

Demonstration of a partially cryptic epitope of the major cat allergen Fel d 1: Consequences for mAb-based standardization of cat extracts

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Background: Antiallergen mAbs that do not recognize clinically important isoforms have been described, raising the question of the selection of mAbs for quantifying major allergens in order to standardize allergenic extracts. This question is even more critical if mAbs can discriminate between different forms of allergen molecules with the same amino acid sequence.

Objective: We sought to demonstrate that an anti-Fel d 1 mAb was able to discriminate between two forms of the major cat allergen independently of its amino acid sequence and to determine the relative importance and stability of both forms in various cat extracts.

Methods: Anti-Fel d 1 mAbs were raised in mice and characterized. By using two of these mAbs, a two-site ELISA was developed to quantify Fel d 1 in mass units.

Results: One of the anti-Fel d 1 mAbs developed was shown to specifically recognize a particular form of Fel d 1. A two-site ELISA with this mAb to capture Fel d 1 was able to quantify the allergen specifically in this form. It was then shown that (1) the quantitative importance of this form of Fel d 1 could vary from one cat extract to another, (2) Fel d 1 was converted into this form under certain conditions, and (3) both converted and unconverted forms of Fel d 1 may bear IgE epitopes that are specific.

Conclusion: Although the present study emphasizes the issue of selecting mAbs that are not too specific to standardize allergenic extracts, it also demonstrates that very specific mAbs can be of interest, especially to verify the stability of allergens in extracts, since this stability might have clinical implications. (*J Allergy Clin Immunol* 2000;106:669-76.)

Key words: Cat extract, Fel d 1, monoclonal antibody, ELISA, specificity, standardization

It is largely recognized that standardization of allergenic extracts is significantly improved by the quantification of major allergens,¹ which provides a better knowledge of the composition and potential efficacy of allergenic extracts for both diagnosis and treatment. Essentially two types of techniques are used to assay

Abbreviation used

EAST: Enzyme allergosorbent test

major allergens: (1) polyclonal antibody-based techniques that are performed in gel, such as radial immunodiffusion and rocket immunoelectrophoresis, and (2) mAb-based sandwich ELISA. The latter technique is increasingly used because mAbs are well-defined reagents with constant quality and virtually unlimited availability. However, their very high specificity is sometimes a limiting factor because various allergens are present in different isoforms, some of which are not recognized by related antiallergen mAbs, although they are able to bind IgE and must therefore be taken into account in standardization.¹ As an example, an isoform of the mite major allergen Der p 2 did not bind 5 anti-Der p 2 mAbs, only because it had an aspartic acid instead of an asparagin at position 114 of the amino acid sequence, although this difference did not significantly influence the binding of human IgE.²

Some mAbs might also discriminate between different forms of an allergen if they bind to an epitope on some forms that is cryptic (ie, inaccessible) on other forms, although all forms exhibit the same amino acid sequence. For example, an mAb has been shown to discriminate between the normal (PrP^C) and pathogenic (PrP^{Sc}) forms of the PrP prion protein.³ The present study describes an mAb that is capable of a similar discrimination between two forms of the cat allergen Fel d 1. This mAb can be used to specifically quantify the particular form of the allergen recognized when combined with another anti-Fel d 1 mAb in a two-site sandwich ELISA, thereby allowing determination of the relative importance, as well as the stability, of both forms in cat allergenic extracts. Information concerning the allergenicity of both forms of Fel d 1 was also obtained.

METHODS

Cat allergen sources and extracts

Cat hair and two samples of cat anal gland secretions were obtained from IFFA-CREDO (L'Arbresle, France). The first sample of anal gland secretions consisted of a pool obtained from 7 female

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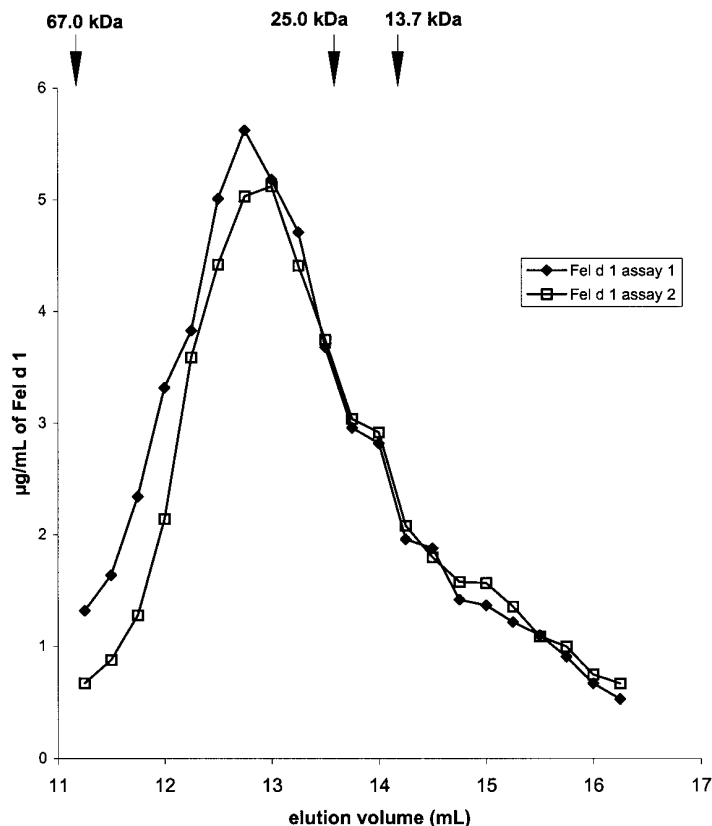


FIG 1. Size-exclusion chromatography of purified Fel d 1 (see the "Methods" section for details).

cats. The other sample was a pool obtained from 8 female and 2 male cats. A freeze-dried cat hair extract was obtained from Laboratorium Diephuis (Houten, The Netherlands). This extract, cat hair, and cat anal gland secretions were extracted by overnight incubation at 4°C with 50 mmol/L ammonium bicarbonate (pH 7.8) at extracting ratios of 20% (m/v), 10% (m/v), and 40% to 55% (m/v), respectively. After centrifugation, supernatants were collected and are hereafter called *concentrated cat extract*, *cat hair extract*, and *anal gland secretion extract*, respectively.

For stability studies, extracts were sterile filtered at 0.2 µm, sampled under sterile conditions, and incubated at 4°C. Samples were transferred at -20°C at defined intervals. After transfer of all samples corresponding to a given extract at -20°C, they were thawed and tested at the same time.

For size-exclusion analysis, the concentrated cat extract was extracted with 0.05 mol/L sodium phosphate buffer and 0.15 mol/L NaCl (pH 7.0) instead of ammonium bicarbonate buffer and then diluted 10 times in the same buffer.

Serum pool from subjects with cat allergy

Sera from 9 subjects with cat allergy were pooled. Sera were RAST class 3 or more (>3.5 kIU/L) to cat allergens. The final serum pool was class 4 (49 kIU/L) to cat allergens and negative for tree pollen, grass pollen, and molds (*Alternaria* species, *Aspergillus* species, *Cladosporium* species, and *Penicillium* species).

Purified Fel d 1

Purified Fel d 1 was provided by ASTER Biotechnologies (La Gaude, France). Briefly, a concentrated cat extract was dialyzed

overnight at 4°C against 0.02 mol/L TRIS and 0.15 mol/L NaCl (pH 7.2). It was then loaded onto a column containing Sepharose 4B gel (Pharmacia, Uppsala, Sweden) coupled through cyanogen bromide with anti-Fel d 1 mAb 1G9 (University of Virginia) and after equilibration of the gel with 0.02 mol/L TRIS and 0.15 mol/L NaCl (pH 7.2). Immobilized Fel d 1 was eluted with 0.5 mol/L glycine (pH 2.5) and neutralized with 3 mol/L TRIS (pH 8.0).

For size-exclusion chromatography, lyophilized purified Fel d 1 was reconstituted at 0.5 mg/mL by using 0.05 mol/L sodium phosphate buffer and 0.15 mol/L NaCl (pH 7.0).

Monoclonal antibodies

Monoclonal antibodies were obtained as follows. Three BALB/c mice were first immunized subcutaneously with 50 µg of purified Fel d 1 in complete Freund's adjuvant and then boosted 4 times intraperitoneally with 50 µg per injection of purified Fel d 1 in incomplete Freund's adjuvant. The mouse showing the highest anti-Fel d 1 serum antibody titer against purified Fel d 1, as measured by direct ELISA, was selected for hybridization. An intraperitoneal booster with 50 µg of purified Fel d 1 in incomplete Freund's adjuvant was administered 4 days before splenectomy. Hybridization was performed, as described by Köhler and Milstein.⁴ Antibody-producing hybrids were selected by an ELISA with adsorbed purified Fel d 1 and peroxidase-conjugated goat anti-mouse Ig. Cloning was performed by repeated limiting dilution. Ascitic fluid was obtained from Pristane-treated BALB/c mice and then purified on Sepharose protein A gel. Three stable clones producing anti-Fel d 1 antibodies were obtained and coded as 3G9, 7B6, and 1C12. They were biotinylated by using a biotinylation kit (Sigma, St Louis, Mo) according to the manufacturer's instructions.

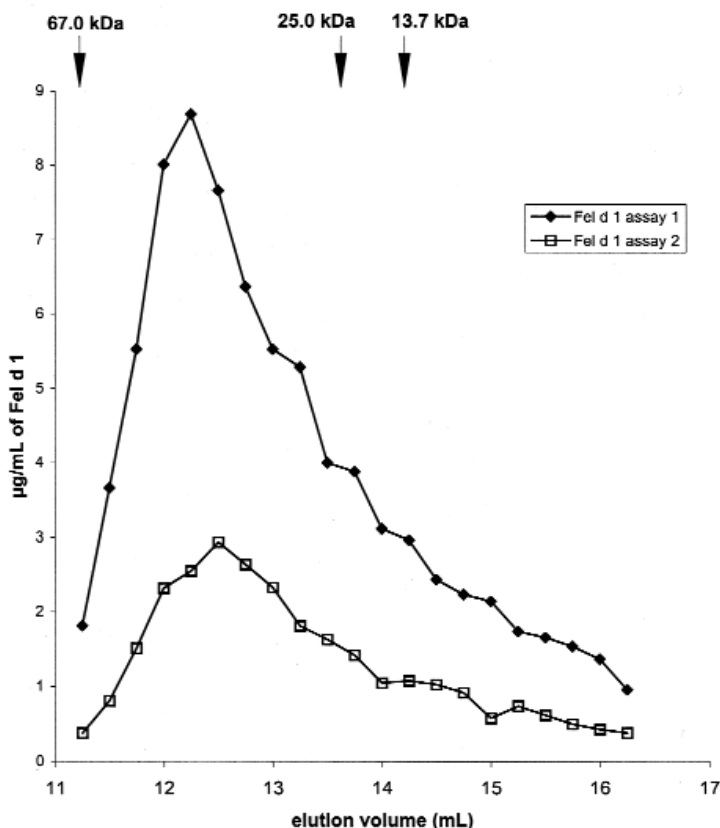


FIG 2. Size-exclusion chromatography of a concentrated cat extract (see the "Methods" section for details).

Epitope specificity of anti-Fel d 1 mAbs

The following ELISA experiment was carried out to determine whether any two mAbs bound different, identical, or overlapping epitopes of Fel d 1. Ninety-six-well microtiter plates (Nunc, Denmark) were coated with 10 µg/mL mAb 6F9 from the Fel d 1 ELISA kit purchased from Indoor Biotechnologies (Charlottesville, Va) in 0.1 mol/L sodium carbonate-bicarbonate buffer (pH 9.6) or 10 µg/mL mAbs 3G9 and 7B6 in PBS. After blocking with 0.1 mol/L potassium phosphate buffer (pH 7.4) containing 0.4 mol/L NaCl, 1 mmol/L EDTA, and 0.1% (m/v) BSA (blocking buffer) and washing, 1 µg/mL purified Fel d 1 in blocking buffer was added to the wells to saturate the available antibody-binding sites. Wells were incubated for 2 hours at 37°C and washed. Biotinylated 3E4 from the Fel d 1 ELISA kit from Indoor Biotechnologies (Charlottesville) and biotinylated 3G9 and 7B6 were then incubated at concentrations of 0.01 to 1.2 µg/mL in blocking buffer for 1 hour at 37°C. After a further wash cycle, biotinylated mAbs were detected by using streptavidin-biotinylated horseradish peroxidase (Amersham, UK). Peroxidase activity was detected by using hydrogen peroxide plus orthophenylenediamine dihydrochloride (bioMérieux, Marcy l'Etoile, France) blocked with 1.8 N H₂SO₄ and read at 492 nm with a reference at 620 nm (Titertek Multiskan reader, Flow Laboratories).

Size-exclusion chromatography

Samples were separated by size exclusion on a Superdex 75 HR 10/30 column (10 × 300-310 mm, Amersham Pharmacia Biotech) by using a Waters 650E protein purification system, with a Waters 600E system controller and a Waters 486E detector (Millipore). The

column was calibrated with albumin (molecular mass = 67.0 kd), chymotrypsinogen A (molecular mass = 25.0 kd), and ribonuclease A (molecular mass = 13.7 kd, Amersham Pharmacia Biotech). Samples were isocratically analyzed by injecting 100 µL onto the column and eluting with 0.05 mol/L sodium phosphate buffer and 0.15 mol/L NaCl (pH 7.0) at a flow rate of 0.5 mL/min (132 psi). Fractions of 0.25 mL were collected and assayed with Fel d 1 assays 1 and 2, as described below.

Fel d 1 assays

Fel d 1 assay 1 was performed by using the commercial kit from Indoor Biotechnologies (Charlottesville), according to the manufacturer's instructions. A Fel d 1 immunoassay, hereafter referred to as Fel d 1 assay 2, was developed by using mAbs 3G9 and 7B6 to Fel d 1. Briefly, 96-well microtiter plates (Nunc) were coated with 10 µg/mL mAb 3G9 in PBS. After a 1-hour incubation with blocking buffer (see above) and washes, 2-fold dilutions of purified Fel d 1 or samples to be tested at given dilutions in blocking buffer were added to the wells, and the plates were then incubated for 2 hours at 37°C. After washes, biotinylated 7B6 was incubated for 1 hour at 37°C and, after a final wash, detected by using streptavidin-biotinylated horseradish peroxidase (Amersham). Peroxidase activity was detected, blocked, and read, as described above.

Competition between mAb 3G9 and IgE from patients with cat allergy

Ninety-six-well microtiter plates (Nunc) were coated with 1 µg/mL purified Fel d 1 in PBS. After incubation with blocking

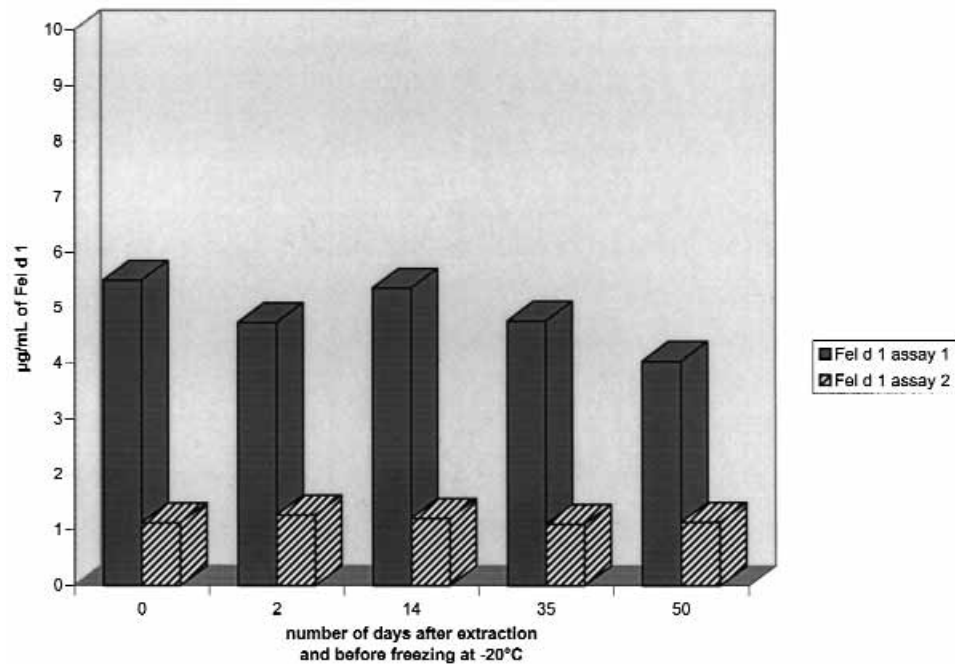


FIG 3. Fel d 1 concentration in a cat hair extract incubated at 4°C, as measured with Fel d 1 assays 1 and 2.

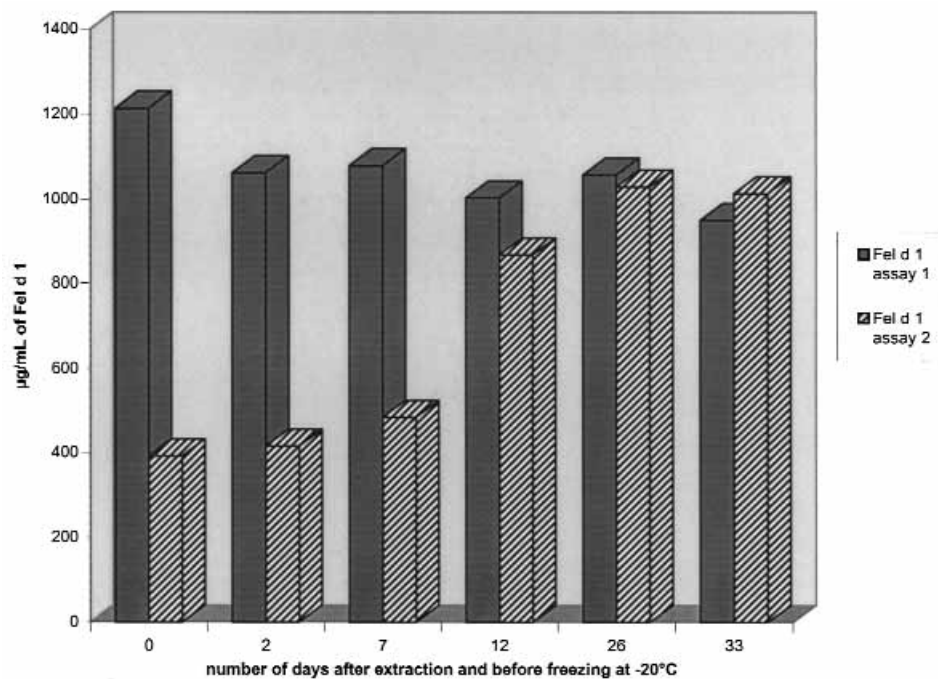


FIG 4. Fel d 1 concentration in a concentrated cat extract incubated at 4°C, as measured with Fel d 1 assays 1 and 2.

buffer (see above) and washing, one volume of the serum pool from patients with cat allergy diluted to 1:10 in blocking buffer and one volume of mAb 3G9 at given concentrations were added to the wells, and the plates were incubated for 3 hours at 37°C. After washing, a peroxidase-linked mouse monoclonal anti-human IgE (bioMérieux) was incubated for 1 hour at 37°C. After a final wash, peroxidase activity was detected, blocked, and read, as described above.

Enzyme allergosorbent test inhibition

Enzyme allergosorbent test (EAST) inhibition experiments were performed by using the VIDAS automat (bioMérieux), in which compartments were coated with cat hair extract. For the experiments, various dilutions of concentrated cat extract stored at either 4°C or -20°C were coincubated in the compartments with the serum pool from patients with cat allergy at a 1:5 dilution.

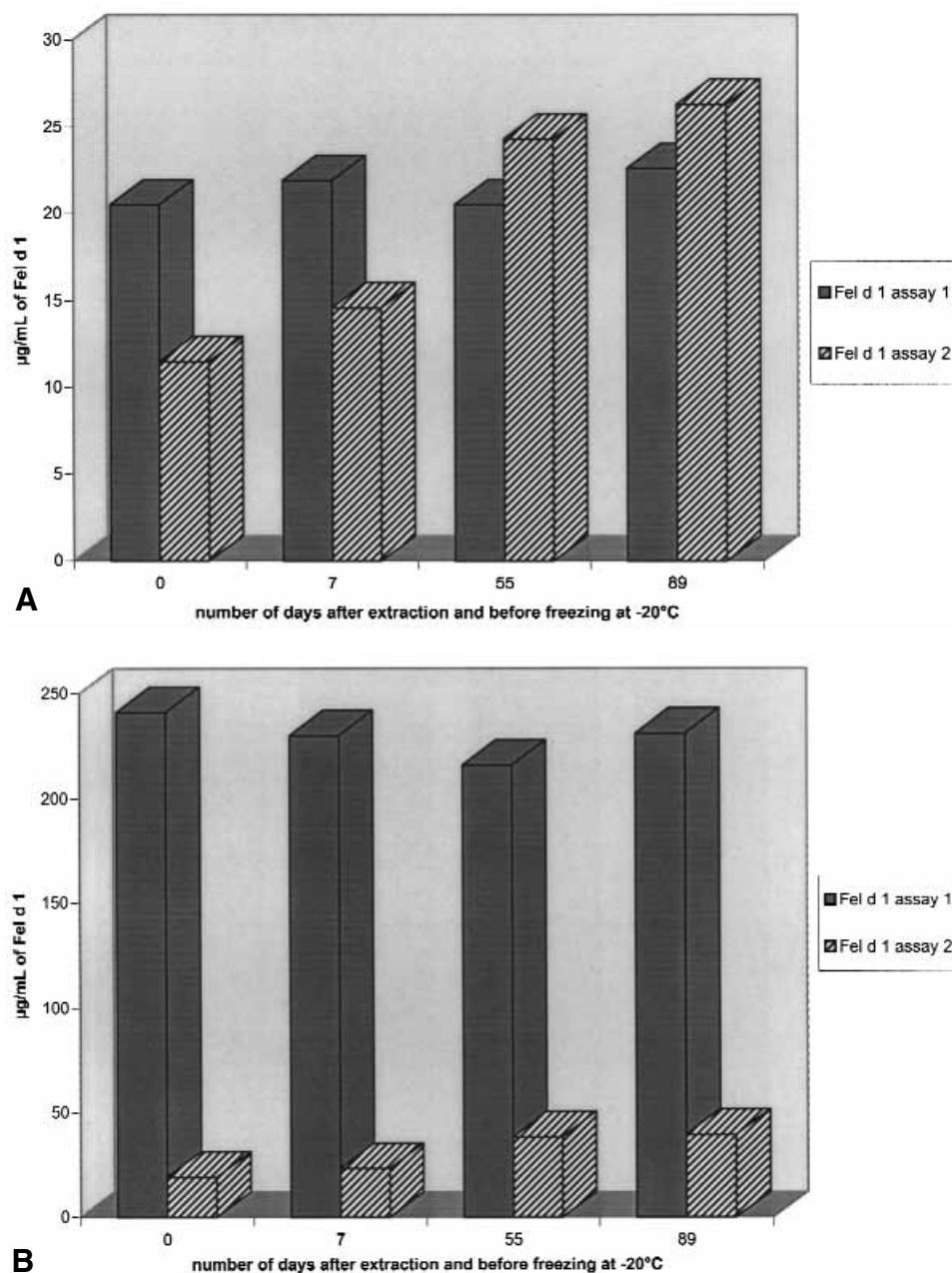


FIG 5. Fel d 1 concentrations in two samples (**A** and **B**) of anal gland secretion extract, as measured with Fel d 1 assays 1 and 2.

RESULTS

Epitope specificity of the mAbs

Among the mAbs obtained from a mouse immunized with purified Fel d 1, two were studied more specifically, namely mAbs 3G9 and 7B6. Both mAbs were able to form a sandwich with purified Fel d 1, either when combined together or when combined with immobilized mAb 6F9 from the Fel d 1 ELISA kit from Indoor Biotechnologies (Table I). Therefore these 3 mAbs defined 3 different epitopes on the Fel d 1 molecule.

TABLE I. Binding combination of various anti-Fel d 1 mAbs in a "sandwich" ELISA*

Immobilized mAb	Biotinylated mAb		
	3G9	7B6	3E4
3G9	–	+	–
7B6	–	–	–
6F9	+	+	+

*Binding of biotinylated mAb (1–120 ng/well) Fel d 1 (100 ng/well) presented by immobilized mAb (1 µg/well) was expressed as the percentage of the response obtained for the pair which gave maximal absorbance for the given biotinylated mAb. The + sign indicates that the binding was higher than 75%, and the – sign indicates that the binding was lower than 25%.

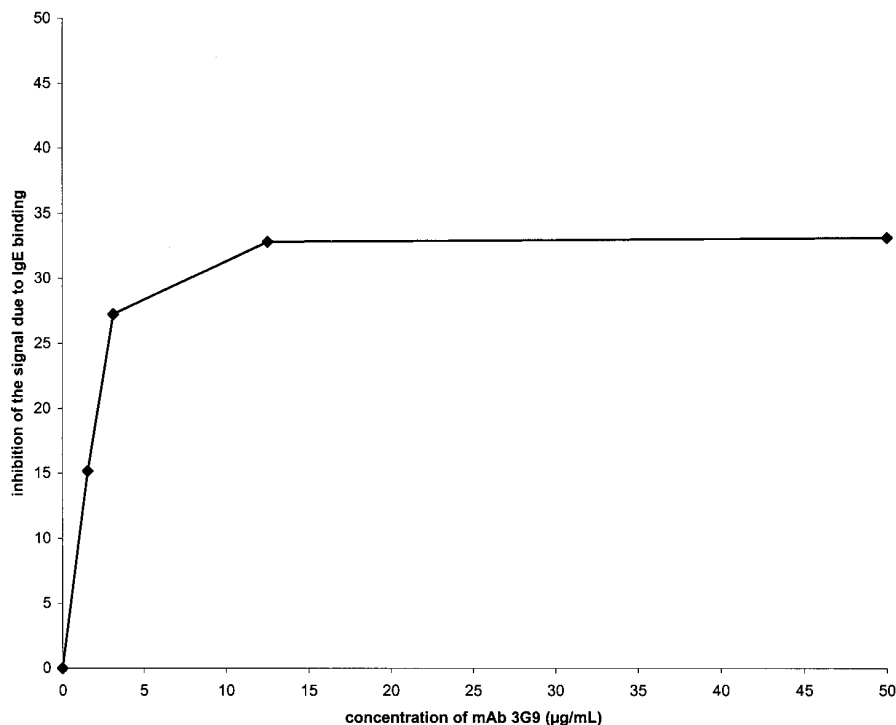


FIG 6. Inhibition of IgE binding to immobilized Fel d 1 by mAb 3G9.

Fel d 1 concentrations in fractions of size-exclusion chromatography and in cat allergen extracts

In size-exclusion experiments, the measured Fel d 1 concentration peaked in fractions corresponding to a molecular mass of approximately 40 kd for both Fel d 1 assays 1 and 2 (Figs 1 and 2).

As shown in Fig 3, Fel d 1 concentrations measured by either Fel d 1 assay 1 or Fel d 1 assay 2 remained stable over time in a cat hair extract stored at 4°C. This extract contained 4.9 µg/mL Fel d 1, as measured by using Fel d 1 assay 1, only a quarter of which (1.2 µg/mL) was measured by using Fel d 1 assay 2.

In a concentrated cat extract incubated at 4°C, although the Fel d 1 concentration measured by Fel d 1 assay 1 did not significantly change, it increased with time when measured with the Fel d 1 assay 2. It was one third of the value given by the Fel d 1 assay 1 on day 0 and finally reached almost 100% of that value (Fig 4). When testing anal gland secretion extracts in the same conditions, a marked (Fig 5, A) and slight increase (Fig 5, B) was observed with Fel d 1 assay 2, but no change was observed with Fel d 1 assay 1. The ratio between the two measured Fel d 1 concentrations in these extracts was 1:2 and 1:13, respectively, on day 0.

Inhibition of IgE binding to immobilized Fel d 1 by mAb 3G9 and EAST inhibition

As shown in Fig 6, mAb 3G9 competed with IgE from patients with cat allergy for binding to Fel d 1.

The concentrated cat extract stored at -20°C induced 100% EAST inhibition, whereas that incubated at 4°C failed to induce 100% EAST inhibition (Fig 7).

DISCUSSION

Both mAbs 3G9 and 7B6 were specific for Fel d 1 because each mAb bound to Fel d 1 presented by immobilized mAb 6F9 (Table I), which was previously characterized as specific for the major cat allergen.⁵ This binding cannot be due to recognition of a molecule that complexes with the presented Fel d 1 because, when combined in a two-site sandwich ELISA (Fel d 1 assay 2), mAbs 3G9 and 7B6 bound to a molecule with a similar molecular mass to the molecule bound by anti-Fel d 1 mAbs 6F9 and 3E4 (Fel d 1 assay 1; ie, a molecule of approximately 40 kd [Figs 1 and 2], the molecular mass previously obtained for Fel d 1 by using size-exclusion chromatography).^{6,7}

The fact that the Fel d 1 concentration measured with Fel d 1 assay 2 in a cat hair extract stored at 4°C was only a quarter of the concentration measured by Fel d 1 assay 1 cannot be due to a difference in the standard because the purified Fel d 1 concentration was verified by Fel d 1 assay 1 and its own standard before it was used as a standard for Fel d 1 assay 2. In fact, both assays gave similar concentrations in fractions obtained from size exclusion of purified Fel d 1 (Fig 1). Fel d 1 assay 2 was therefore able to measure only part of the Fel d 1 contained in the cat hair extract stored at 4°C, indicating that it discriminated between two forms of the allergenic molecule.

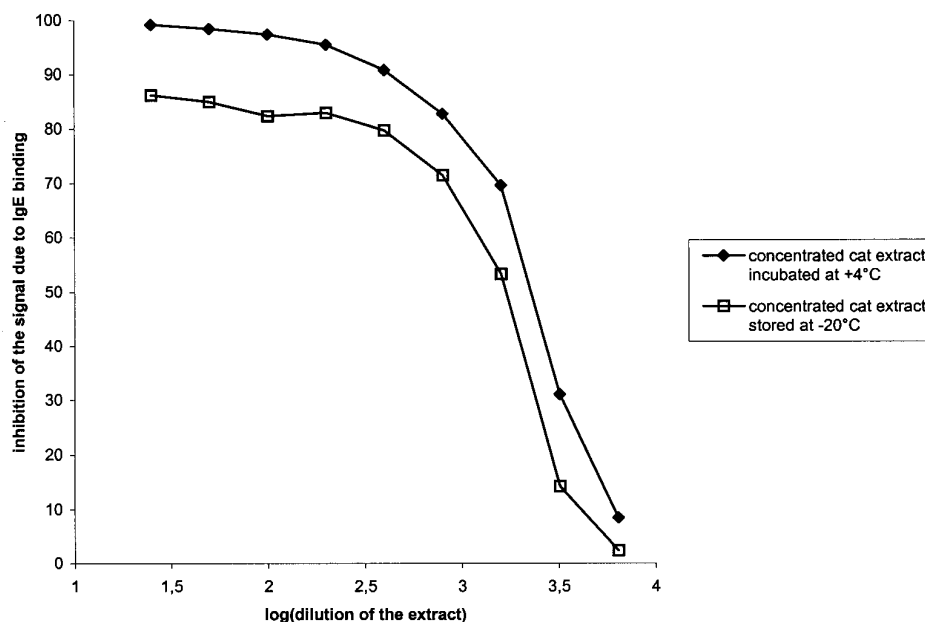


FIG 7. Inhibition of binding of IgE from subjects with cat allergy to immobilized cat hair extract obtained with concentrated cat extracts either stored at -20°C or incubated at 4°C .

The fact that the Fel d 1 concentration measured by Fel d 1 assay 2 in concentrated cat extract stored at 4°C increased with time from one third up to 100% of the value given by Fel d 1 assay 1 is not attributable to a lack of reproducibility of Fel d 1 assay 2 because all samples were assayed at the same time. Moreover, when testing anal gland secretion extracts under the same conditions, time-related increases of Fel d 1 concentration were again observed with Fel d 1 assay 2, whereas Fel d 1 assay 1 still did not show any change (Fig 5). Finally, when a cat hair extract was incubated for several days at 45°C , the Fel d 1 levels measured by Fel d 1 assay 2 increased up to 100% of the value given by Fel d 1 assay 1 (results not shown).

In Fel d 1 assay 2 only mAb 3G9 and not mAb 7B6 was able to discriminate between the two forms of Fel d 1. Indeed, when mAb 7B6 was combined with mAb 6F9 in a sandwich ELISA, no change in measured concentration of Fel d 1 was observed with time in a concentrated cat extract stored at 4°C , whereas an increase was still observed when mAb 3G9 was combined with biotinylated anti-Fel d 1 mAb 1C12 (obtained from the same mouse as mAbs 3G9 and 7B6), which itself did not detect any increase when combined with mAb 6F9 (results not shown).

Overall, these results indicate that mAb 3G9 binds to a partially cryptic epitope; that is, an epitope that, in various cat extracts, is already accessible on some of the Fel d 1 molecules and potentially accessible on all molecules. This partially cryptic epitope cannot be isoform specific because if this were the case, the time-related change in binding of mAb 3G9 would indicate a spontaneous

change in the Fel d 1 amino acid sequence. The authors first hypothesized that appearance of a new epitope on Fel d 1 was due to splitting of Fel d 1 dimers (approximately 40 kd) into monomers (approximately 20 kd) and that mAb 3G9 bound to an epitope on the monomers involved in the binding into dimers that would then be hidden in the dimer. But size-exclusion experiments indicated that Fel d 1 assay 2 detected only approximately 40-kd dimers in tested extracts (Fig 2). This also indicates that the appearance of a new epitope is not caused by Fel d 1 aggregation. However, the mechanism of Fel d 1 conversion from one form to another remains to be elucidated. It is likely that the carbohydrates that compose 20% of the Fel d 1 molecules are involved because they probably play a role in many biologic interactions.⁶ It is possible that Fel d 1 assay 2 detects a denatured form of Fel d 1, as has been shown previously by van Milligen et al⁸ with anti-Fel d 1 mAbs. However, it should be noted that Fel d 1 assay 2 was able to measure Fel d 1 in a cat hair extract, which is a crude extract, and Fel d 1 assay 2 detected increasing amounts of Fel d 1 in a concentrated cat extract stored at the gentle temperature of 4°C .

Conversion of Fel d 1 did not occur at 4°C in a cat hair extract with a Fel d 1 concentration close to $5\text{ }\mu\text{g/mL}$ but occurred in a concentrated cat extract containing more than $1000\text{ }\mu\text{g/mL}$ Fel d 1. It might then be concluded that this conversion is facilitated in the presence of a high concentration of Fel d 1. However, only very slight conversion was observed when an anal gland secretion extract with a high Fel d 1 concentration ($250\text{ }\mu\text{g/mL}$) was incubated at 4°C . Moreover, under the same conditions, complete conversion was observed in another anal

gland secretion extract, despite a more than 10-fold lower Fel d 1 concentration (Fig 5). The mean Fel d 1 concentrations measured by Fel d 1 assay 1 in these two extracts (60 ng/mg and 410 ng/mg) were in the range found by Dornelas de Andrade et al⁹ in similar extracts and using the same assay (ie, approximately 10-1360 ng/mg). This represents a very wide range. It is noteworthy that the concentrations of converted Fel d 1 in the two anal gland secretion extracts assayed in the present study were situated in a much narrower range (ie, 32-34 ng/mg on day 0 and 70-71 ng/mg after 89 days of incubation at 4°C).

Results obtained in the present study confirm that the selection of mAbs to construct assays designed to standardize allergenic extracts constitutes a critical step. Because of their very high specificity, not only can some antiallergen mAbs be nonspecific for all important isoforms, but they might also discriminate between two molecules with the same amino acid sequence. However, very specific tools, such as mAb 3G9, may be of interest because they provide information about stability of certain forms of allergenic molecules. This would only be truly relevant if it had clinical implications. In this respect mAb 3G9 inhibited the binding of IgE from patients with cat allergy to Fel d 1 (Fig 6). These IgEs probably recognize the same epitope as mAb 3G9 or overlapping epitopes. However, it cannot be excluded that inhibition was due to a conformational change of Fel d 1 induced by the binding of mAb 3G9 because most of the IgE epitopes of Fel d 1 are probably conformational. In any case the gain in 3G9 reactivity is not reflected in a gain in IgE reactivity. In this respect an EAST-inhibition assay suggested that some IgE epitopes disappeared after conversion of Fel d 1 because a concentrated cat extract incubated at 4°C and containing a high level of

converted Fel d 1 (Fig 4) failed to inhibit 100% of IgE binding to an immobilized cat hair extract (Fig 7). In contrast, when the same extract was stored at -20°C, it induced 100% inhibition. These results indicate that the two forms of Fel d 1 demonstrated in the present study might behave differently in a clinical point of view. In this case it would be of importance to quantify these forms specifically, as made possible for one of them by using anti-Fel d 1 mAb 3G9.

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