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Structure of the Major Cat Allergen *Fel d I* in Different Allergen Sources: An Immunoblotting Analysis with Monoclonal Antibodies against Denatured *Fel d I* and Human IgE

Key Words

Cat allergen
Fel d I
Monoclonal antibodies
IgE

Abstract

In this paper we show the reactivity of monoclonal antibodies (mAbs) and human IgE with *Fel d I* from different allergen sources in reduced SDS-PAGE immunoblots. By SDS-PAGE analysis of affinity-purified ^{125}I -*Fel d I*, a 14- to 20-kD band was found, which dissociated under reducing conditions into a 4- to 5-kD chain (chain 1) and a 11- to 15-kD chain (chain 2). In initial immunoblotting experiments with mAbs against *Fel d I* however, only chain 1 was detected, while the mAbs lost activity upon reduction of *Fel d I*. Therefore mAbs were raised against reduced and alkylated *Fel d I*. Two of the four mAbs to 'denatured' *Fel d I* that were obtained did react with chain 2 on an immunoblot under reducing conditions; the other two reacted with chain 1. The mAbs did not react with native *Fel d I*. With these mAbs and human IgE, differences between allergen source materials in blot patterns of *Fel d I* were detected. A variable molecular weight for the protein stained with mAb antichain 2 was found, and occasionally the presence of a 12-kD band stained with mAb antichain 1. Human IgE strongly bound to chain 1 of *Fel d I*, while only 2 out of 6 sera gave a strong reaction with chain 2. The additional 12-kD band was also recognized by human IgE. In a competitive radioimmunoassay with mAb antichain 1, differences in levels of 'denatured' *Fel d I* between commercial extracts were quantitated. In vitro 'denatured' *Fel d I* was generated under high pH conditions. The reactivity of human IgE with this 'denatured' *Fel d I* was demonstrated in indirect RAST experiments with mAb antichain 1. We conclude that mAb antichain 1 recognizes a form of *Fel d I* that is not detected by mAb antichain 2, but does react with human IgE.

Introduction

Cats are an important source of indoor allergens. Sensitization to cat allergens is an important cause of asthma and/or rhinitis; 20–30% of the asthmatic patients respond with immediate hypersensitivity upon skin prick tests with cat allergens [1, 2]. A large part of the IgE antibodies in patients allergic to cats is directed against the cat allergen *Fel d I* (*Felis domesticus* allergen I) [3, 4]. Sources of *Fel d I* are cat saliva [5–8] and cat pelt, in which the presence of *Fel d I* is probably caused by production of *Fel d I* by cat skin [6, 7] and by deposition of the allergen during grooming. Recently, we found lacrimal fluid to contain high concentration of *Fel d I*, whereas cat milk contained only little allergen [9].

Fel d I is a relatively stable molecule. Only a modest reduction in *Fel d I* activity is found after heating [10, 15], treatment at pH 12.0 or with 6 M guanidine [15]. Reduction and alkylation (R & A) of *Fel d I*, however, causes a > 80% loss of its antigenic and allergenic activity [10, 15, 16].

Fel d I consists of an acidic protein dimer, with three isoallergenic forms in the pH range 3.5–4.1 [10]. There is no agreement about the molecular weight (MW) of *Fel d I* under physiological conditions. Values reported vary from 35 to 55 kD [10–13]. On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), affinity-purified *Fel d I* from cat dander [10] and house dust [12] has a MW of approximately 17 kD, suggesting that *Fel d I* is a non-covalently linked homodimer. Chapman et al. [12] reported three faint bands at 11–14 kD for *Fel d I* in addition to the major band of 17 kD. Using immunoblotting techniques, Duffort et al. [14] found a MW of 10–20 kD for *Fel d I* from cat dander extract. Under reducing SDS-PAGE conditions, Chapman et al. [15] noticed that the 17-kD band of *Fel d I* may dissociate into a heavily stained band at 5–6 kD. By gel filtration, Stokes and Turner [13] found that reduced and alkylated (R & A) *Fel d I* was eluted with an apparent MW of 6–15 kD (55 and 27 kD under nonreducing conditions). Recently, Duffort et al. [16] showed that *Fel d I* (affinity purified from cat dander extract with mAb C5/8) is composed of two separate chains with apparent MWs of 4 and 14 kD.

In this immunoblotting study, we compared the reactivity of human IgE and mAbs towards *Fel d I* in commercial cat extracts. Because the mAbs described by ourselves as well as those described by others [12, 14, 17, 18] have a restricted specificity, there is no indication that these mAbs recognize chain 2 on reduced SDS-PAGE immunoblots and these mAbs loose reactivity with *Fel d I* after reduction in radioimmunoassays (RIA) [14, 15], mAbs were

raised against reduced *Fel d I*. In immunoblotting experiments, we demonstrated that in various allergen sources, apart from chain 2 and chain 1, subunits are present that are recognized by these mAbs and by human IgE. As allergen sources, different commercial cat extracts, house dust and cat saliva were used. To ensure that every protein band recognized by human IgE was indeed *Fel d I*, an immunoprecipitation with mAb Fd1a was performed, prior to SDS-PAGE. In a competitive RIA with mAb antidenatured *Fel d I*, we also demonstrated the presence of varying concentrations of 'denatured' *Fel d I* in different cat extracts. Moreover, with this immunoassay generation of denatured *Fel d I* under high pH conditions was detected. In indirect RAST experiments with mAb antidenatured *Fel d I*, the reactivity of patient IgE with this denatured *Fel d I* was studied.

Materials and Methods

Affinity Purification of *Fel d I*

Fel d I was isolated from house dust by affinity purification with mAb Fd1a [18]. House dust extract, 200 mg/ml (w/v), containing 6,800 U of *Fel d I* in 400 ml as determined by a polyclonal RIA, was added to a 20-ml phenyl-Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) in 1 M (NH₄)₂SO₄. After washing with 200 ml (NH₄)₂SO₄, the column was eluted with H₂O and the eluate, containing 5,715 U of *Fel d I*, was applied onto monoclonal anti-*Fel d I* coupled to Sepharose 4B (volume of the column: 40 ml; 360 mg of mAb coupled to 13 g of Sepharose). The column was washed with PBS and subsequently eluted with 0.5 M glycine-HCl, pH 2.5. Fractions obtained were immediately neutralized with 0.1 M NH₄ acetate, pH 7.0, and dialyzed against H₂O. Finally the eluate (2,398 U *Fel d I*) was lyophilized, reconstituted in 2 ml of H₂O and stored at –20 °C.

Allergen Sources

Cat dander extract 1 was a 10% (w/v) extract, obtained from HAL (Haarlems Allergen Laboratorium, Haarlem, The Netherlands), charge 92242; it contained 160 U/ml of *Fel d I*; cat dander extract 2 was a 10% (w/v) extract, obtained from ARTU Biologicals NV (Lelystad, The Netherlands), charge 88E06/06-001; it contained 50 U/ml of *Fel d I*; cat dander extract 3 was obtained from ALK (Allergologisk Laboratorium Copenhagen, Copenhagen, Denmark), batch No. 8005/90705202; it contained 80 U/ml of *Fel d I*; cat dander extract 4 was a gift from Dr. M. Lombardero (Alergia e Immunologia Abello, Santa Leonor, Madrid, Spain) and contained 9 U/mg of *Fel d I*.

To obtain saliva, cats were anesthetized with 0.6 ml aescoket (100 mg/ml ketamine HCl; Harlan CPB, Austerlitz, The Netherlands) intramuscularly. To obtain a clear fluid, saliva was centrifuged through a 3-cm layer of G-25 Sephadex (Pharmacia). The *Fel d I* concentration was 3 U/ml. Subsequently, saliva was concentrated 3-fold by a Centricon 10 microconcentrator (Amicon, Division of W.R. Grace, Danvers, Mass., USA).

Reduction of Fel d I and Subsequent Alkylation or Renaturation

To immunize BALB/c mice with R & A *Fel d I*, *Fel d I* was reduced and alkylated as follows: 1 vol of affinity purified *Fel d I* (1–4 mg/ml) was incubated with 1 vol of 1.1 M Tris-HCl (pH 8.2) – 6 M guanidine – 200 mM dithiothreitol (DTT; Bio-Rad Laboratories, Richmond, Calif., USA) at room temperature. After 2 h 2 vol of 250 mM iodoacetamide (Aldrich-Chemie, Steinheim, FRG) were added and alkylation was performed during 2 h at 0 °C in the dark. Subsequently, the sample was dialyzed overnight against PBS. *Fel d I* thus treated is referred to as R & A *Fel d I*.

For screening the supernatants of hybridomas, a different alkylating reagent, N-ethylmaleimide (NEM; BDH Chemicals, Poole, UK), was used, to prevent selection of antibodies specific for the alkylating agent.

For renaturation of reduced *Fel d I*, 1 vol of reduced *Fel d I* was incubated during 30 min with 1 vol of 400 mM $K_3Fe(CN)_6$ or PBS and subsequently dialyzed against PBS. Alternatively reduced *Fel d I* was only dialyzed against PBS.

Production of mAbs against Denatured Fel d I

Five BALB/c mice were immunized with an intraperitoneal injection of 30 µg of R & A *Fel d I* in Freund's complete adjuvant. The mice were boosted 3 times with 30 µg of R & A *Fel d I* in Freund's incomplete adjuvant at intervals of 4 weeks. Sera of all mice elicited a positive response to R & A *Fel d I* when tested with goat anti-mouse IgG coupled to Sepharose and ^{125}I -labeled R & A *Fel d I*. To the mouse with the highest antibody titer, an intravenous boost consisting of the same amount of allergen was administered. After 3 days, the spleen of this mouse was removed, and spleen cells were fused with myeloma SP₂/0 cells. Hybridoma supernatants were screened by a radioimmunosorbent test procedure with reduced and NEM-alkylated *Fel d I* coupled to Sepharose and ^{125}I -labeled goat anti-mouse IgG. Antibody-producing hybrids were cloned by limiting dilution and cultured in 500-ml roller bottles in Iscove's modified Dulbecco's medium (Gibco, Paisley, UK), containing fetal calf serum (5% v/v; Sera-Lab, UK), hybridoma growing factor (HGF/IL-6; CLB, Amsterdam, The Netherlands) at a concentration of 80 U/ml, 50 µM 2-mercaptoethanol, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The culture supernatant was precipitated with 50% ammonium sulfate, dissolved in 40 ml of PBS (pH 7.4) and extensively dialyzed against PBS. The total protein yield was 0.17–0.28 mg/ml culture supernatant. The obtained mAbs were coupled to CNBr-activated Sepharose 4B (3 mg of a 50% SAS fraction to 100 mg of Sepharose) for mutual competition experiments.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed according to the procedure of Laemmli [19] with 15% (w/v) polyacrylamide slab gels (12.5 by 14.5 cm). For the characterization of the mAbs directed against denatured *Fel d I*, 35 U of affinity-purified *Fel d I* were added to a gel under reducing conditions or after R & A. For the characterization of *Fel d I* in different allergen sources, 30 µl of allergen sample were applied to the gel under nonreducing conditions. Cat dander extracts 1 and 2 were first dialyzed overnight against PBS to remove phenol, prior to being applied to a gel. Each sample contained about 50 U/ml of *Fel d I*, with the exception of cat saliva which contained only 9 U/ml of *Fel d I*. Before application, each sample was mixed with an equal volume of sample mix [125 mM Tris-HCl, pH 6.8, containing 6 M ureum, 20% (w/v) glycerol, 4% (w/v) SDS and 0.05% (w/v) bromophenol blue]. For reducing conditions, 40 mM DTT (final concentra-

tion) were added. After an incubation for 2 min at 100 °C, another 20 mM DTT were added to the reduced samples, and all samples were applied to the gel.

After electrophoresis at 30 mA for 4 h, separated protein bands were electrophoretically transferred to a nitrocellulose sheet (45 µm; Schleicher & Schuell, Dassel, FRG) by the Western blotting technique according to Burnette [20], with a Consort E 554 power supply (16 h, 250 mA). The blots were saturated for 2 h in 100 ml of PBS, containing 10 mM EDTA, 0.1% (w/v) Tween-20, 0.45% (w/v) BSA and 0.05% (w/v) NaN_3 . Blots of affinity-purified *Fel d I* were cut into 0.7-cm strips and incubated with mAb Fd1a ascites (0.35 µg/ml), mouse immune serum (0.02% v/v), preimmune serum (0.16% v/v) and mAbs antidenatured *Fel d I* (200 µl of unfractionated hybridoma supernatant). Blots of different *Fel d I* sources were incubated with mAb Fd1a (2.3 µg/ml), mAb FdR-2b (3.4 µg/ml) and mAb FdR-1a (1.9 µg/ml). As second antibody ^{125}I -goat anti-mouse IgG was used (50,000 cpm per strip or 250,000 cpm per blot). Both incubations (first and second antibody) were performed overnight in 4 ml of PBS, containing 10 mM EDTA, 0.1% Tween-20, 0.3% BSA and 0.05% NaN_3 . Autoradiography was performed by exposing immunoblots to X-ray films (Kodak X-Omat S, Eastman Kodak, Rochester, N.Y., USA) at –70 °C with an intensifying screen.

Molecular weights of *Fel d I* were determined by a MW-SDS-17 kit (Sigma, St. Louis, Mo., USA; with the corrected MW for the fastest moving band) or by a prestained low MW marker from BRL (Bethesda Research Laboratories, Life Technologies, Breda, The Netherlands).

Immunoprecipitation of Fel d I from Different Extracts, SDS-PAGE and Immunoblotting

150 µl of cat dander extract 1, 300 µl of cat dander extract 3, 20 U of affinity-purified *Fel d I* and 1 ml of cat saliva were incubated overnight with 0.4 ml of packed mAb Fd1a-Sepharose. After washing, the Sepharose was suspended in 375 µl of H₂O, and 375 µl of sample mix, containing 80 mM DTT, were added. After heating of the samples for 2 min at 100 °C, another 20 mM DTT (final concentration) were added. Subsequently, the Sepharose was removed by centrifugation (5 min at 12,000 g), and the supernatant was applied to a 15% (w/v) polyacrylamide gel. For each sample, half of a gel was used. After electrophoresis, proteins were transferred to a nitrocellulose sheet, which was saturated for 2 h. Of each blot, 0.5-cm nitrocellulose strips were cut, which were incubated with mAb Fd1a (0.6 µg/ml), mAb FdR-1a (0.25 µg/ml), mAb FdR-2b (2.2 µg/ml) and 200 µl of serum of 6 cat allergic patients respectively. As second antibodies, ^{125}I -labeled goat anti-mouse IgG and ^{125}I -labeled sheep anti-human IgE were used. Negative control strips were only incubated with the second antibodies. The procedure was performed as described above.

Analysis of Affinity Purified ^{125}I -Labeled Fel d I

Fifty microliters of ^{125}I -*Fel d I* (4.63×10^5 Bq) were incubated during 4 h with 50 µl of a 1:1,000 dilution of mAb Fd1a or mAb Fd1b and with 0.5 ml of goat anti-mouse Sepharose (1 mg/ml). After a washing procedure, the Sepharose was suspended in 50 µl of sample mix. For reducing conditions, 40 mM DTT was added. After heating of the samples for 2 min at 100 °C, the Sepharose was removed by centrifugation and the supernatant was applied to a 15% (w/v) polyacrylamide gel. After electrophoresis, the gel was fixed with 30% methanol and 10% acetic acid (v/v, aqueous), dried and exposed to an X-ray film. MWs were determined by a prestained low MW marker from BRL.

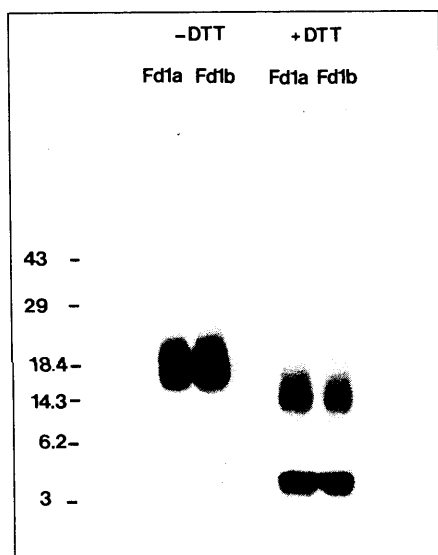


Fig. 1. Radioimmunoprecipitation of affinity-purified *Fel d I* from house dust, followed by SDS-PAGE. ^{125}I -*Fel d I* was immunoprecipitated with mAb Fd1a or Fd1b and applied to a 15% polyacrylamide gel. Migration patterns under nonreducing and reducing conditions are shown.

Generation of Denatured *Fel d I* in vitro by High pH Treatment

1 vol of affinity-purified *Fel d I* (280 U/ml) was incubated during 2 h with 3 vol of 0.1 M Tris-HCl (pH 7, 12, 12.5 or 13), containing 0.01% ascorbic acid. The reaction was blocked with 4 vol of 0.2 M NaH_2PO_4 , and the samples were subsequently dialyzed against PBS.

RIA Procedures

A polyclonal inhibition assay for *Fel d I* was performed according to the method described by De Groot et al [18]. A 1:1,000 dilution of rabbit antiserum directed to cat dander from ALK was used and ^{125}I -*Fel d I* (specific activity 4.63 MBq/U *Fel d I*; 10^{-4} U *Fel d I* per test).

Competition Experiments between mAbs Directed to Denatured *Fel d I*

Fifty microliters of mAb dilution (in PBS) were preincubated with 50 μl of ^{125}I -R & A *Fel d I* (specific activity 4.63 MBq/U *Fel d I*). After 2 h, 0.5 ml of mAb-Sepharose (2 mg/ml) was added, and the mixture was incubated overnight. After a washing procedure, radioactivity bound to the Sepharose was measured.

Activity of mAbs against Denatured *Fel d I*

RIA. Fifty microliters of mAb dilution were added to 0.5 ml of goat anti-mouse Sepharose (2 mg/ml) and 50 μl of ^{125}I -*Fel d I* (native or R & A). After an overnight incubation, the Sepharose was washed, and radioactivity bound to the Sepharose was measured.

RAST. Fifty microliters of mAb dilution were incubated overnight with 250 μl of *Fel d I*-Sepharose (2 mg/ml; 50 U of affinity-purified *Fel d I*, native or R & A, coupled per 100 mg of CNBr-activated Sepharose 4B). Then, the Sepharose was washed, 50 μl of ^{125}I -goat anti-

mouse IgG (specific activity 0.37 MBq/ μg ; 1.2 ng per test) were added, and the suspension was incubated overnight. Finally, the Sepharose was washed and bound radioactivity was measured.

Indirect RAST Experiments with mAb Antidenatured *Fel d I* and mAb Fd1a

Fifty microliters of sample dilutions were incubated overnight with 250 μl of mAb-Sepharose. After extensive washing of the Sepharose, 50 μl of IgE serum were added, and another incubation overnight followed. IgE bound to the Sepharose was detected by a third incubation overnight with 1 ng ^{125}I -anti-serum IgE (0.4 MBq/ μg). For base-treated *Fel d I*, negative control experiments were performed with mAb anti-yellow jacket Ag 5 coupled to Sepharose.

Competitive RIA with mAb against Denatured *Fel d I* and ^{125}I -R & A *Fel d I*

Fifty microliters of allergen sample were preincubated with 50 μl of mAb FdR-1a (0.19 mg/ml). After 2 h 0.5 ml of goat anti-mouse Sepharose (2 mg/ml) and 50 μl of ^{125}I -R & A *Fel d I* were added. Then the mixture was incubated overnight. Finally the Sepharose was washed, and bound radioactivity was measured.

Results

mAbs Directed to Denatured *Fel d I*

Affinity-purified *Fel d I* from house dust was radiolabeled, immunoprecipitated with mAb Fd1a or Fd1b and subjected to SDS-PAGE. Under nonreducing conditions, *Fel d I* migrated as a diffuse band with a MW of 14–20 kD. After reduction, this band dissociated into a 11- to 15-kD chain (chain 2) and a 4- to 5-kD chain (chain 1; fig. 1). To obtain mAbs reactive with the 11- to 15-kD chain, mice were immunized with R & A *Fel d I*. MAb were selected by screening with R & A *Fel d I*-Sepharose and ^{125}I -goat anti-mouse IgG. To prevent selection of antibodies directed against iodoacetamide (used to alkylate *Fel d I* that was used for immunization), *Fel d I* used for screening was alkylated with NEM. From 1 mouse four mAbs were obtained: mAb FdR-1a, FdR-1b, FdR-1a and FdR-2b. All mAbs were of the IgG1 subclass.

In allergen binding experiments with goat anti-mouse Sepharose and ^{125}I -*Fel d I* (native or R & A), all mAbs strongly bound to R & A *Fel d I*, but hardly to the native molecule (fig. 2). In indirect RAST experiments with mAb Fd1a (raised against native *Fel d I*), reactivity was almost completely (> 99%) abolished after reduction and subsequent alkylation of *Fel d I*. Under renaturing conditions however, reactivity with mAb Fd1a was partially regained (fig. 3).

In reduced SDS-PAGE immunoblots with affinity-purified *Fel d I* (fig. 4), two Mabs showed reactivity with chain 2: Mab FdR-2a and FdR-2b. MAb FdR-1a and

FdR-1b gave a reaction with chain 1. As expected, mouse immune serum reacted with both the 4- to 6-kD and the 11- to 15-kD bands. Reactivity with *Fel d I* was observed under reducing conditions (fig. 4a) as well as after reduction and alkylation of *Fel d I* (fig. 4b). No reaction was found under nonreducing conditions. MAb Fd1a reacted with the 4- to 6-kD band of *Fel d I*, which reaction disappeared after alkylation of the molecule.

In competition experiments with mAb-Sepharose and ^{125}I -R & A *Fel d I*, mAb FdR-1a showed a strong inhibitory effect (90%) on allergen binding to mAb FdR-1b, vice versa. The mAbs FdR-1a and FdR-1b did not inhibit allergen binding to mAb FdR-2b, and only mAb FdR-1a showed a weak competition with mAb FdR-2a (2% inhibition activity compared to homologous competition). The inhibitory activity of mAb FdR-2a on allergen binding to mAb FdR-1a and FdR-1b was 0.4 and 1%, respectively compared to homologous competition (probably no real competition), and mAb FdR-2b only showed homologous competition.

Reactivity of mAbs against *Fel d I* and of Human IgE with *Fel d I* in Different Allergen Sources:

An Immunoblotting Study

With mAb antinative *Fel d I*, Fd1a, under nonreducing conditions, variations in MW of *Fel d I* between the different extracts were noted (fig. 5a). Under reducing conditions (fig. 5b), chain 1 was detected (except in cat saliva) and a component that migrated as native *Fel d I*. With mAb FdR-2b (fig. 5c), in all allergen sources, except in cat

dander extract 2 (lane 2), a component smaller than native *Fel d I* was detected that varied in MW in the different extracts, chain 2. MAb FdR-1a recognized chain 1 in all extracts (fig. 5d). In table 1, the MWs of *Fel d I* and *Fel d I* components are listed.

To study the reactivity of human IgE with *Fel d I* from different allergen sources, *Fel d I* was immunoprecipitated with mAb Fd1a, prior to SDS-PAGE. Figure 6 shows the immunoblotting patterns of human IgE, mAb Fd1a and

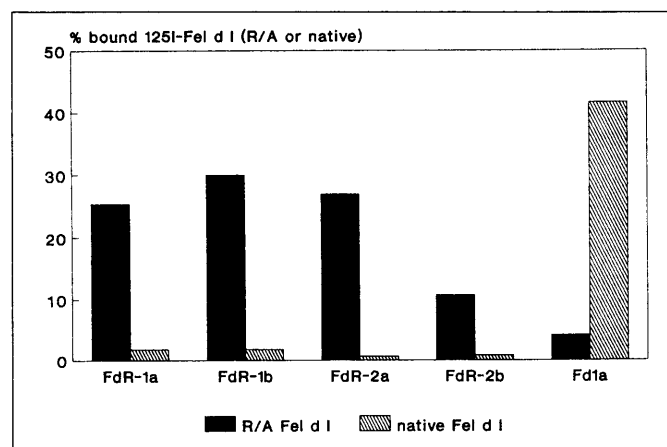
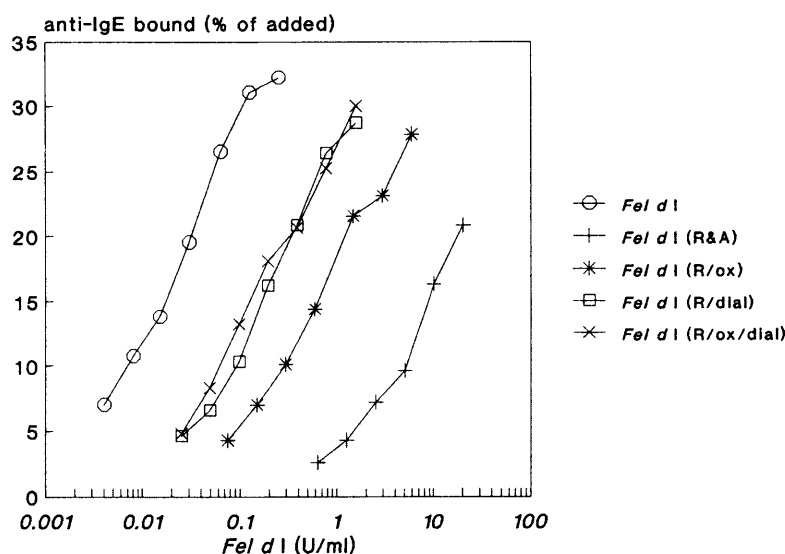


Fig. 2. Activity of mAbs anti-denatured *Fel d I* and mAb Fd1a towards native and R & A *Fel d I*. 50 μl of a diluted 50% SAS fraction of hybridoma supernatant or 50 μl diluted mAb Fd1a ascites were incubated overnight with 0.5 ml goat anti-mouse Sepharose (2 mg/ml) and 50 μl ^{125}I -*Fel d I* (native or R & A). After washing, radioactivity bound to the Sepharose was measured.

Fig. 3. Activity of *Fel d I* in an indirect RAST with mAb Fd1a after R & A or renaturation. 50- μl dilutions of affinity-purified *Fel d I* were incubated overnight with 250 μl of mAb Fd1a-Sepharose. After washing, 12.5 μl of IgE serum were added during 6 h. IgE bound to the Sepharose was detected by an overnight incubation with ^{125}I -anti-human IgE.



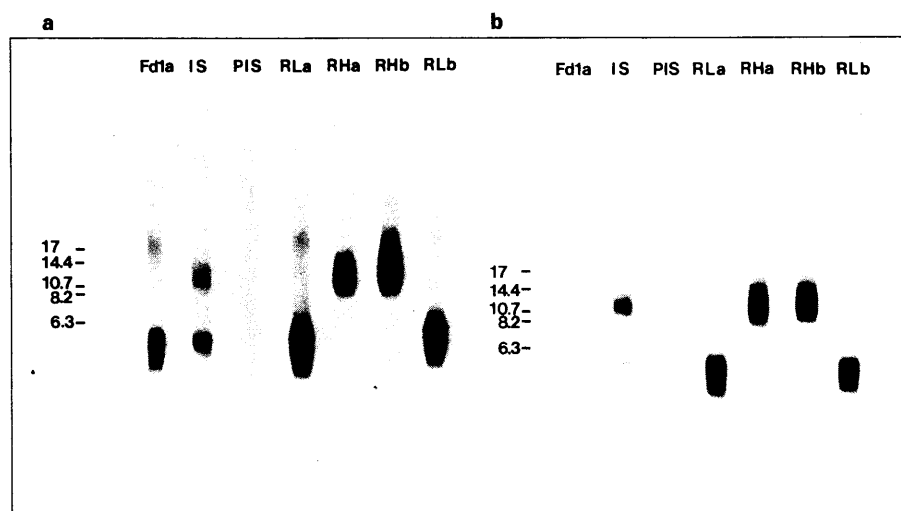


Fig. 4. Immunoblot of affinity-purified *Fel d I* with mAbs anti-denatured *Fel d I*. Affinity-purified *Fel d I* was subjected to SDS-PAGE [15% (w/v) polyacrylamide gel] under reducing conditions (a) or after R & A (b). After transfer of protein onto a nitrocellulose sheet, blots were cut into 0.7-cm strips and incubated overnight with mAb Fd1a (antinative *Fel d I*), mouse immune serum (IS), mouse preimmune serum (PIS) and mAbs antidenatured *Fel d I* (FdR-1a, FdR-2a, FdR-2b, FdR-1b). *Fel d I* bands were visualized by ^{125} I-goat anti-mouse IgG. Autoradiography was performed by exposing the strips for 5 days to an X-ray film. MWs were determined by a MW-SDS-17 kit from Sigma.

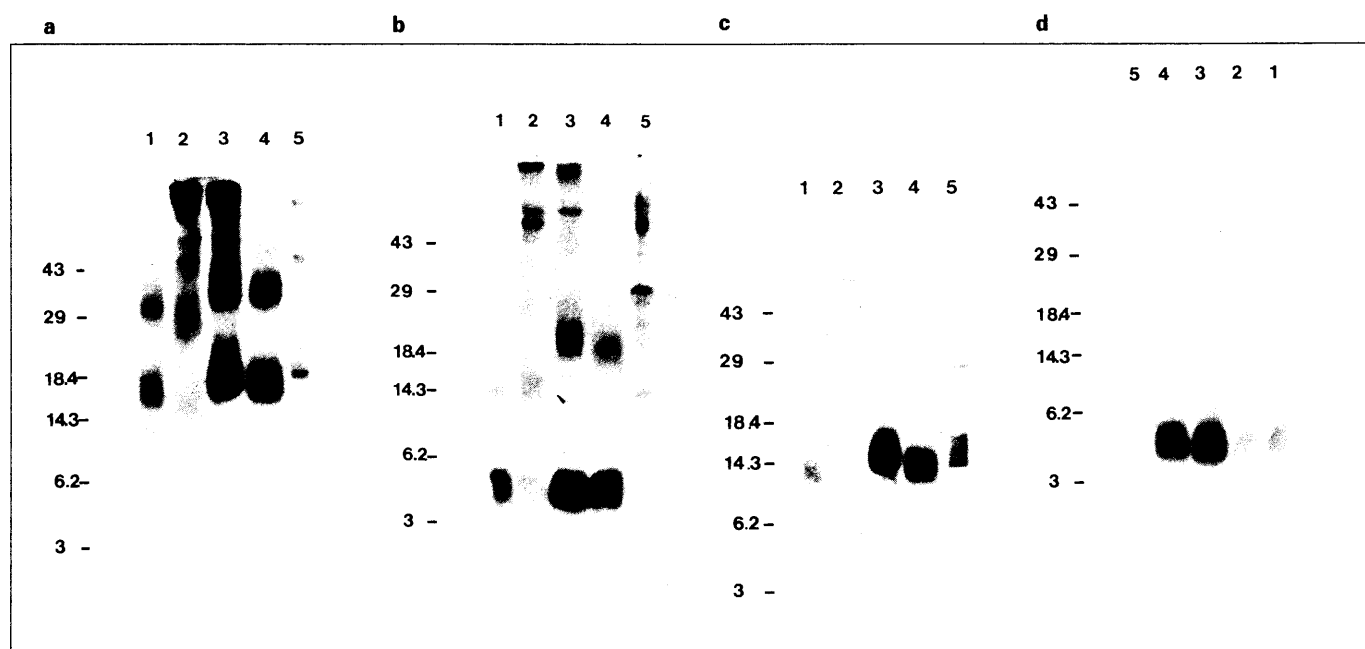


Fig. 5. Immunoblot of various allergen sources with mAbs directed to *Fel d I*. 30- μ l samples of cat dander extract 1 (lane 1), extract 2 (lane 2) and extract 3 (lane 3), of affinity-purified *Fel d I* from house dust (lane 4) and cat saliva (lane 5) were subjected to SDS-PAGE [15% (w/v) polyacrylamide gel] under nonreducing (a) and reducing conditions (b-d; the order of the extracts in d has been inverted). Protein was transferred onto a nitrocellulose sheet, which was incubated

overnight with mAb Fd1a (a, b) mAb antichain 2 FdR-2b (c) and mAb antichain 1 FdR-1a (d). *Fel d I* was visualized by ^{125} I-goat anti-mouse IgG. Autoradiography was performed by exposing the blots for 5, 10, 2 and 5 days, respectively to an X-ray film. Lane 2 in c was exposed for 5 days to an X-ray film instead of 2 days. MWs were determined by a prestained MW marker from BRL.

Table 1. MW of *Fel d I* in various allergen sources, determined by the reaction of the allergen with mAb Fd1a (antigenic *Fel d I*), mAb FdR-2b (antichain 2) and mAb FdR-1a (antichain 1) in immunoblotting experiments

Allergen source	Nonreducing conditions mAb Fd1a	Reducing conditions		
		Fd1a	FdR-2b	FdR-1a
Extract 1	30-33/15-18/12	13/13-17/5-6	11-13	5-6
Extract 2	28-31/13-16	13-14/5-6		5-6
Extract 3	33-42/16-21	18-21/5-6	12-15	5-6
House dust	36-40/16-19	17-19/5-6	11-14	5-6
Cat saliva	/18-19	27	27/12-15	5-6

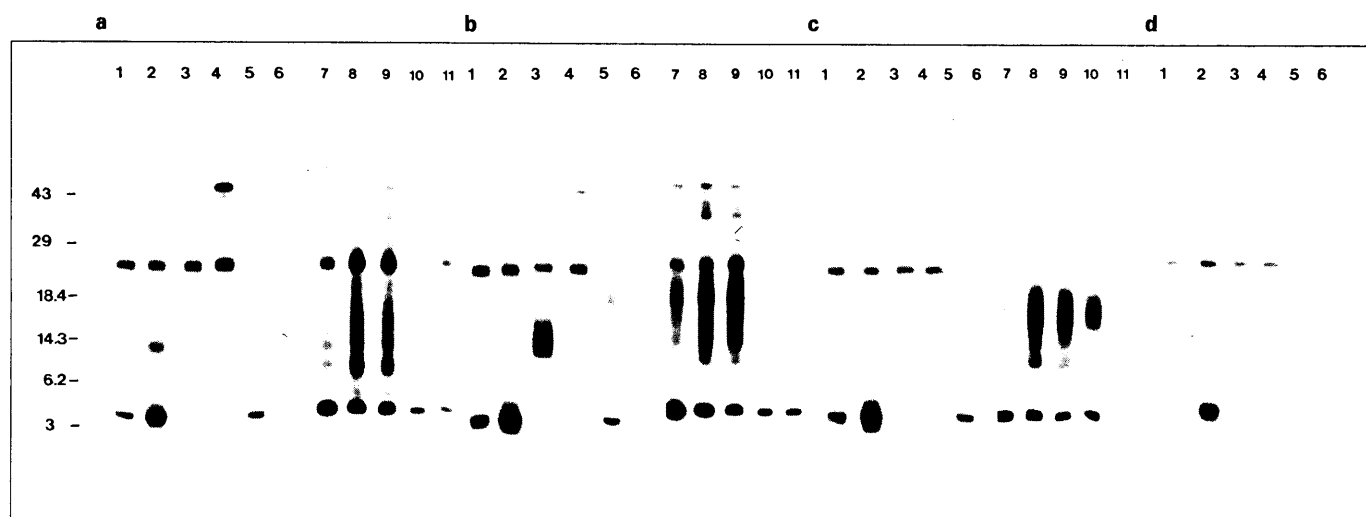


Fig. 6. Immunoblot of *Fel d I*, immunoprecipitated from various allergen sources, with mAb Fd1a, mAb anti-denatured *Fel d I* and human IgE. Cat dander extract 1 (**a**), extract 3 (**b**), affinity-purified *Fel d I* from house dust (**c**) and cat saliva (**d**) were immunoprecipitated with mAb Fd1a and subjected to SDS-PAGE [15% (w/v) polyacrylamide gel]. Proteins were transferred onto a nitrocellulose sheet, which was incubated with mAb Fd1a (lane 1), mAb anti-chain 1 FdR-1a (lane 2), mAb anti-chain 2 FdR-2b (lane 3) and sera of 6 cat-allergic patients (lane 5, 7-11). As second antibodies ^{125}I -goat anti-mouse IgG (lane 1-4) and ^{125}I -sheep anti-human IgE (lane 5 and 6) were used. Control strips were incubated with only the second antibodies (lane 4 and 6). Autoradiography was performed during 4 days (lanes 1-6) and 20 days (lanes 7-11).

the mAbs antidenatured *Fel d I* with immunoprecipitated *Fel d I* from cat dander extracts, affinity-purified *Fel d I* from house dust and cat saliva.

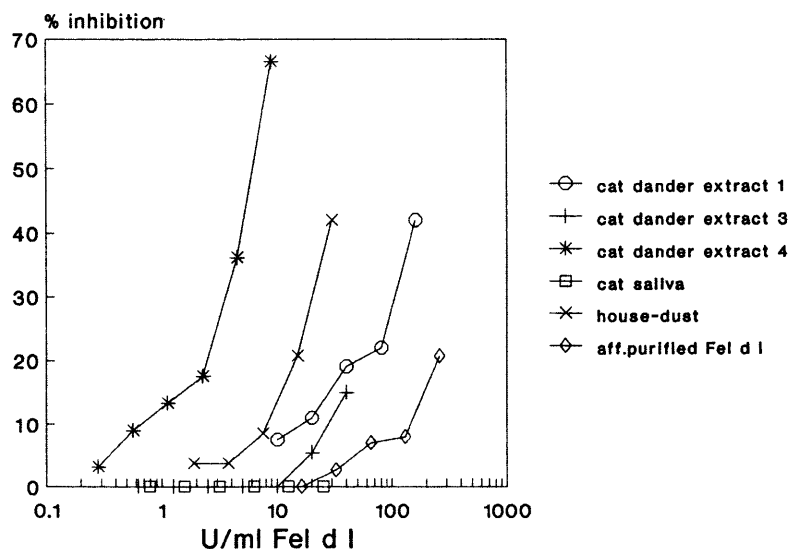
Presence of 'Denatured' Fel d I in Cat Extracts and after Generation in vitro

In a competitive RIA with mAb FdR-1a and ^{125}I -R & A *Fel d I*, the amount of denatured *Fel d I* in different allergen sources was measured. Figure 7 shows the presence

of considerable quantities of denatured *Fel d I* in cat dander extract 4 and in house dust. After affinity purification of *Fel d I* only little denatured *Fel d I* was left, while in cat saliva no denatured *Fel d I* was detected.

In vitro denatured *Fel d I* was generated at high pH (fig. 8a). Under these conditions, reactivity with mAb antigenic *Fel d I* was reduced (fig. 8b). In indirect RAST experiments with mAb FdR-1a reactivity of human IgE with this denatured *Fel d I* was demonstrated (fig. 8c).

Fig. 7. Denatured *Fel d I* in different allergen sources. 50 μ l of cat extract were preincubated with 50 μ l of mAb FdR-1a (0.19 mg/ml). After 2 h 0.5 ml of goat anti-mouse Sepharose (1 mg/ml) and 50 μ l of ^{125} I-R & A *Fel d I* were added, and an incubation overnight followed. After washing, radioactivity bound to the Sepharose was measured. Results are expressed as percentage inhibition.



Discussion

In this study, we examined the reactivity of mAbs and human IgE with *Fel d I* from different allergen sources in reduced SDS-PAGE immunoblots. To obtain mAbs reactive with reduced *Fel d I* and recognizing chain 2 in immunoblotting experiments, mAbs were raised against R & A *Fel d I*.

The mAbs to denatured *Fel d I* bound, in a RIA, very poorly, if at all, to the native form of the molecule, but strongly to the reduced form (fig. 2). On the other hand, mAbs directed to native *Fel d I* (e.g. mAb Fd1a) hardly react with reduced *Fel d I* [10, 15, 16]. So, the new mAbs are more likely to be useful for detecting allergen fragments and recombinant protein. Similar conformation-dependent properties are described for the ragweed pollen allergen *Ambrosia artemisiifolia* I (*Amb a I*) by Smith et al. [21].

Immunoblot analysis of affinity-purified *Fel d I* revealed two fragments recognized by the mAbs under reducing conditions (fig. 4). mAbs FdR-2a and FdR-2b recognized a component smaller than nonreduced *Fel d I* (i.e. recognized by mAb Fd1a under nonreducing conditions). mAbs FdR-1a and FdR-1b reacted with the 5- to 6-kD chain of *Fel d I*. Recent protein sequence analysis and cDNA cloning data [22, 23] show that these two polypeptide chains of *Fel d I* are encoded by two different genes and not generated by RNA splicing or proteolysis. mAb antinative *Fel d I*, Fd1a, recognized chain 1 of *Fel d I*. Only after alkylation of *Fel d I*, this reaction disappeared, which

indicates that under standard reducing conditions also conformational epitopes can be recognized. This implies refolding of reduced *Fel d I* on the nitrocellulose membrane. Indirect RAST experiments with mAb Fd1a (antinative *Fel d I*) on Sepharose also demonstrated renaturation of reduced *Fel d I* after removal of the reducing agent (fig. 3).

With the mAbs directed to *Fel d I*, differences between allergen source materials in blot patterns of *Fel d I* were detected (fig. 5). This variability was revealed more strikingly with antibodies to denatured *Fel d I* than with antibodies to native *Fel d I*. Under nonreducing conditions, a variable MW for the protein stained with mAb Fd1a was found. Under reducing conditions, mAb Fd1a recognized in all extracts, except in cat saliva (but this might be due to a concentration effect), chain 1 and a component that migrated as native *Fel d I* (fig. 5b). This latter component might represent nonreduced *Fel d I*, or alternatively, point to cross-linking of the *Fel d I* subunits other than by cysteine residues. An example of cross-linking through carbohydrate residues is described by Heimgartner et al. [24] for immunoglobulin heavy chains. With mAb FdR-2b, under reducing conditions, a variable MW for chain 2 was found (fig. 5c). These variations in MW could be explained by the recent work of Rogers et al. [23], in which they demonstrate that chain 2 is encoded by two separate genes, differently expressed in skin and salivary glands and showing sequence heterogeneity. This group also shows that chain 2 is modified by N-linked oligosaccharides [22]. Differences in glycosylation of chain 2 could also explain

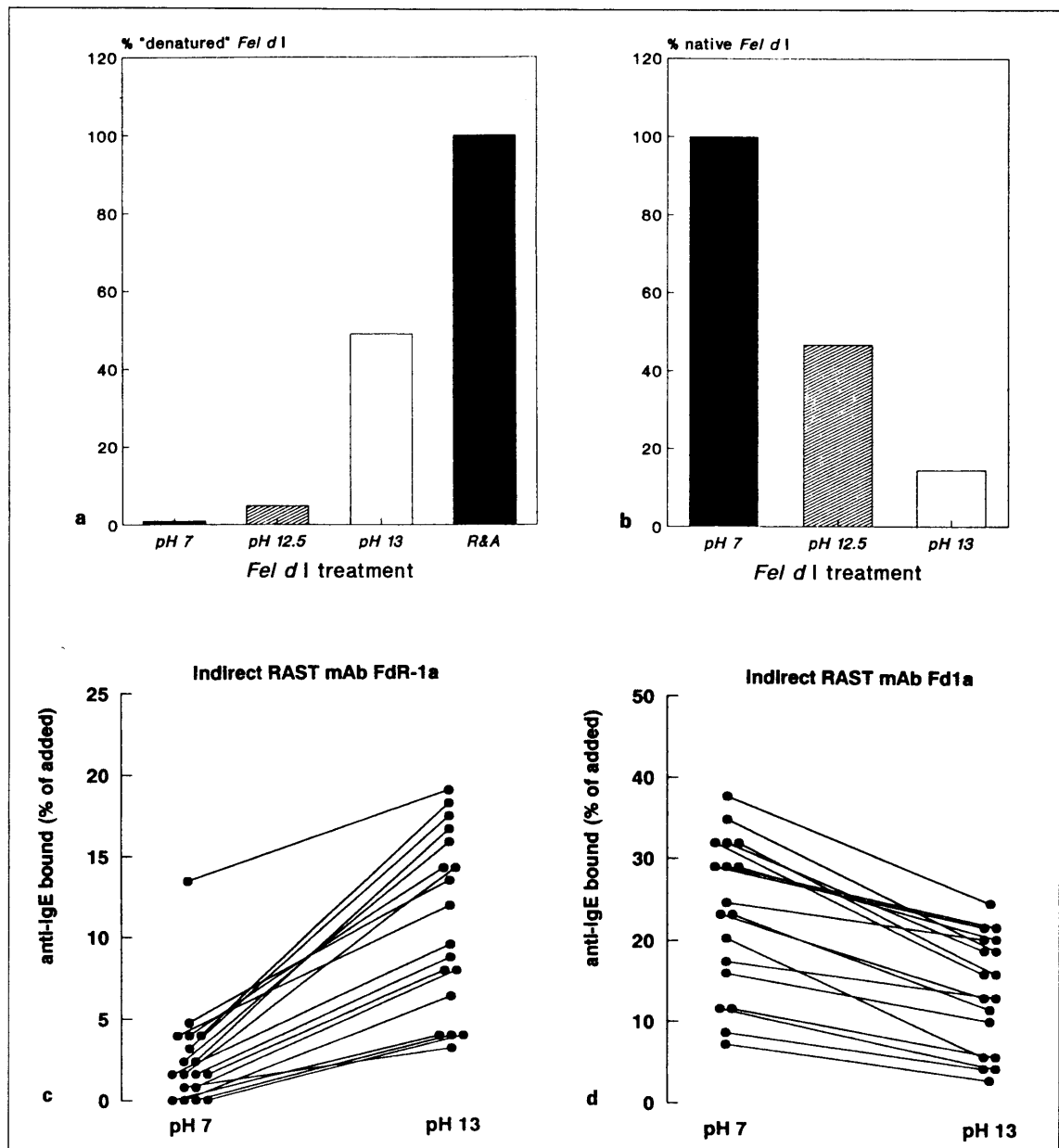


Fig. 8. Generation of denatured *Fel d I* in vitro and reactivity with human IgE. Affinity-purified *Fel d I* was treated at pH 7, 12.5 and 13 and tested for denatured *Fel d I* activity (a) and native *Fel d I* activity (b) in competitive RIAs. In indirect RAST experiments with mAb FdR-1a (c) the reactivity of human IgE with this denatured *Fel d I* was demonstrated. Indirect RAST experiments with mAb Fd1a (d) showed a decrease in native *Fel d I* activity.

the differences found between the cat extracts. The absence in reactivity of mAb FdR-2b with *Fel d I* in cat dander extract 2 indicates a difference in antigenic or allotypic structure of this *Fel d I* molecule. MAb antichain 1 FdR-1a recognized chain 1 in all extracts.

Studying the reaction of human IgE with *Fel d I* from different allergen sources, we were confronted with the

problem of the origin of the protein bands recognized by these patients; obviously not every protein detected is necessarily *Fel d I*. Therefore *Fel d I* was immunoprecipitated from the various allergen sources with mAb Fd1a, prior to SDS-PAGE and immunoblotting (fig. 6). These experiments confirmed that the additional bands found were indeed *Fel d I* related. Moreover, marked differences be-

tween patients and between extracts were found in blot patterns of *Fel d I*. In the cat extracts (except for cat saliva), all patients showed a strong reaction with chain 1 of *Fel d I* (lane 5, 7–11). In cat dander extract 1 (fig. 6a) 3 patients (lane 7–9) showed detectable IgE binding to the protein band stained by mAb FdR-2b. However, reactivity with this chain was weaker than with chain 1. In this extract, mAb FdR-1a and Fd1a recognized apart from chain 1 an additional 12-kD band. This *Fel d I* component was also recognized by human IgE. In cat dander extract 3 (fig. 6b), all sera showed some response with a component migrating as native *Fel d I*. Only 2 sera (lane 8, 9) gave a clear reaction with chain 2, while this chain was heavily stained by mAb FdR-2b. Also in *Fel d I* from house dust (fig. 6c) only these 2 sera gave an obvious reaction with chain 2 (lane 8, 9). All patients reacted with a component migrating as native *Fel d I*, but only by 4 patients (lane 8–11) this component was recognized equally well as chain 1. In cat saliva, hardly any reaction with chain 1 was found for mAb Fd1a and 1 patient. The heavily stained protein bands at 50 and 25 kD in all cat extracts represent the heavy and light chain of the mouse mAbs, used for immunoprecipitation.

In all extracts (saliva excluded), mAb antinative *Fel d I* and human IgE strongly reacted with chain 1 of *Fel d I*. Chain 2 was less well recognized by human IgE, while mAb antinative *Fel d I* did not show any reactivity with this chain. Probably in the native molecule, chain 1 is better exposed than chain 2. The additional protein bands recognized by the mAbs and by human IgE may represent fragments of *Fel d I* present in the cat extracts. We conclude that in immunoblotting studies protein bands with MWs distinct from chain 1 and chain 2 cannot be regarded as being not *Fel d I*.

The immunoprecipitation blot resulted in more discrete protein bands than the direct immunoblotting experiments, and with this procedure *Fel d I* fragments were detected which remained unnoted in the classical immunoblotting experiments. This suggests that the immunoprecipitation blot is a good procedure for analyzing allergen extracts. Other advantages of this procedure are: concentration of the allergen and the elimination of interfering factors.

In a competitive RIA with mAb FdR-1a and ^{125}I -R & A *Fel d I*, differences in concentration of denatured *Fel d I* between the extracts were also found. These differences may be of importance with regard to the quality of a cat extract. Affinity purification of *Fel d I* from house dust with mAb Fd1a, reduced the concentration of denatured *Fel d I* about 40-fold, implying that this form of *Fel d I* is not rec-

ognized by mAb Fd1a. This can have consequences with regard to the standardization of cat extracts.

In vitro denatured *Fel d I* was generated under high pH conditions (fig. 8). After base treatment, a loss in reactivity with mAb anti-native *Fel d I* was found, which is probably due to a reduction in disulfide bonds. We cannot exclude deamidation of Asn residues [25]. In indirect RAST experiments with mAb antichain 1, activity was generated after base treatment, demonstrating that this denatured *Fel d I* is recognized by cat allergic patients. In indirect RAST experiments with mAb antinative *Fel d I*, IgE binding was reduced at high pH. This study demonstrates that mAb anti-denatured *Fel d I* FdR-1a recognizes a form of *Fel d I* that is not reactive with mAb antinative *Fel d I*, but does react with human IgE.

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