who did not respond to saline but to the cat allergen in the titrated SPT. The high degree of concordant results between specific serum IgE and positive SPT to cat allergen might be a result of the same allergen source used for the preparation of RAST paper disks and the SPT extract (Allergen Laboratories, Sweden).

Anti-IgE and Cat-Induced HR

In this study, all cell donors were found to release significant amounts of histamine (>10%) after anti-IgE stimulation. The capability of secreting inflammatory mediators after this kind of immunological stimulation – the phenomenon of so-called ‘responding basophils’ – is usually only found in 80% of a group of unselected cell donors [20]. However, only 30 of 40 cat-sensitized individuals were found to release more than 30% histamine after antigen stimulation. Nevertheless, the similar amounts of HR found after stimulation by either anti-IgE or antigen reflect the basophil ‘intrinsic reactivity’ induced by the individual strength of signal transduction in the mediator-containing cells [21]. The rising portions of these histamine curves varied over a large range (approximately 4 log), which can be interpreted as a result of different quantities of cell-bound specific IgE, but also as a result of different patterns of specific sensitization to a variety of single allergens [13].

Inhibition of HR

The significant inhibition of the mediator response after absorbance by anti-*Fel d I* of the major allergen of the cat extract confirmed the specificity of the polyclonal antibody, which has been found useful in another investigation using CIE as a specific immunoassay for single cat allergens [22]. While the amount of the antibody was found to be quite similar in producing a defined inhibition (IC_{30} levels of 25 individuals were between 0.1 and 1.0 $\mu\text{g/ml}$), different maxima for the inhibition curves were obtained. Indirectly, the individual degree of inhibition reflects the differential relevance of *Fel d I* in triggering IgE-mediated HR based on the sensitization pattern of the patient. The highest concentration of 1 $\mu\text{g/ml}$ anti-*Fel d I* used for inhibition of HR stimulated with 10 SQ/ml of the cat extract (containing approximately 4.3 ng *Fel d I*) [15] reached nearly total inhibition (> 85%) only in 2 cases. In spite of the antibody excess, approximated from the concentrations used, the ratio between the amount of cat antigen and the anti-*Fel d I* antibody appeared suboptimal for complete S-shaped inhibition curves. This phenomenon might be explained by those antibody regions responsible for specific binding to *Fel d I* which represent only a small part of the antibody and its total weight. Unfortunately, the small amount of this antibody which was available did not allow experiments with higher concentrations than 1 $\mu\text{g/ml}$. Reduction of allergen dose used <10 SQ/ml, required for the maximum response, is another possible way to optimize the antigen-antibody ratio. However, significant mediator release induced by a cat allergen concentration below

10 SQ/ml will only be found in cases of extremely strong basophil sensitization, limiting this option.

In another study, no significant differences in the histamine levels obtained were found after triggering of leukocyte mediator release by either a cat allergen extract or the equivalent amount of the purified major allergen, *Fel d I*. Preincubation with an anti-*Fel d I* antibody produced a 300-fold shift in the cat-allergen-induced histamine curves, calculated by measuring the basophil sensitivity to cat allergens with and without previous antibody preincubation [23]. The significant relationship between cellular sensitivity to cat allergens and the inhibition induced by anti-*Fel d I* indicates that in cases of large amounts of cell-bound specific IgE minor allergens might play a significant role [5, 24, 25].

In addition with house dust mite allergens, the effect of major allergen (*Der p I*) depletion by monoclonal and monospecific antibodies on the reactivity in the HR assay has been used to evaluate the contribution of *Der p I* to the biologic activity of house dust mite extracts [26]. In this study, removal of the major allergen caused no significant change in the result of mite-induced HR, and the activity of the purified *Der p I* appeared to be lower than expected on the basis of *Der p I*-specific IgE response. In contrast to our experimental design, however, the investigators used only one fixed concentration of the antibodies to deplete their house dust mite allergen extract.

The primary importance of the major allergen, *Alt a I*, in patients sensitized to *Alternaria*, was emphasized by significant inhibition of leukocyte HR and RAST after *Alt a I* depletion using a monospecific antibody [27]. IgE binding to the major allergen shown by immunoblot experiments in all subjects confirmed the results of the HR experiments. Similar results were obtained in another study, comparing the biologic activity of the purified major allergen *Alt a I* and a crude *Alternaria* allergen extract in the HR assay, showing a good correlation between the Ag50 of both allergen sources [28].

SDS-PAGE/Western Blot

Five different locations of IgE binding could be demonstrated after electrophoretic separation of the cat allergen extract. The number of individual IgE binding bands was positively related to the specific serum IgE levels, but not to the specific sensitivity determined by SPT and HR. This might be explained by the larger variability of cell-bound IgE, which is not only related to the serum levels of specific IgE, but also to the number of Fc ϵ RI receptors on mediator-containing cells, like mast cells and basophils, and their occupation by allergen-specific IgE

antibodies [21]. Furthermore, specific IgE determinations and Western blot experiments were performed with identical serum samples, which might be another explanation for the positive correlation between these results.

A similar pattern of IgE binding was obtained in another study using SDS-PAGE and the same cat allergen extract [29]. Electrophoretic analysis of a cat allergen extract resulted in 9 different polypeptides with significant affinity for specific serum IgE from allergic donors [25]. One of these proteins with a MW of about 18 kD was found to bind specific IgE frequently with high affinity, indicating a major role among the numerous single cat allergens. We could not show any protein at 18 kD on the NC, which might be explained by our cat extract containing only small amounts of the major allergen, *Fel d I*. Nevertheless, the majority of the sera investigated by Western blot had diffuse, but strong IgE binding at 18 kD, confirming previous findings, since purified *Fel d I* had been shown at this molecular weight by SDS-PAGE [30]. Several authors described two active components as the major allergen at either 18 or 35 kD [6, 10, 11]; recent findings from two independent groups demonstrate that the larger molecule is a noncovalently linked dimer of the 18-kD protein [11, 13]. Protein sequencing of *Fel d I* from an immunoaffinity-purified extract of house dust revealed that the allergen is composed of two polypeptide chains [31]. This might explain the variable results after electrophoretic separation of the major allergen due to nonreducing or reducing conditions.

The IgE binding at 46 and 54 kD demonstrated in 4 sera might correspond to fragmented cat IgG, which has been shown to bind IgE after SDS-PAGE at a MW of 50 kD in a few cat-allergic subjects [32]. Cat albumin (approximately 66 kD), which has recently been purified and analyzed for its primary structure [33], might also belong to the allergens of minor importance [25, 32]. The role of other minor cat allergens characterized by different immunoelectrophoretic techniques [5] and found in cat extracts from different sources [8] still remains to be determined.

The binding pattern of anti-*Fel d I*, used in this study, was equally strong with both *Fel d I* molecules, the 18-kD monomer and the 35- to 39-kD dimer [11, 31, 34]. That the anti-*Fel d I* binding did not appear as a sharp band despite using different antibody concentrations and several allergen preparations [10] might reflect characteristics of the protein itself. However, the high frequency of IgE binding at 18 and/or 35 kD in all patients investigated underlines the predominant role of the major cat allergen in cases of allergic sensitization. This conclusion might be important for selecting the composition of commercial available al-

lergen extracts for diagnostic and therapeutic purposes. Despite our increasing knowledge regarding molecular characterization and environmental distribution of *Fel d I*, more investigation is necessary to define the role of *Fel d I* in cat-allergic individuals and its contribution to the biologic activity of cat allergen extracts.

Acknowledgement

We gratefully acknowledge Dr. Anne Kagey-Sobotka (Johns Hopkins Asthma and Allergy Center, Allergy and Clinical Immunology Division, Baltimore, Md., USA) for editorial assistance.

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