

Formation of Disulfide Bonds and Homodimers of the Major Cat Allergen Fel d 1 Equivalent to the Natural Allergen by Expression in *Escherichia coli**

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Hans Grönlund‡§, Tomas Bergman¶, Kristofer Sandström¶, Gunvor Alvelius¶, Renate Reininger**, Petra Verdino‡‡, Alexander Hauswirth§§, Karin Liderot‡, Peter Valent§§, Susanne Spitzauer**, Walter Keller‡‡, Rudolf Valenta¶¶, and Marianne van Hage-Hamsten‡

From the ‡Unit of Clinical Immunology and Allergy and the ¶Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 76 Stockholm, Sweden, the §Department of Biotechnology, Royal Institute of Technology, S-106 91 Stockholm, Sweden, the ‡‡Division of Structural Biology, Institute of Chemistry, Karl Franzens University Graz, A-8010 Graz, Austria, and the **Clinical Institute for Medical and Chemical Laboratory Diagnostics, the §§Division of Hematology, Department of Internal Medicine, and the ¶¶Department of Pathophysiology, Vienna General Hospital, University of Vienna, A-1090 Vienna, Austria

Dander from the domestic cat (*Felis domesticus*) is one of the most common causes of IgE-mediated allergy. Attempts to produce tetrameric folded major allergen Fel d 1 by recombinant methods with structural features similar to the natural allergen have been only partially successful. In this study, a recombinant folded Fel d 1 with molecular and biological properties similar to the natural counterpart was produced. A synthetic gene coding for direct fusion of the Fel d 1 chain 2 N-terminally to chain 1 was constructed by overlapping oligonucleotides in PCR. *Escherichia coli* expression resulted in a non-covalently associated homodimer with an apparent molecular mass of 30 kDa defined by size exclusion chromatography. Furthermore, each 19,177-Da subunit displayed a disulfide pattern identical to that found in the natural Fel d 1, i.e. Cys³(1)-Cys⁷³(2), Cys⁴⁴(1)-Cys⁴⁸(2), Cys⁷⁰(1)-Cys⁷(2), as determined by electrospray mass spectrometry after tryptic digestion. Circular dichroism analysis showed identical folds of natural and recombinant Fel d 1. Furthermore, recombinant Fel d 1 reacted specifically with serum IgE, inducing expression of CD203c on basophils and lymphoproliferative responses in cat-allergic patients. The results show that the overall fold and immunological properties of the recombinant Fel d 1 are very similar to those of natural Fel d 1. Moreover, the recombinant Fel d 1 construct provides a tool for defining the three-dimensional structure of Fel d 1 and represents a reagent for diagnosis and allergen-specific immunotherapy of cat allergy.

Exposure to airborne particles derived from household cats (*Felis domesticus*) is a common cause of IgE-mediated allergy in

Europe and elsewhere (1–3). The severity of symptoms range from relatively mild rhinitis and conjunctivitis to potentially life-threatening asthmatic exacerbation. Treatment of cat allergy by allergen injections is often employed, but clinical results are, in part, controversial. Because only crude dander extract is available for the treatment of cat allergy (4, 5), alternative formulations have been proposed (6, 7).

Although patients are occasionally sensitized to several different molecules in cat dander (8) and pelts, e.g. albumin (9) and cystatin (10), the importance of the ubiquitous major allergen Fel d 1 has been emphasized in numerous studies. In fact, >80% of cat-allergic patients exhibit IgE antibodies to this potent allergen (11, 12). Fel d 1, (formerly Cat 1) was first described 25 years ago as the dominant cat allergen (13), and several subsequent studies have characterized the biochemical and immunological nature of Fel d 1 (14–23). The allergen, a 35–39-kDa acidic glycoprotein containing 10–20% N-linked carbohydrates (15, 16, 22), is found in the pelt, saliva, and lacrimal glands of cats (24–26). It is formed by two non-covalently linked heterodimers (16), each consisting of one 70-residue peptide and one 85-, 90-, or 92-residue peptide (17) (i.e. chain 1 and chain 2, respectively) encoded by separate genes (22, 27). Chain 1 shares limited sequence homology with the rabbit uteroglobulin/human Clara cell 10-kDa protein (28, 29), and the mature natural Fel d 1 (nFel d 1)¹ has been associated with gelatin- and fibronectin-degrading activity (30). Furthermore, three interchain disulfide bonds linking the two peptides in native Fel d 1 have been identified, i.e. Cys³(1)-Cys⁷³(2), Cys⁴⁴(1)-Cys⁴⁸(2), and Cys⁷⁰(1)-Cys⁷(2) (22), suggesting an anti-parallel orientation of Fel d 1 peptides. Several attempts have been made to associate the separate peptides into a native-like allergen in *Escherichia coli* with only partial success (31–33), and recently a soluble and immunoreactive chain 1-linker-chain 2 fusion expressed in baculovirus was described (34). A mix of the separate chains has proven to be useful for *in vitro* allergy diagnosis (12, 33), but to date no soluble, stable, and correctly folded recombinant Fel d 1 (rFel d

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§ To whom correspondence should be addressed. Tel.: 46-8-51776698; Fax: 46-8-335724; E-mail: hans.gronlund@ks.se.

¹ The abbreviations used are: nFel d 1, natural Fel d 1; BSA, bovine serum albumin; EDC, N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; kU_A, kilounits of allergen; NHS, N-hydroxysuccinimide; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; rFel d 1, recombinant Fel d 1; RU, response units; SEC, size exclusion chromatography.

1) homodimer with retained disulfide formation has been obtained by expression in *E. coli*.

In this study, experiments were performed to find out if a protein derived from a direct fusion of the genes coding for the two polypeptide chains constituting Fel d 1 can be folded to mimic the structure and allergenic activity of natural Fel d 1. Synthetic genes coding for the two Fel d 1 chains were produced and joined in PCR by overlapping oligonucleotides and expressed as refolded His-tagged proteins in *E. coli*. Two constructs were produced, one with chain 1 and the other with chain 2 in the N-terminal position. Both constructs were purified to homogeneity and analyzed for intramolecular disulfide bonds and homodimer formation using size exclusion chromatography, mass spectrometry, and surface plasmon resonance. The 2 + 1 construct revealed greater capacity to inhibit IgE antibodies to Fel d 1 from sensitized individuals and was, therefore, studied further. The structure of the purified recombinant Fel d 1(2 + 1) fusion molecule was compared with that of natural Fel d 1 by CD spectroscopy, and the analysis of IgE antibody responses in direct and competition ELISA assays using sera from individuals sensitized to cat were carried out. The biological activity was demonstrated by the induction of CD203c on basophils and T cell proliferation in cat-allergic patients.

Based on the findings that the recombinant Fel d 1(2 + 1) fusion molecule exhibits the same disulfide bonding pattern and homodimer formation and reveals an almost identical CD spectrum, similar immunoreactivity, and comparable biological activity as does the natural Fel d 1, we suggest that the recombinant allergen is suitable for structural analysis and development of diagnostics and specific immunotherapy of allergy to cat.

EXPERIMENTAL PROCEDURES

Natural and Recombinant Fel d 1—Standard (35) or manufacturers' protocols were used for DNA manipulations. From published amino acid sequences of Fel d 1 chains 1 and 2 (15–17), synthetic genes were made by PCR-amplification (Eppendorf Mastercycler, Hamburg, Germany) using overlapping DNA primers from DNA Technology A/S (Århus, Denmark) (Table I). PCR reactions (10 μ l) containing 1 pmol of each primer, primers 1–4 for chain 1 and 5–10 for chain 2, using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), were used. The reactions proceeded for 1 min at 94 °C, 1.5 min at 65 °C, and 2 min at 68 °C for 30 cycles. The PCR products were ligated into pT7Blue Blunt vector and transformed into Nova Blue Single Competent cells using the Perfectly Blunt cloning kit (Novagen Inc., Madison, WI). Single colonies were grown in 2.5-ml LB medium containing 100 μ g/ml ampicillin, and plasmids were purified (Qiagen, GmbH, Hilden, Germany) and cut with the restriction enzymes *Nde*I and *Xho*I followed by electrophoretic analysis in 0.3 μ g/ml ethidium bromide in 1% agarose gels. Clones with the right sized insert (210 and 276 bp for chains 1 and 2, respectively) were sequenced (ABI PRISM® 377 DNA sequencer, Applied Biosystems). Verified plasmids were used as templates to join chains 2 and 1, using primers 4, 5, and 11 as described for the single chains. After sequencing, the fusion constructs were subcloned into pET 20b (Novagen) using the *Nde*I/*Xho*I restriction sites and transformed into *E. coli* strain BL-21(DE3)pLysS (Novagen). Clones were selected on LB agar plates with ampicillin (100 mg/liter) and chloramphenicol (30 mg/liter) and grown in LB medium containing 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol. pET 20b inserts of chain 1, chain 2, and rFel d 1(2 + 1) were sequence-verified (Applied Biosystems). Expression of protein was induced for 3 h in mid-log phase using 0.4 mM isopropyl thiogalactoside. The cells were pelleted using a J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA) at 1000 $\times g$ for 10 min and stored at –20 °C. Natural Fel d 1 (>90% pure) from Indoor Biotechnologies (Charlottesville, VA) was used for comparison of immunoreactivity and biological activity between samples.

Purification and Characterization of Recombinant Fel d 1—Protein purification was performed using fast protein liquid chromatography (Amersham Biosciences). The same purification protocol was used for all recombinant constructs. The *E. coli* pellets containing the expressed recombinant construct were resuspended in 20 mM Tris-HCl, 0.2 M

NaCl, and 1 mM EDTA, pH 7.4, and disrupted via 8 $\times$ 10 s sonication bursts on ice (Soniprep 150 ultrasonic disintegrator, Sanyo Gallenkamp, Uxbridge, UK) followed by centrifugation at 12,000 $\times g$ for 25 min (Beckman Instruments). This procedure was repeated twice, after which the pelleted inclusion body preparation was solubilized in 6 M guanidine-HCl, 20 mM Tris-HCl, 0.5 M NaCl, and 5 mM imidazole, pH 8.0, and loaded onto a 5-ml Ni²⁺-HiTrap affinity column (Amersham Biosciences) operated at 5 ml/min. The column buffer was changed to 6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8.0, and a linear gradient was formed to reach 20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8.0, after 12 column volumes. The protein was eluted with 20 mM Tris-HCl, 0.5 M NaCl, and 0.5 M imidazole, pH 8.0. The enriched rFel d 1 preparation was purified by size exclusion chromatography (SEC) (HiLoad® 16/60 Superdex 200 pg, Amersham Biosciences), equilibrated in PBS, and in PBS with 0.1% SDS at 1 ml/min. Molecular weight calibration of the column was carried out using 67-kDa bovine serum albumin, 43-kDa ovalbumin, 25-kDa chymotrypsinogen A, and 13.7-kDa ribonuclease A (Amersham Biosciences) dissolved in PBS or PBS with 0.1% SDS. The relative elution of reference proteins and rFel d 1(2 + 1) was calculated according to the formula $K_{av} = V_e - V_o/V_t - V_o$ (36). Purified proteins were stored at –80 °C until use.

The protein concentration of rFel d 1(2 + 1) was analyzed by amino acid analysis using a Biochrom 20 Plus ninhydrin-based analyzer (Amersham Biosciences) after hydrolysis at 110 °C for 24 h in evacuated tubes with 6 M HCl containing 0.5% (w/v) phenol. The BCA protein assay (Pierce) was sometimes used to estimate the protein concentration. Purity was judged by SDS-PAGE using 15% homogeneous gels and low molecular weight markers (Amersham Biosciences). Samples were denatured at 98 °C for 5 min in SDS sample buffer with or without β -mercaptoethanol (35).

Electrospray Mass Spectrometry and Determination of Disulfide Bonds—For mass determination of rFel d 1(2 + 1), the folded protein was dissolved at 27 pmol/ μ l in 10 mM ammonium acetate (pH 7.3) and applied to electrospray ionization (ESI) mass spectrometry (see below) via direct infusion using a syringe pump at 2–5 μ l/min (Harvard Apparatus, Holliston, MA).

To localize disulfide bonds, 2.7 nmol of the folded rFel d 1(2 + 1) was dissolved in 10 μ l of 9 M urea and incubated for 30 min under vortex at 45 °C, after which 10 μ l of water was added. Modified trypsin (5 μ g; Promega) and 10 μ l of 0.5 M ammonium bicarbonate (pH 8.0) were added, followed by water to yield a final volume of 100 μ l. Digestion proceeded overnight under vortex at 37 °C. The reaction was quenched by adding 1 μ l of neat trifluoroacetic acid to the sample which was stored at –20 °C until analyzed. Before mass spectrometry, aliquots of the tryptic digest (10 μ l) were desalted on μ -C₁₈ ZipTips (Millipore, Bedford, MA) and eluted in 60% acetonitrile containing 1% acetic acid for nano-ESI mass spectrometry. To make sure that no free sulphydryl groups existed in rFel d 1(2 + 1), alkylation was carried out on the non-reduced recombinant preparation (5.4 nmol) using iodoacetamide (Sigma) at 5.5 mM in 20 mM ammonium bicarbonate (pH 8.0) for 15 min at room temperature followed by desalting on μ -C₄ ZipTips and nano-ESI mass spectrometry.

Mass spectra were recorded using a quadrupole time-of-flight tandem mass spectrometer, Q-TOF (Micromass, Altrincham, UK). The instrument was equipped with an orthogonal sampling ESI-interface (Z-spray, Micromass). Metal-coated nano-ESI needles (Protana, Odense, Denmark) were used and manually opened on the stage of a light microscope to give a spraying orifice of about 5 μ m. This resulted in a flow of ~20–50 nl/min when a capillary voltage of 0.8–1.2 kV was applied. A nitrogen counter-current drying gas facilitated desolvation. The cone voltage was set at 40 V.

Homodimer Dissociation Constant Analysis—A BIACORE®2000 instrument (Biacore AB, Uppsala, Sweden) was employed to investigate homodimer formation of rFel d 1(2 + 1) by evaluation of the decrease in response relative to maximum binding to the chip surface and the associated dissociation constant. rFel d 1(2 + 1) and, for control purposes, a monomer protein, BB (39), were immobilized onto the surface of CM-5 chips (research grade) via amine coupling to a carboxylated dextran layer using NHS/EDC chemistry according to the manufacturer's recommendations. The time intervals between surface activation (240–660 s, 35 μ l), protein immobilization (900–1140 s, 20 μ l), dissociation phase (1140–1940 s, 67 μ l), and surface deactivation (1940–2060 s, 10 μ l) were kept constant. In the immobilization step, 20 μ l of a protein solution containing 0.05 μ g/ μ l in 10 mM sodium acetate (pH 4.5) was injected over the NHS/EDC-activated surface. After the dissociation phase, the surface was deactivated by injection of 10 μ l of ethanolamine. The decrease in the percentage of protein initially attached to the chip surface was calculated as follows: (response units

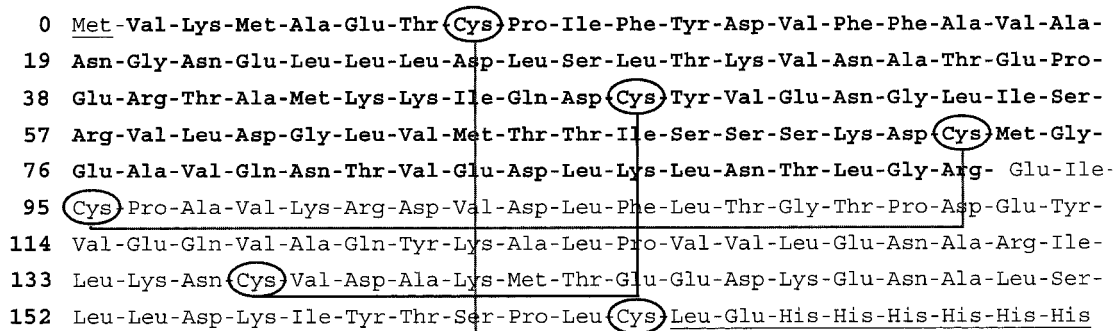


FIG. 1. The primary structure and disulfide bonds of the recombinant His-tagged rFel d 1(2 + 1) fusion molecule. Chain 2, shown in **bold**, is positioned N-terminally of chain 1. Underlined amino acids are the *NdeI/XhoI* restriction site and the derived vector. Linked circles show the cysteine disulfide bonds as determined by mass spectrometry of tryptic fragments.

(RU) after protein immobilization at 1230 s – RU after deactivation/RU after protein immobilization) $\times 100$. All experiments were performed at 25 °C and 5 μ l/min. The running buffer was 10 mM Hepes (pH 7.4), 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20. The dissociation constant analyzed at 1230–1235 s was based on the equilibrium responses and calculated using the 3.0 software.

CD Measurements—CD measurements of the natural and recombinant Fel d 1 were performed in MilliQ water with protein concentrations of 1.56×10^{-5} M (here determined using the Bio-Rad protein assay). The investigations were carried out on a Jasco J-715 spectropolarimeter (JASCO Labor-und-Datentechnik GmbH, Gross-Umstadt, Germany) using a 0.1-cm pathlength cell equilibrated at 20 °C. Spectra were recorded with 0.5-nm resolution at a scan speed of 100 nm/min and resulted from averaging three scans. The final spectra were baseline-corrected by subtracting the corresponding MilliQ spectra obtained under identical conditions. Results were expressed as the molar mean residue ellipticity (θ) at a given wavelength. The data were fitted with the secondary structure estimation programs Dicroprot (37) and J-700 (JASCO) using miscellaneous data deconvolution algorithms.

ELISA Analysis—Serum specimens from 15 individuals were selected on the basis of positive IgE responses to cat dander (range 0.45–38 kilounits of allergen (kU_A)/liter using the Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden)). For control purposes, a pool of 20 non-cat-allergic patients was used. The serum samples were analyzed in duplicates by ELISA for IgE antibody binding to rFel d 1(2 + 1), nFel d 1, or a mix of rFel d 1 chain 1 and chain 2. The assay was performed as a sequential, solid phase adsorption of allergens, serum sample, primary antibody, antibody conjugate, and finally substrate, and included rinsing four times with 250 μ l of PBS containing 0.05% Tween 20 (PBS-T) between incubations. Unless stated otherwise, all steps were performed at room temperature. Microtiter plates (96 wells; Nunc, Roskilde, Denmark) were coated with 100 μ l of rFel d 1(2 + 1) solution and, for comparison, also nFel d 1 and an equimolar mixture of chains 1 and 2 to final concentrations of 5 μ g/ml in 0.1 M carbonate buffer, pH 9.6. After overnight adsorption at 4 °C, the plates were emptied, and the remaining protein binding sites were blocked with 200 μ l of PBS-T containing 1% bovine serum albumin for 2.5 h at room temperature (20–22 °C). Each serum sample (100 μ l) was diluted 1:1 in PBS (duplicates) and incubated for 2 h at room temperature, after which 100 μ l of rabbit anti-human IgE (Miab, Uppsala, Sweden, diluted 1:2000 (v/v)) was added, and incubation was continued for 2 h. Finally, 100 μ l of goat anti-rabbit (Dako, Denmark, diluted 1:2000 (v/v)) conjugated to alkaline phosphatase was added, and incubation continued for 1 h. Alkaline phosphatase substrate tablets (Sigma 104® Diagnostics) were used, and the color reaction monitored at 405 nm was registered in an automated ELISA reader (Multiskan RC, Labsystems, Helsinki, Finland). A competition assay of serum IgE was performed using pooled sera from individuals sensitized to cat with a >10 kU_A /liter response to cat dander (mean concentration, 23 kU_A /liter) (Pharmacia CAP System). Microtiter plates (96 wells) were coated with 100 μ l, 5 μ g/ml nFel d 1. 3-fold serial dilutions in PBS-T of rFel d 1(2 + 1), nFel d 1, and an equimolar mixture of chains 1 and 2 were incubated at a 1:1 volume ratio with the serum pool diluted 1:2 (v/v) in PBS for 2 h at room temperature and thereafter added to the wells. The subsequent steps were as described for the direct ELISA.

CD203c Assay—The expression of CD203c was performed as described (38). Briefly, heparinized blood samples were taken from two cat-allergic patients. Blood aliquots (100 μ l) were incubated with dilutions of recombinant and natural Fel d 1 (1 and 10 μ g/ml), anti-IgE

antibody (1 μ g/ml), or PBS for 15 min (37 °C). After incubation, cells were washed in PBS/EDTA and then incubated with 10 μ l of phycoerythrin-labeled CD203c monoclonal antibody 97A6 (Immunotech, Marseille, France) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with 2 ml of FACSTM lysing solution (BD Biosciences). Cells were then washed, resuspended in PBS, and analyzed by two-color flow cytometry on a FACScan (BD Biosciences).

Lymphoproliferation Assay—Peripheral blood mononuclear cells (PBMCs) were isolated from cat-allergic patients by Ficoll (Amersham Biosciences) density gradient centrifugation. PBMCs (2×10^5) were cultured in triplicate in 96-well Nunclone plates (Nunc) in 200 μ l of serum-free Ultra Culture medium (BioWhittaker, Rockland, ME) supplemented with 2 mM L-glutamine (Sigma), 50 μ M β -mercaptoethanol (Sigma), and 0.1 mg gentamicin per milliliter (Sigma) at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were stimulated with different concentrations (5, 2.5, 1.25, and 0.6 μ g per well) of rFel d 1, nFel d 1, and, for control purposes, recombinant birch pollen allergen Bet v 1, 4 units of interleukin-2 (IL-2) per well (Roche Applied Science), and medium alone. After 6 days of culture, 0.5 μ Ci per well of [³H]thymidine (Amersham Biosciences) was added, and, 16 h thereafter, incorporated radioactivity was measured by liquid scintillation counting using a Microbeta scintillation counter (Wallac ADL, Freiburg, Germany), and mean counts per minute were calculated from the triplicates. The stimulation index was calculated as the quotient of the counts per minute obtained by antigen or interleukin-2 stimulation and the unstimulated control.

Statistical Analysis—Serological results using rFel d 1(2 + 1), nFel d 1, and the Fel d 1 peptide mixture in the direct ELISA were compared employing analysis of variance (ANOVA) repeated measures. A *p* value of <0.05 was considered statistically significant.

RESULTS

rFel d 1(2 + 1) Forms Stable Disulfide-bonded Homodimers—The amino acid sequence (17) and the disulfide bonds as determined by nano-ESI mass spectrometry after tryptic digestion of the *E. coli* expressed (His)₆-tagged rFel d 1(2 + 1) fusion protein is shown in Fig. 1. Following protein purification and simultaneous renaturation by Ni²⁺-chelate chromatography, dominant fractions in SEC with apparent molecular masses of 30 and 51 kDa (Fig. 2) were recovered for further analysis. The 51-kDa fraction, which, in part, could be reassembled to 30 kDa by reduction and re-oxidation, was comprised of two SS-linked rFel d 1(2 + 1) molecules as shown by a 35-kDa band in non-reducing SDS-PAGE and a 20-kDa band in reducing SDS-PAGE (Fig. 2). The 51-kDa fraction was therefore not further analyzed. The 30-kDa fraction showed a single 30-kDa peak upon re-chromatography and exhibited 20- and 16-kDa bands by reducing and non-reducing SDS-PAGE, respectively, (Fig. 2). The yield after SEC of pure 30-kDa fractions typically was 40–60% between different batches. When subjected to electrospray mass spectrometry, rFel d 1(2 + 1) revealed a molecular mass of 19,177 Da, which corresponds to the average molecular mass (19,183 Da) minus 6 Da, indicating the existence of three disulfide bonds in the structure (Fig. 3). Also seen is an additional peak at 19,046 Da, which corresponds to the full-length rFel d 1 without the initiating methionine (Table I, residue 0 in Fig. 1). The purity of both the

FIG. 2. Size exclusion chromatography of rFel d 1(2 + 1). The SDS-PAGE lanes, from left to right, are molecular weight (MW) markers (lane 1), total *E. coli* cell content after Fel d 1(2 + 1) expression (lane 2), 51-kDa fraction reduced (lane 3), 51-kDa fraction non-reduced (lane 4), 30-kDa fraction reduced (lane 5), and 30-kDa fraction non-reduced (lane 6). The markers denote the following: 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen A; and 13.7 kDa, ribonuclease A. The symbol *R* denotes the correlation coefficient for the reference proteins.

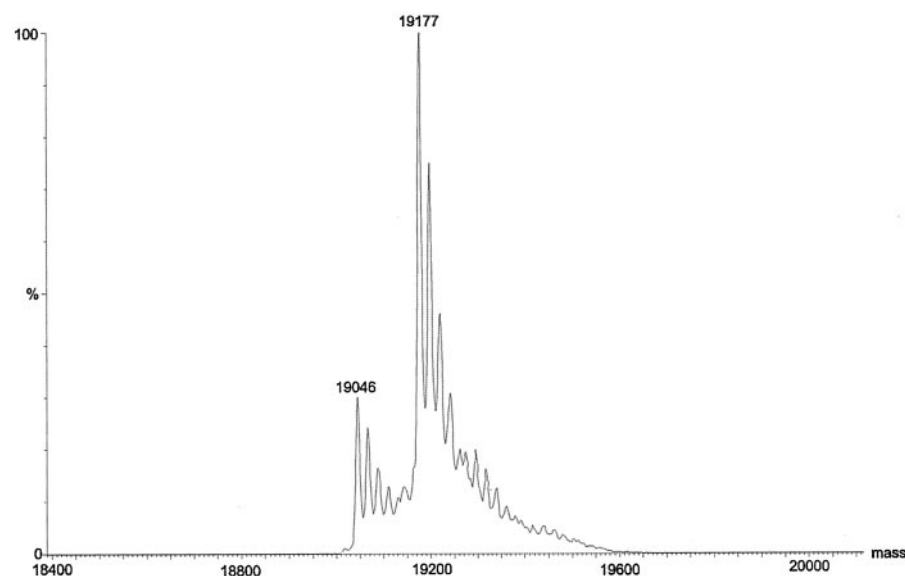
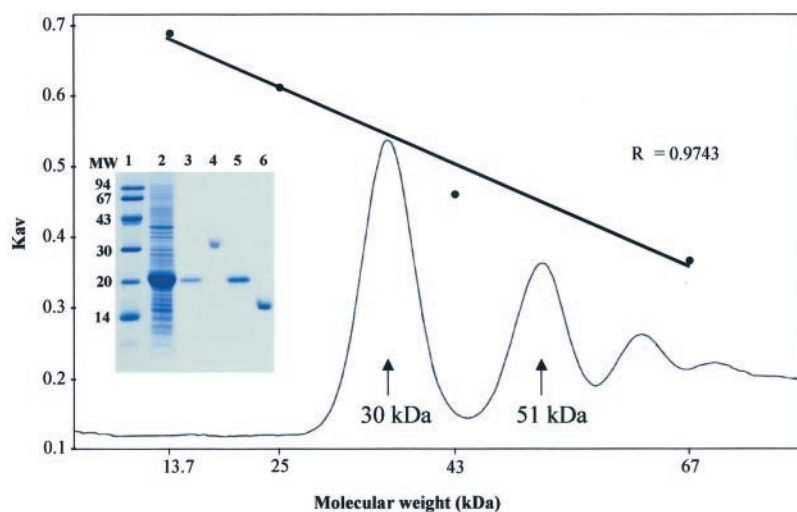


FIG. 3. Deconvoluted electrospray mass spectrum from analysis of rFel d 1(2 + 1). The protein preparation contains the full-length recombinant protein, both with (mass 19,177 Da) and without (mass 19,046 Da) the initiator Met residue present (residue 0 in Fig. 1). The smaller signals following each of the two major signals mainly correspond to sodium adducts.

natural and recombinant preparations was >95% as judged by SDS-PAGE (data not shown).

CD Analysis Shows That rFel d 1 Represents a Folded, Mainly α -Helical Protein—The 20 °C CD spectra of natural and recombinant Fel d 1 are nearly identical, characterized by two minima at 208 and 222 nm and a characteristic maximum at ~195 nm (Fig. 4). The shape of the spectrum is indicative for a well folded protein with a significant α -helical secondary structure content. The secondary structure estimation resulting from the fitting procedures yields 35–40% α -helix and 7–16% β -sheet structures with root mean square deviations ($CD_{calc} - CD_{exp}$) in the range 4–11%.

rFel d 1(2 + 1) Forms Homodimers—The 30-kDa molecular size detected for rFel d 1(2 + 1) suggests a non-covalent dimerization similar to that exhibited by natural Fel d 1. This was investigated by Biacore analysis and SEC under dissociating elution conditions. In the latter case, the 30-kDa rFel d 1(2 + 1) fraction produced a single peak corresponding to a molecular mass of 15 kDa using PBS with 0.1% SDS in the running buffer (data not shown). The 30-kDa fraction was further analyzed by surface plasmon resonance with the assumption that dissociation of the two subunits can be recorded. As a control, a monomeric protein, BB (39), was used, (Fig. 5). The rFel d 1(2 + 1) construct and the BB monomer bound to the sensor chip in a similar manner. The time-dependent decrease in RU after immobilization of the rFel d 1(2 + 1) molecule to the chip surface

was 53%. In contrast, the BB monomer exhibited a stable association to the chip surface during the same time period. In addition, the dissociation constant was determined shortly after the immobilization phase to be $8.74 \cdot 10^{-4} \text{ s}^{-1}$.

Analysis of Disulfide Bonding Pattern by Mass Spectrometry—Correctly paired disulfide bonds are important for protein structure and stability. We analyzed the disulfide bond formation in rFel d 1(2 + 1) by nano-ESI mass spectrometry after trypsin digestion of the non-reduced preparation (Fig. 6 and Table II). The tryptic peptide mass map revealed that all six cysteine residues are engaged in disulfide bonds in the pattern Cys³(1)-Cys⁷³(2), Cys⁴⁴(1)-Cys⁴⁸(2), Cys⁷⁰(1)-Cys⁷(2), because the corresponding mass values for the cysteine-linked peptides were found in the mass spectrum (Fig. 6), whereas the masses of the non-linked individual peptides could not be detected (Table II). This pattern, which is identical to that in nFel d 1 (22), generates an anti-parallel orientation of the two chains. Furthermore, upon alkylation of the non-reduced rFel d 1(2 + 1) preparation using iodoacetamide, we could not detect free sulfhydryl groups (data not shown), a finding in agreement with the mass spectrometry data from analysis of the tryptic digest (Fig. 6).

rFel d 1(2 + 1) Binds IgE from Cat-allergic Patients—The diagnostic relevance of a recombinant allergen lies in its ability to specifically bind IgE antibodies in body fluids or tissues from allergic patients in a manner similar to the natural counter-

TABLE I
Overlapping primers used for the construction of rFel d 1(2 + 1)

Restriction enzyme sites (*Nde*I and *Xho*I) are underlined. Forward (F) and reverse (R) primers are indicated.

Primer no.	Sequence
1 (F)	5'-gtacatatgg aaatctgccc ggctgttaaa cgtgacgttg acctgttcct gaccgggtacc ccggacgaat acgttgaaca ggttg-3'
2 (R)	5'-ggcagagcgtt tgtactgagc aacctgttca acgtattcgt ccgggtgagc aacctgttca acgtattc-3'
3 (F)	5'-tgctcagtag aaagctctgc cggttgttct ggaaaacgct cgtatcctga aaaactcgtg tgacgctaaa atgacc-3'
4 (R)	5'-cctctcagagg cacagcgggg aggtgttagat ttgttccagc agggacagag cgttttcttt gtcttcttcg gtcattttag cgtcaacgc-3'
5 (F)	5'-gtacatatgg ttaaaatggc tgaaacctgc ccgatcttct acgacgtttt cttcgtctgtt gtaacggta acgaac-3'
6 (R)	5'-ggtagcgttcc gggttcgtag cggttaacttt ggtcaggagc aggtccagca gcagttcgtt accgttagca acagc-3'
7 (F)	5'-ctaccgaacc ggaacgtacc gctatgaaaa aaatccagga ctgctacgtt gaaaacggtc tgatctccc tgttctggac-3'
8 (R)	5'-gcttcaccca tgcagtcctt ggaggaggag atggtgggtca taaccagacc gtccagaaca cgggagatca g-3'
9 (F)	5'-caaagactgc atgggtgaag ctgttcagaa caccgttgaa gacctgaac tgaacaccct gggtcgctcg agagg-3'
10 (R)	5'-cctctcagagg cgaccacggg tg-3'
11 (linker)	5'-cgtttaacag ccgggcagat ttcacgaccc aggtgtgttca gtttc-3'

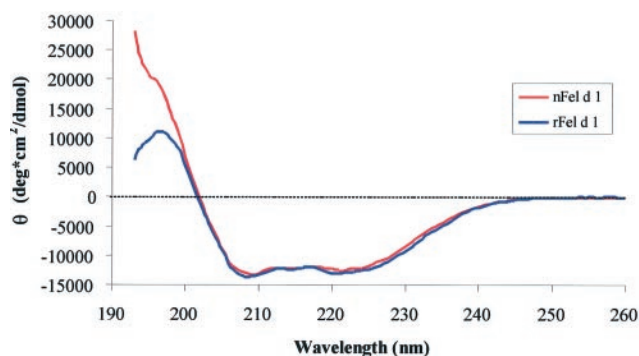


FIG. 4. Far-UV CD analysis of rFel d 1 and nFel d 1. The spectra are expressed as the mean residue ellipticities (θ) at a given wavelength.

part. We compared this ability of IgE antibodies in sera from 15 subjects sensitized to cat to detect rFel d 1(2 + 1), nFel d 1, and a Fel d 1 peptide mixture using ELISA. All sera from cat-allergic patients showed elevated IgE concentrations compared with a pool of serum from non-cat-allergic patients (Fig. 7). We observed similar responses for rFel d 1(2 + 1) (OD mean, 0.412) and nFel d 1 (OD mean, 0.384). There was a significantly lower IgE response to the peptide mixture (OD mean, 0.288) compared with rFel d 1(2 + 1) and nFel d 1, (analysis of variance, $p < 0.001$ and $p < 0.01$, respectively).

rFel d 1(2 + 1) Contains the IgE Epitopes of nFel d 1—The capacity of serially diluted rFel d 1(2 + 1), nFel d 1 and a mixture of chains 1 and 2 to compete with the binding of patient IgE to microtiter plate-bound nFel d 1 was compared using ELISA. All three Fel d 1 preparations exhibited competing activity. The rFel d 1(2 + 1) fusion protein inhibited IgE similarly as nFel d 1, as shown by the proximity and slopes of the dose-dependent inhibition curves in the sensitive range (0.01–0.33 μ g/ml) (Fig. 8). The mixture of chains 1 and 2 exhibited >25-fold reduced capacity to compete with IgE binding. By homologous inhibition of nFel d 1, a residual capacity to block IgE was evident using 1 and 3 μ g/ml.

Biological Activity of rFel d 1—The biological activity of rFel d 1(2 + 1) and nFel d 1 was evaluated in cell preparations donated by two cat-allergic patients. The surface marker CD203c is up-regulated exclusively on basophils in response to allergen cross-linking of the high affinity IgE receptor, FcεRI (38). The capacity of rFel d 1(2 + 1) and nFel d 1 to activate expression of CD203c on basophils was similar and compared well to that of anti-IgE, which was used as a positive control (Fig. 9a).

The lymphoproliferative responses after the challenge of cultured PBMCs with rFel d 1(2 + 1) and nFel d 1 were analyzed

by cell incorporation of [^{3} H]thymidine. Both rFel d 1(2 + 1) and nFel d 1 exhibited equally good proliferation (Fig. 9b).

DISCUSSION

The aim of this study was to generate recombinant major cat allergen Fel d 1 with biochemical and immunological properties comparable with the natural counterpart. Attempts to reconstitute a mixture of two isolated polypeptides comprising three disulfide bonds produced in *E. coli* have sometimes been only partially successful (32, 33, 40). Therefore we engineered a head-to-tail construct of the two polypeptide chains of Fel d 1, which we considered would facilitate a uniform and natural-like folding. Interestingly, a high yield of properly refolded protein from the inclusion body was achieved using a similar single polypeptide strategy in the production of recombinant human insulin produced in *E. coli* (41, 42). Similarly, a high level expression *E. coli* system, which produces proteins without carbohydrates, was also used for the Fel d 1(2 + 1) hybrid molecule because there is evidence that deglycosylated nFel d 1 is still IgE reactive (20). Furthermore, nonspecific IgG antibody binding to rFel d 1 expressed in insect cells has been observed, possibly directed to the exogenous carbohydrate moiety associated with chain 2 (34). On the one hand, a potential advantage of direct fusion is that no extra amino acids are included within the molecule that otherwise might compromise the use of the molecule in diagnosis and treatment. On the other hand, concerns can be raised regarding whether a direct fusion induces constraints or unfolding, an aspect analyzed in this study and found to be insignificant for rFel d 1(2 + 1) by comparison with nFel d 1 in CD and ELISA measurements.

To produce a dimer composed of the two Fel d 1 chains that closely mimics the natural counterpart, two constructs were prepared. The first construct (rFel d 1(1 + 2)) contained an N-terminal chain 1 fused to a C-terminal chain 2. The analysis of this construct and another construct (rFel d 1(2 + 1)) consisting of an N-terminal chain 2 fused to a C-terminal chain 1 for specific IgE reactivity showed that the rFel d 1(2 + 1) construct exhibited superior IgE reactivity. In the case of rFel d 1(1 + 2), the lower IgE binding may be explained by tension induced by linking the two chains, because mass spectrometry indicated that the SS linkages were the same in the two Fel d 1 constructs. Because IgE antibodies from allergic patients are formed by sensitization against the wild type natural Fel d 1, it was assumed that the rFel d 1(2 + 1) construct represents a closer mimic of the natural Fel d 1 than the rFel d 1(1 + 2) construct.

An important structural feature of nFel d 1 is the formation of stable non-covalently associated homodimers. The ability of the rFel d 1(2 + 1) construct to form dimers was investigated by several methods. In early experiments, the 30-kDa rFel d 1(2 + 1) fraction isolated from SEC indicated a homodimer by virtue

FIG. 5. **Time-dependent dissociation analysis of immobilized rFel d 1(2 + 1) by surface plasmon resonance.** The dissociation constant was analyzed over a period of 5 s, starting at 1230 s and 2780 RU (arrow 1). The relative loss of mass after immobilization of rFel d 1(2 + 1) (arrow 1) and deactivation (arrow 2, 2200 s) was 1487 RU. A monomeric control protein, BB, showed no loss of response during the same time period (3055–3057 RU). Lane 1, NHS/EDC activation; lane 2, protein immobilization; lane 3, dissociation phase; and lane 4, ethanolamine deactivation.

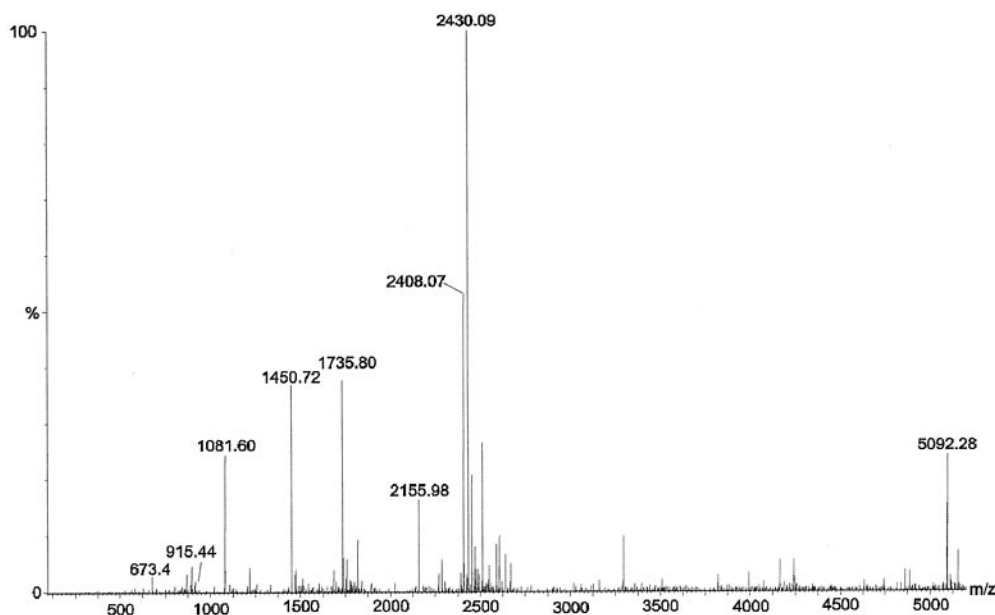
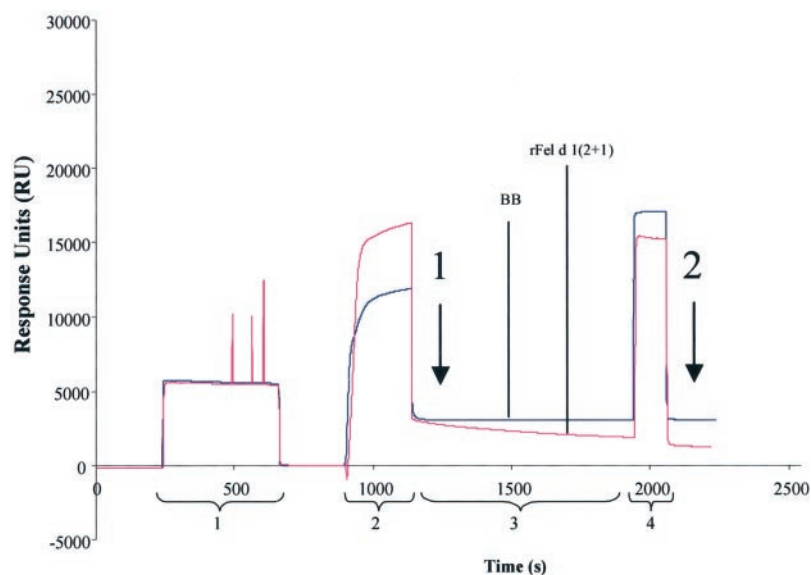


FIG. 6. **Electrospray mass spectrum from analysis of non-reduced rFel d 1(2 + 1) digested with trypsin.** The data have been processed using the software (MaxEnt 3, Micromass) to convert multiply charged ions to singly charged species only. The masses detected are summarized and interpreted in Table II.

of its elution position and corresponding molecular mass. The difference in molecular mass to the cat dander-derived 35–38-kDa nFel d 1 may be explained by the presence of 10–20% *N*-linked carbohydrates (16, 22) in the natural allergen. We further investigated the possible homodimer formation via re-chromatography of the isolated 30-kDa fraction by SEC under dissociating conditions. Now the corresponding component eluted as a 15-kDa peak in agreement with the findings from SDS-PAGE using a non-reduced sample, suggesting a non-covalently associated dimer. Finally, the rFel d 1(2 + 1) was analyzed by surface plasmon resonance with the assumption that a dissociation rate should be possible to calculate if a dimer was attached to the chip. The time-dependent dissociation indicated a tight protein-protein association, which was also supported by the fact that no peak corresponding to the size of a monomer could be detected in SEC (Fig. 2). Furthermore, the sensorgram obtained suggested a dimer by the roughly 50% decrease in response measured after deactivation.

Chain 1 of Fel d 1 shares 30% sequence identity to the Clara

cell 10-kDa protein (CC10) (17), which supports the notion that Fel d 1 is structurally similar to this secretoglobin protein. Interestingly, a notable difference in the molecular mass of rFel d 1(2 + 1) was evident in non-reduced SDS-PAGE (16 kDa) (Fig. 2, lane 6) compared with the calculated molecular mass (19.2 kDa). Such anomalous migration has also been observed for CC10 (28).

Biological recognition of proteins is dependent on the primary structures, displayed as linear T cell epitopes in the cavity of MHC molecules on antigen-presenting cells. Equally important for the biological functions are the three-dimensional structures, which, in turn, depend on secondary structure and, frequently, on correct and stable disulfide bonds. Therefore, we analyzed and compared the primary structure and biologic activity of rFel d 1(2 + 1) and nFel d 1 by lymphoproliferation and CD203c assays and the secondary structure by CD measurements and determined the intra-chain disulfide linking through trypsin cleavage and mass spectrometry. The secondary structure (20) and disulfide bond pattern (22) of nFel

TABLE II
Tryptic fragments and the corresponding mass values for the rFel d 1(2 + 1) structure

The experimental values were determined by nano-ESI mass spectrometry after tryptic digestion of the non-reduced protein preparation (cf. Fig. 6). The fragment pairs T6/T15, T8/T10, and T2/T18 were found to be linked via disulfide bridges between cysteine residues in these segments (linked Cys residues are in bold and underlined, corresponding mass values are underlined; see also Fig. 1). The masses of the individual, non-linked fragments could not be detected.

Tryptic fragment	Residues	Sequence	Theoretical mass [M + H]	Experimental mass [M + H]
T5	44-44	(K)K(I)	147.11	Not found
T11	100-100	(K)R(D)	175.12	Not found
T14	132-134	(R)ILK(N)	373.28	Not found
T1	0-2	(-)MVK(M)	377.22	Not found
T4	40-43	(R)TAMK(K)	450.24	450.75
T9	87-92	(K)LNTLGR(E)	673.40	673.40
T16	141-146	(K)MTEEDK(E)	752.31	Not found ^a
T3	32-39	(K)VNATEPER(T)	915.45	915.44
T17	147-155	(K)ENALSLLDK(I)	1002.55	Not found ^a
T13	122-131	(K)ALPVVLENAR(I)	1081.64	1081.60
T7	58-71	(R)VLDGLVMTTISSSK(D)	1450.78	1450.72
T6	45-57	(K)IQDCYVENGLISR(V)	<u>2156.01</u>	<u>2155.98</u>
T15	135-140	(K)NCVDAK(M)		
T8	72-86	(K)DCMGEAVQNTVEDLK(L)	<u>2408.11</u>	<u>2408.07</u>
T10	93-99	(R)EICPAVK(R)		
T12	101-121	(R)DVDLFLTGTPEYVEQVAQYK(A)	2430.17	2430.09
T2	3-31	(K)MAETCPIFYDVFFAVANGNELLLDLSLTK(V)	<u>5092.46</u>	<u>5092.28</u>
T18	156-170	(K)IYTSPLCLEHHHHHH(-)		
T16 + T17	141-155	(K)MTEEDKENALSLLDK(I)	1735.86	1735.80

^a Not found as a separate fragment. Detected as a part of the larger fragment T16 + T17 with [M + H] 1735.80, resulting from a miscleavage at Lys¹⁴⁶ which is surrounded by acidic residues; see Fig. 1.

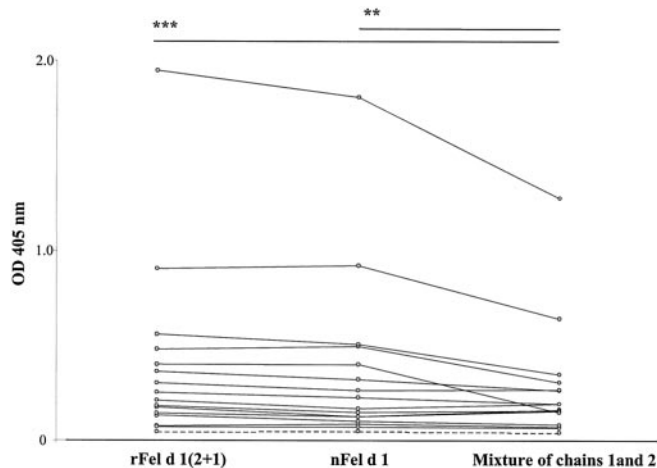


FIG. 7. IgE responses to Fel d 1 in individuals sensitized to cat in direct ELISA. A comparison of IgE binding to a microtiter plate coated with rFel d 1(2 + 1), nFel d 1, or a mixture of Fel d 1 chains 1 and 2 were made. A serum pool of non-cat-allergic patients is included (dotted line). Analysis of variance: **, $p < 0.01$; and ***, $p < 0.001$.

d 1 as well as the proliferation of cultured PBMC in the presence of nFel d 1 were found to correspond well to those observed for rFel d 1(2 + 1). Thus, the structure of rFel d 1(2 + 1) forms a basis for a stable and immunoreactive allergen with an anti-parallel orientation of the polypeptide chains (17, 22).

From an epitope-probing as well as clinical point of view, it is important to accurately establish levels of allergen-specific antibodies in serum from, for example, cat-allergic patients. The ability to detect allergen-specific IgE in serum from 15 cat-allergic patients to rFel d 1(2 + 1), nFel d 1, and a mixture of Fel d 1 peptides was evaluated using direct ELISA. No significant difference in response to IgE was detected for recombinant and natural Fel d 1, indicating that all relevant IgE epitopes are present in the rFel d 1(2 + 1) structure. Also, the results suggest that the carbohydrate side chain is not crucial for the folding or else serves as an epitope of nFel d 1. The comparable behavior of rFel d 1 and nFel d 1 was also implied in the competition ELISA. The two protein preparations revealed the same capacity to compete with IgE in serum for

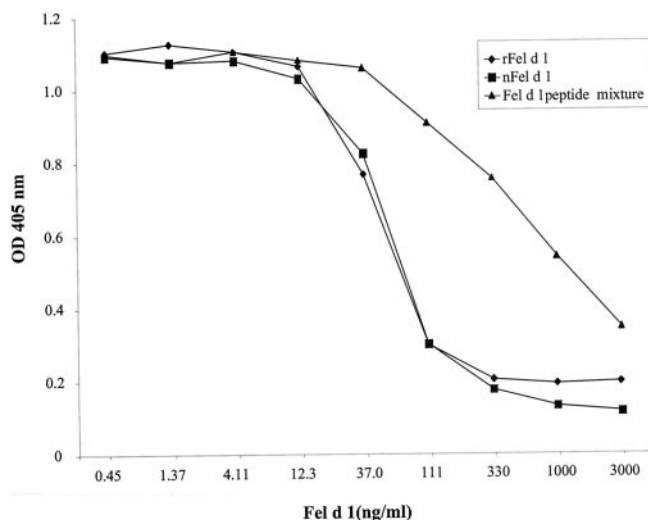


FIG. 8. Dose-dependent inhibition of serum IgE from a pool of individuals sensitized to cat. Pooled sera from 15 individuals were pre-incubated with 3-fold dilutions of rFel d 1(2 + 1) (◆), nFel d 1 (■), and a mixture of chains 1 and 2 (▲) and subsequently added to ELISA plate-bound nFel d 1.

binding to microtiter plate-bound nFel d 1 in ELISA. The somewhat better homologous inhibition achieved using high concentrations of nFel d 1 is likely to be caused by the inhibition of antibodies present in the serum pool by matching impurities in the nFel d 1 preparation. The mixture of chains 1 and 2 showed significantly lower IgE binding capacity in direct ELISA and a lower specific activity in the inhibition assay, which is consistent with previous findings (32, 33) suggesting a distorted protein preparation with fewer exposed epitopes.

In conclusion, we have constructed and, for the first time, produced in *E. coli* a recombinant Fel d 1 molecule by direct fusion of chain 2 and chain 1 with structural features mimicking that of the natural allergen. Thus, we propose this novel molecule as a suitable candidate for solving the three-dimensional structure of Fel d 1 and, furthermore, as a candidate for diagnosis and therapy of cat-allergic patients.

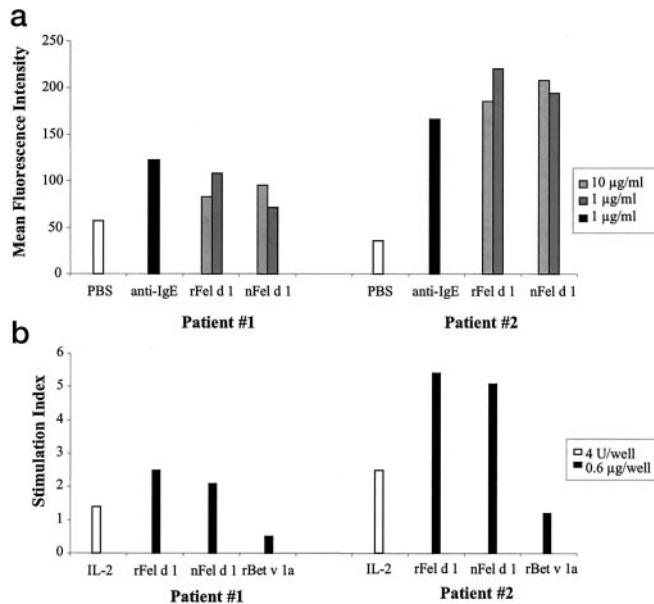


FIG. 9. Biologic activity of rFel d 1(2 + 1) and nFel d 1 in two patients allergic to cat. The induction of CD203c expression on basophils after incubation of blood samples with rFel d 1, nFel d 1, and, for control purposes, PBS and anti-IgE (a). Specific inductions of lymphoproliferative responses were obtained in PBMCs using rFel d 1, nFel d 1, and, for control purposes, with recombinant birch pollen allergen rBet v 1 and interleukin-2 (b).

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