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EXPRESSION OF HETERODIMER BY USE OF A BACULOVIRUS EXPRESSION SYSTEM*

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Fel d 1 is a major cat allergen inducing allergic rhinitis and asthma in sensitized individuals. It has a more complex structure when compared with other allergens and therefore expression of recombinant Fel d 1 has been considered a challenge. The present study shows for the first time that a Baculovirus expression system is able to produce an intact rFel d 1 molecule that is glycosylated and structurally equivalent to the natural cat allergen, nFel d 1. Enzymatic digestion of rFel d 1 and further analysis by use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) resulted in a complete coverage of the amino acid sequence of rFel d 1. In addition, the three disulfide bridges at the positions α70-β77, α44-β48, and α3-β73 were verified. The N-glycan structure of rFel d 1 was investigated by a combination of MALDI-TOF MS and monosaccharide analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAC). The N-glycosylation analyses of rFel d 1 refer to a pattern of glycoforms including core α1.3-fucosylation that is different from nFel d 1. Further characterization by use of human serum IgE, histamine release, and lymphocyte proliferation assays demonstrated that the immunological characteristics of rFel d 1 are similar to those of nFel d 1. Detailed characterization of both natural and recombinant allergens provides tools to explore immunological mechanisms associated with allergen sensitization and desensitization.

* The abbreviations used are: MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPAEC-PAC,

Effective in modulating allergic responses and resulting in down-regulation of allergen-specific T-cell responses (3). Conventional specific allergen immunotherapy is performed by the use of standardized natural allergen extracts (4). Cat allergen extract contains a number of allergenic molecules, such as Fel d 1 (5–7), cat albumin (Fel d 2) (8), and cystatin (Fel d 3) (9). Fel d 1, however, has been considered to be the major allergen (1). Immunodominant characteristics have made it a prominent candidate in efforts to develop novel vaccines for treatment of cat allergy (10–14). A foremost ongoing approach is the development of hypoallergenic vaccines, which are based on recombinant technology (3).

Natural Fel d 1 is a noncovalently linked ~38-kDa dimer that is composed of two ~18-kDa subunits. Each subunit comprises α- and β-chains that are encoded by two separate genes (15, 16). Natural Fel d 1 isolated from cat dander is composed of a mixture of variants comprising both full-length and truncated versions of β-chain (17–19). The folding of the polypeptide chains results in an anti-parallel orientation of the α- and β-chain(s) held together with three disulfide bridges (20). Correct orientation of the α- and β-chain(s) is considered to be critical because IgE-related antigenic determinants are conformation-dependent (17–23). Further analyses of the natural molecule have verified that the single N-glycosylation site in the β-chain is carrying heterogeneous tetrantenary complex type of structures. This heterogeneity is caused by terminal sialic acids, fucose, and β-galactose residues (20).

The anti-parallel orientation of the α- and β-chains together with conformation-dependent allergenicity has caused major challenges in efforts to express Fel d 1 as a recombinant (r) allergen. Both the α- and β-chain(s) have been expressed separately in Escherichia coli (18, 19, 24, 25). Individual chains, however, show markedly reduced immunogenicity (17–19, 24, 25). Recent studies have focused on expression of Fel d 1 as a fusion protein in a Baculovirus expression system (12) and as a His-tagged homodimer in E. coli (14). The recently solved three-dimensional structure of a head-to-tail construct expressed in E. coli suggests that Fel d 1 belongs to the secreto-
diation was combined to analyze the amino acid sequence and disulfide bridge formation in rFel d 1. The N-glycan structure of rFel d 1 was investigated by combination of MALDI-TOF MS and monosaccharide analysis by HPAEC-PAD. The immunobiological characterization and comparison with nFel d 1 was performed in vitro using IgE inhibition, histamine release, and lymphocyte proliferation assays.

**EXPERIMENTAL PROCEDURES**

**Construction of the Synthetic Genes Encoding Fel d 1 Polypeptide Chains**—Synthetic genes encoding Fel d 1 α- and β-chains (α: M74952/β: M77341) were assembled by PCR using overlapping oligonucleotides (Table I). In short, primers A1+A2 and A3+A4 for the α-chain and B1+B2 and B3+B4 for the β-chain were annealed and amplified in six cycles. Extension primers α-chain (ARF/ARR) and β-chain (BRF/BRF) with specific restriction sites were used to rescue the full-length products. The fragments were then cloned into pCR4-TOPO vector (Invitrogen), and their sequences were confirmed (ABI PRISM® 377 DNA sequencer; Applied Biosystems, Framingham, MA).

**Construction of Recombinant Baculovirus Vector**—The construction of the recombinant Baculovirus vector and the expression of the rFel d 1 was performed by CeNeS Pharmaceuticals plc (Cambridge, UK). The cDNA encoding the mature α- and β-chains were subcloned into Baculovirus shuttle vector pFastBacDUAL (Invitrogen). The mellitin signal sequence was engineered to the N-terminal of both genes by PCR (Fig. 1). The recombinant dual vector was then transformed into MAX efficiency DH10Bac™ competent cells (Invitrogen) containing the baculovirus genome. Within the cell a transposition takes place between a mini-AttR7 target site and the mini-Tn7 element on the vector when recombinant virus is generated. The resulting recombinant bacmid was confirmed according to the suppliers’ manual (Invitrogen).

**Expression of the rFel d 1 —Spodoptera frugiperda** (SF21) (Invitrogen) cells were used to generate the primary virus titer for the expression of rFel d 1. The SF21 cells were grown in 47.5% ExCell 401/HiRH Biosciences, 47.5% TC100 (Invitrogen) and 5% heat-inactivated fetal bovine serum (Invitrogen) as suspension cultures in shaker flask(s) (24). SF21 cells were transfected with recombinant bacmid DNA in the presence of Lipofectin (Invitrogen) according to the supplier’s manual (Invitrogen). The culture medium was collected 7 days post-transfection. The virus titer was assessed by plaque assay (29). For large virus stock production SF21 cells were infected with the recombinant virus at a multiplicity of infection of 0.5 at a cell density of 1 × 10⁶ cells/ml in a spinner bottle. The virus was harvested 7 days post-infection and titrated again by plaque assay. High Five™ cells (Invitrogen) were used for protein production and cultured according to the manufacturer’s instruction. For protein production the cells were infected at a multiplicity of infection of 10. The cell supernatant was harvested 96 h post-infection.

**Purification of Natural and rFel d 1**—Natural Fel d 1 was isolated by use of monoclonal immunoaffinity chromatography from dried cat allergen extract (ALK-Abello®) as described (20). Natural Fel d 1 was then subjected to HR 5/5 Mono Q (Amersham Biosciences) column and eluted with a linear gradient of 0–100% 20 mM Tris-Cl, 0.5 mM NaCl, pH 7.5, in 20 min. Eluted fractions containing nFel d 1 were pooled and subjected to size exclusion chromatography (Amersham Biosciences) in a 10 mm NH₂/HCO₃ buffer and freeze dried. For the immunological assays, rFel d 1 was dissolved into sterile Dulbecco’s phosphate-buffered saline (Innovit). Endotoxin levels in the purified Fel d 1 preparations were <15 EU/mg as determined by Limulus amoebocyte lysate assay (Bio Whittaker, Walkersville, MD).

Recombinant Fel d 1 was isolated from the High Five™ cell culture supernatant. The cells were gently centrifuged, and the culture supernatant was collected and sterile filtered. The purification of rFel d 1 followed the protocol described for the nFel d 1.

**SDS-PAGE Analysis of Affinity Purified rFel d 1**—1–16 Tris-Tricine SDS-PAGE (Invitrogen) was used to analyze collected fractions (10 μl/10 ml) from monoclonal affinity chromatography. The electrophoresis was performed in reducing conditions according to the manufacturer’s instructions and stained with silver (Invitrogen).

**Protein Measurements**—A Lambda 800 UV-visible spectrophotometer (PerkinElmer Life Sciences) was used to measure protein concentration at 280 nm using the absorption coefficient A₅₅₀ (1 mg/ml⁻¹ cm⁻¹) = 0.356 for both rFel d 1 and nFel d 1.

**Reversed-phase Chromatography**—Lyophilized rFel d 1 was first dissolved (47 pmol/μl) into Milli-Q grade water (Millipore, Bedford, MA). 5 nmol of rFel d 1 in 0.06% trifluoroacetic acid (Rathburn Chemicals, Peeblesshire, UK) was subjected to a 4.6 × 250-mm Jupiter, C₄ reversed-phase column (Phenomenex, Torrence, CA). The column was equilibrated with 0.06% trifluoroacetic acid, and the sample was eluted with 0.05% trifluoroacetic acid, 80% acetonitrile (Riedel-de-Hil, Seelze, Germany) in a gradient of 25–55% in 15 column volume and 55–80% in 5 column volume. Recombinant Fel d 1 eluted as a single peak and was collected and dried in a Speedvac. The dried fraction was redissolved into water, divided into 1-nmol aliquots, dried, and stored at −20 °C.

**Mass Spectrometric Analyses**—Mass spectrometric analysis was performed on a Voyager-DE™ STR Biospectrometry™ (Applied Biosystems, Foster City, CA) MALDI-TOF MS instrument by use of laser-ablation voltage of 25 kV and a nitrogen laser at 337 nm. The spectra were acquired in the positive ion mode and calibrated externally. MS/MS of selected peptides was performed on a Micromass® Q-Tof Ultima MALDI mass spectrometer (Waters Corporation, Manchester, UK).

20 pmol of intact rFel d 1 was desalted by custom-made micro columns packed with POROS R1 (Applied Biosystems, Framingham, MA) reversed-phase resin (3 μm). The samples were eluted directly on MALDI targets with 1.0 μl of matrix solution containing 10 μg/μl α-cyano-4-hydroxy-cinnamic acid or 2,5-dihydroxybenzoic acid in 70% acetonitrile, 0.1% trifluoroacetic acid. To analyze the molecular mass of the α- and β-chain, 20 pmol of rFel d 1 was reduced by 45 mM dithiothreitol (Sigma) at 56 °C for 30 min and alkylated by 100 μM iodoacetamide (Sigma) for 30 min at room temperature in the dark. Desalting columns packed with MALDI-TOF MS instrument of the sample was performed as described for the intact protein. Trypsin (NovoNordisk A/S, Bagsvaerd, Denmark), acyl-CoA-biding protein (Sigma), and a Sequazyme™ peptide mass standard kit (Applied Biosystems) were used as external calibrants. Theoretical masses were calculated by GPMAW (Lighthouse Data, Odense, Denmark).

**Proteolytic Digestions**—500 pmol of rFel d 1 was reduced and alkylated as described above. The sample(s) were dissolved into 50 μM high performance anion exchange chromatography with pulsed amperometric detection; Tricine, N-[2-hydroxy-1,1-bia(hydroxymethyl)ethyl]glycine; Pipes, 1,4-piperazinediethanesulfonic acid; PBMC, peripheral blood mononuclear cell.
Assignment of the disulfide bridges was performed by enzymatic digestion employing endoproteinase Asp-N (Calbiochem) in 50 mM Tris-HCl, 0.2 M NaCl, 1 M urea, pH 8.5, and by sequential digestion with 3% (w/w) trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI) and 3% (w/w) chymotrypsin (Wako Chemicals GmbH, Richmond, VA) (20) in 50 mM NH₄Ac, pH 6.0.

**Edman Degradation**—Sequencing of the rFel d 1-derived peptides was performed with Hewlett Packard G1000A sequencer (Palo Alto, CA) equipped with a Hewlett Packard 1090 series II liquid chromatograph and run as described by the manufacturer.

**Acid Hydrolysis and Monosaccharide Analysis**—1 mg of rFel d 1 was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 4 h, subsequently lyophilized in a Speedvac, and resuspended in water. The monosaccharides were analyzed by HPAEC-PAD using a Dionex (Sunnyvale, CA) P-500 chromatographic system consisting of Carbo PacPA-10 preanalytical and analytical columns, an AS50 auto sampler, a GP40 gradient pump, and an ED40 electrochemical detector. The flow rate was 1 ml/min, and the injection volume was 10 μl. Separation was achieved isocratically with water as eluent. A pump with 600 mM NaOH as eluent was used to improve the sensitivity of detection. L-fucose (Merck), D-xylose (Fluka), D-mannose (Merck), D-glucose (Merck), D-galactose (BDH Chemicals Ltd., Poole, UK), GlcNAc (BDH Chemicals Ltd.), N-acetylgalactosamine (BDH Chemicals Ltd.), and D-arabinose (Sigma) were treated as described above and used as standards.

**Structural Analysis of the N-Glycan**—Removal of N-glycan was assessed with recombinant N-glycosidase F from *Flavobacterium meningosepticum* (Roche Applied Science) and N-glycosidase A from almonds (Roche Applied Science) at 37 °C for 18 h. The resulting products were then analyzed by MALDI-TOF MS (Applied Biosystems) (30). The sequential digestions with glycosidases were performed following the procedure of Kroll-Kristensen et al. (20).

**IgE Inhibition Analyses**—The IgE inhibition experiments were performed on an ADVIA Centaur Immunoassay system (Bayer Diagnostics, Denmark) (31). Pooled human serum IgE from cat-allergic individuals (n = 4) was coupled to the solid phase absorbed IgE was determined as the number of relative light units after the addition of ADVIA Centaur Lite reagent (Universal reagent pack). All of the inhibition experiments were performed as triplicates, and the data sets (log₁₀(concentration), mean (DoB)) were fitted to a four-parameter logistic function using GraphPad Prism version 4.01 (GraphPad Software, San Diego, CA).

**Histamine Release Assay**—Histamine release assay was performed using freshly drawn blood from cat-allergic individuals (n = 4) and controls (n = 4). The tested individuals gave their informed consent to donate blood for research purposes. The antigens, rFel d 1 and nFel d 1,
were diluted into Pipes buffer (Invitrogen). Antigens were then mixed with the blood samples and incubated for 30 min at 37 °C. Analyses were performed in a final concentration ranging from 1.5 pg/ml to 500 ng/ml of antigen(s). The samples were then centrifuged, and the supernatants were analyzed by enzyme-linked immunosorbent assay (kit IM 2015; Immunotech). The release of histamine was measured at 405 nm by an EL 340 Biokinetics reader (Bio-Tek Instruments, Winooski, VT).

Lymphocyte Proliferation—T-cell lines specific to Fel d 1 were established from peripheral blood mononuclear cells (PBMCs) of cat-allergic patients (n = 5) as described previously for grass allergen-specific T-cell lines (32). In short, freshly isolated PBMCs (2 × 10^6/ml) were stimulated in 1-ml bulk cultures with natural or rFeld 1 (2 μg/ml) for 14 days with the addition of recombinant interleukin-2 from day 5. After 14 days T-cells were restimulated with irradiated autologous PBMCs, Fel d 1 (2 μg/ml), and 0.05 μg/ml phytohemagglutinin-P (Difco, Detroit, MI), and recombinant interleukin 2 was added at days 3, 4, and 5.

T-cell Stimulation Assay—On day 10 after restimulation, T-cells (2 × 10^4/well) were cultured with autologous PBMCs (105/well, irradiated 2500 Rad) in 200 ml of RPMI 1640 medium supplemented with 5% v/v AB serum (Cambrex Bio Sciences), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). The culture was performed with or without antigen in 96-well round-bottomed microtiter plates (Nunc). The cells were cultured for 72 h in a humidified atmosphere at 37 °C and 5% CO₂ followed by a 16-h pulse with 0.5 C [3H]thymidine/well, and thymidine incorporation was determined by scintillation counting. The results are expressed as the mean cpm values of four replicate cultures.

RESULTS

Purification of rFel d 1—Expression of rFel d 1 was demonstrated from the insect cell culture supernatant by SDS-PAGE and Western blotting (data not shown). Monoclonal affinity chromatography was used to capture rFel 1 from culture supernatant. SDS-PAGE analysis showed that rFel d 1 was bound to the monoclonal antibody column and eluted in a single step. Both α- and the β-chains were detected when stained with silver (Fig. 2). Affinity purified native rFel d 1 was then analyzed by MALDI-TOF MS, which revealed a major peak in the m/z ranging from 18,000 to 19,000. In addition, a peak at m/z 8000 was seen, indicating overexpression and co-purification of free α-chain (data not shown). Anion exchange and size exclusion chromatography were used to remove the free α-chain and other impurities. For MS analyses, rFel d 1 was subjected to reversed-phase chromatography. The total yield of purified dry weight rFel d 1 was from 1 to 3 mg/liter of culture supernatant. The purity of reduced and nonreduced natural and rFel d 1 was verified by MALDI-TOF MS as described below.

Analysis of Native and Reduced rFel d 1—Analysis of rFel d 1 by MALDI-TOF MS revealed several peaks in the m/z range from 18353.48 to 19377.20 (Fig. 3a). A closer inspection of the mass spectrum revealed spacing between peaks that is diagnostic of glycosylation. In addition, a minor peak (Fig. 3a, asterisk) at m/z 17,700 referring to nonglycosylated rFel d 1 was detected. Following reduction and S-carboxymethylation a peak appeared at 8043.60, and several minor peaks appeared in the mass range from 10700.40 to 11385.68 (Fig. 3b). The observed signal at 8043.60 was in agreement with the theoretical value m/z 8043.25 for the α-chain. This result indicates a correct cleavage at the N terminus and overall homogeneous expression of the α-chain.

The peaks in the m/z range 10,700.40 to 11,385.68 showed similar spacing as the native molecule, indicating glycosylation of the β-chain (Fig. 3b). As described below, subsequent diges-
tion with N-glycosidase A confirmed glycosylation at Asn<sup>33</sup>.

**Primary Sequence Analysis and Orientation the rFel d 1 α- and β-Chains**—Reduced and carboxymethylated rFel d 1 was digested with endoproteinase Asp-N, and the peptides were separated by reversed-phase chromatography. The peptides were identified by N-terminal sequencing, MS-MS, and/or MALDI-TOF MS. These peptides are referred to as F-1 to F-20 in a Table II. Enzymatic digestion with Asp-N gave full sequence coverage for the β-chain. The valine in the α-chain at position 10 was verified from the enzymatic cleavage with trypsin and chymotrypsin (data not shown). This finding suggests that a subpopulation of rFel d 1 would have modified N termini.

Partial oxidation of the methionine residues was detected in peptides F-7, F-12, and F-13 when analyzed by MALDI-TOF MS, and partial deamidation was found in peptides F-7, F-12, and F-13 when analyzed by MALDI-TOF MS. The combined digestion with trypsin and chymotrypsin revealed disulfide bond linkages between residue(s) α1-8, β2-83, α36-45, α11-25, α62-70, β26-46, β84-92, β36-46, β60-71, β5-11, αβ11-1, α53-61, α46-61, α19-39, β57-59, β17-47, α19-35, α19-45, β12-25, 1–8, N-terminus (Table II). Analyses of the N-glycan will be described below. Modification referring to γ-carboxylation was found from the N terminus of the α-chain (F-2). In N-terminal sequencing, the peptide was found to be blocked, and it was subsequently identified by MS-MS (data not shown). This finding suggests that a subpopulation of rFel d 1 would have modified N termini.

To determine the positions of the disulfide bond linkages, native rFel d 1 was digested with endoproteinase Asp-N and a combination of trypsin and chymotrypsin. Separation of the peptides was performed by reversed-phase chromatography, and all of the resulting peptides were analyzed by MALDI-TOF MS. The combined digestion with trypsin and chymotrypsin revealed disulfide bond linkages between residue(s) α1.3-linked fucose residue attached to the innermost GlcNAc residue (Fig. 4b). These results suggest that the glycan core is carrying α1.3-fucosylation, which is in correlation with the previous studies of T. ni-expressed recombinant proteins (33).

To further determine the composition of the glycan moiety of rFel d 1, the sample was hydrolyzed, and the released monosaccharides were analyzed by HPAEC-PAD. Fig. 5 shows the HPAEC-PAD chromatogram of monosaccharides released from rFel d 1. The results indicate that the N-glycan is composed of mannose, fucose, GlcNAc, and N-acetylgalactosamine residues. The m/z values and monosaccharide composition of the released glycans was used to predict their structures using GlycoMod (Expasy). The proposed structures are presented in Table IV. Supplementary characterization of the N-glycosidic glycans was performed by MALDI-TOF MS in combination with sequential glycosidase treatment. Removal of the terminal galactose residue was demonstrated by the sequential digestion; however, no evidence of sialic acids was detected (data not shown).

**Competition for Allergen-specific IgE Antibodies**—The presence of conformational specific IgE epitopes on recombinant Fel d 1 was addressed in an IgE inhibition assay using a serum pool derived from four cat-allergic patients. Serum IgE was captured by anti-IgE immobilized on paramagnetic beads. After washing, the binding of biotinylated cat extract to captured IgE was inhibited by the addition of dilution series of recombinant Fel d 1, natural Fel d 1, and extract, respectively. The inhibition curves determined for cat allergen extract, nFel d 1 and rFel d 1 are shown in Fig. 6. Natural Fel d 1 and rFel d 1

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### Table II

| HPLC fraction | Peptide | Theoretical value | Observed value | Observed difference | Verified by sequencing |
|---------------|---------|------------------|----------------|--------------------|-----------------------|
| F-1           | α1-8    | 972.53           | 972.55         | +0.02              | ND<sup>a</sup>         |
| F-2           | α1-8<sup>b</sup> | 1015.55          | 1015.50        | −0.05              | +                     |
| F-3           | β2-83   | 1352.33          | 1352.6         | +0.05              | ND                    |
| F-4           | α36-45  | 1216.64          | 1216.76        | +0.12              | +                     |
| F-5           | α11-25  | 1724.81          | 1724.60        | +0.21              | +                     |
| F-6           | α62-70  | 1098.53          | 1096.57        | +0.04              | +                     |
| F-7           | β26-46  | 2373.28          | 2348.85        | +0.15              | ND                    |
| F-8           | β84-92  | 1029.61          | 1029.60        | −0.01              | ND                    |
| F-9           | β36-46  | 2373.26          | 2373.26        | −0.02              | ND                    |
| F-10          | β60-71  | 1238.63          | 1238.68        | +0.05              | ND                    |
| F-11          | β5-11   | 929.46           | 929.46         | 0.00               | +                     |
| F-12          | β11-1   | 1358.65          | 1358.60        | +0.05              | ND                    |
| F-13          | α53-61  | 1002.55          | 1002.53        | +0.02              | ND                    |
| F-14          | α46-61  | 1806.88          | 1806.89        | +0.01              | ND                    |
| F-15          | α19-39  | 2434.30          | 2434.29        | −0.01              | ND                    |
| F-16          | β57-59  | 1537.77          | 1538.73        | +0.06<sup>b</sup>  | +                     |
| F-17          | β47-59  | 1537.77          | 1537.68        | −0.09              | +                     |
| F-18          | α19-35  | 1964.30          | 1964.05        | −0.25              | ND                    |
| F-19          | α19-45  | 3161.67          | 3161.65        | −0.02              | ND                    |
| F-20          | β12-25  | 1521.79          | 1521.60        | −0.19              | +                     |

<sup>a</sup> ND, not done.

<sup>b</sup> These mass differences can be explained by the reported modifications.

### Table III

| Disulfide bond | Theoretical value | Observed value | Reduced theoretical values | Observed values<sup>c</sup> |
|---------------|------------------|----------------|---------------------------|---------------------------|
| α44–β48      | 2156.01          | 2156.13        | 648.29                    | ND<sup>d</sup>            |
| α3–β73       | 2408.11          | 2408.58        | 759.40                    | ND<sup>d</sup>            |
| α3–β73<sup>d</sup> | 2208.01         | 2208.13        | 914.50                    | 915.51                    |
| α70–β77<sup>d</sup> | 2338.12         | 2338.25        | 1038.51                   | 1038.49                   |

<sup>a</sup> m/z + 1, respectively.

<sup>b</sup> ND, not detected.

<sup>c</sup> Disulfide bonds found from the endoproteinase Asp-N digest.
exhibit parallel inhibition curves indicating that the epitope structures of the natural and recombinant allergens are similar. Inhibition by cat allergen extract showed 100% inhibition, and both recombinant and natural Fel d 1 allergens exhibited equal inhibitions (87%) of the interaction between pooled serum IgE and the cat allergen extract. The EC50 (inhibitor concentration at 50% inhibition) values determined for the natural and recombinant Fel d 1 preparations differ by EC50(rFel d 1)/EC50(nFel d 1), indicating a difference in the concentration of recombinant and nFel d 1 determined at A280.

**Histamine Release Assay**—To investigate the biological effect of recombinant and natural Fel d 1 to stimulate basophil, degranulation was tested in histamine release assay using human basophiles derived from individual cat-allergic patients. The release of histamine was found consistent for both recombinant and nFel d 1 in all the four cat-allergic individuals tested, whereas the controls suffering from pollen allergies showed no release. Even though the molar amount of released histamine varied from patient to patient, the histamine response within the patients remained equal between the recombinant and the natural antigen (Fig. 7).

**T-cell Proliferation**—Allergen-specific T-cell cultures were obtained in most cases when PBMC established from cat-allergic patients were stimulated with natural and recombinant Fel d 1. However, initial stimulation with nFel d 1 resulted in specific T-cell lines in five of five patients, whereas lines were obtained from three of five patients with rFel d 1. When the individual T-cell lines were stimulated with either natural or recombinant Fel d 1, comparable responses were generally obtained even though the response differed between natural and recombinant Fel d 1 in some T-cell lines (Fig. 8). In addi-

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**TABLE IV**

| m/z values | Proposed structure |
|------------|--------------------|
| 1063.36    | [Hex]2[HexNAc]2[Deoxyhexose]2Na |
| 1079.38    | [Hex]3[HexNAc]2[Deoxyhexose]1Na |
| 1184.43    | [Hex]2[HexNAc]2[Deoxyhexose]2K |
| 1241.45    | [Hex]4[HexNAc]2[Deoxyhexose]1Na |
| 1387.51    | [Hex]3[HexNAc]2[Deoxyhexose]2Na |

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tion, all lines examined showed a clear Th2 cytokine profile with a high interleukin 5/interferon-γ ratio (CBA assay; BD Biosciences) (data not shown).

DISCUSSION

The ability to produce recombinant allergens with intact immunochemical properties and correct amino acid sequence and structure is of major importance for their potential use as diagnostic agents and as active ingredients in vaccines. Here we demonstrate the expression of recombinant Fel d 1 as a heterodimer that is structurally and immunochemically equivalent to the naturally occurring cat allergen Fel d 1.

The successful expression of rFel d 1 heterodimer was obtained by cloning the genes encoding Fel d 1 α- and β-chains one by one into a dual gene plasmid (Fig. 1). The expressed α- and β-chains were detected to from the cell culture medium as a mixture of free α-chain and intact αβ-heterodimer. As shown in several previous studies (17–23), monoclonal immunoaffinity chromatography was found to be particularly important in purification of both natural and recombinant Fel d 1. In addition, the co-purification of free α-chain verified that the monoclonal antibody used in the present study was Fel d 1 α-chain-specific. The following purification steps using anion exchange and size exclusion chromatography verified that rFel d 1 shares similar physical and chemical properties with nFel d 1.

The MS analyses of rFel d 1 were designed based on the methods that were previously optimized for nFel d 1 (20). We presumed that expressed as a glycosylated heterodimer, rFel d 1 would show structural characteristics similar to nFel d 1. This was first demonstrated when the three disulfide bridges were assessed. Only after sequential cleavage by trypsin and chymotrypsin were two of the rFel d 1 disulfide bridges (α44-β48 and α3-β73) verified. Similar results were previously obtained with nFel d 1 (20). The third bridge (α70-β7) was demonstrated after cleavage by endoproteinase Asp-N (Table III).

FIG. 6. Inhibition of IgE binding to biotinylated cat allergen extract Fel d by natural Fel d 1, recombinant Fel d 1, and Fel d extract. The individual points are means of triplicate determinations of the degree of binding (DoB) of biotinylated Fel d to IgE, compared with Fel d (■), nFel d 1 (●), and rFel d 1 (▲). The solid curves represent the four-parameter logistic fits determined for the individual data sets. The curves for nFel d 1 and rFel d 1 are parallel (identical Hill Slope) and nonparallel with the curve fitted for the cat allergen extract data.

FIG. 7. Specific histamine release in two representative cat-allergic individuals stimulated with rFel d 1 and nFel d 1. The cat allergen-specific IgE of the two individuals were measured by Magic Light (ALK-Abello). a, 7 SU/ml; b, 34 SU/ml. The release of histamine is comparable with individual total IgE level(s).
Expression of rFel d 1 as a Heterodimer

Table I

| Stim | nFel d 1 | rFel d 1 | rFel d 1 | rFel d 1 |
|------|---------|---------|---------|---------|
| 1    | +       | +++     | -       | -       |
| 2    | ++      | +       | -       | -       |
| 3    | +       | +       | ++      | ++      |
| 4    | +++     | +++     | -       | +       |
| 5    | +       | +       | +       | +       |

Fig. 8. T-cell lines generated from the peripheral blood of two cat-allergic patients through repetitive stimulation with natural or recombinant Fel d 1. Comparable levels of T-cell activation were found when Fel d 1-specific T-cell lines were initiated with natural and recombinant Fel d 1. The lines were subsequently stimulated with each allergen preparation as indicated for comparison. a, T-cell lines from 5 patients scored as follows. b, the exact response pattern of two individual patients (SI = cpm in allergen stimulated/cpm in cultures grown in medium alone (background)). +, SI 3; ++, SI > 10; ++++, SI > 30.

These results indicate that rFel d 1 is a stable molecule resisting enzymatic cleavage in a manner similar to nFel d 1. Demonstration of the disulfide bridges also showed that rFel d 1 α- and β-chains are in anti-parallel orientation as described for the nFel d 1 (20). Correct orientation of the two-polypeptide chains has been shown to be crucial for the allergenicity of this molecule, because the B-cell epitopes of nFel d 1 have shown to be conformation-dependent (17–19, 21–23). In the present study, partial deamidation in rFel d 1 may result from extended expression time (96 h). However, considerable changes for the immunogenicity in vitro were not detected either in IgE inhibition and/or histamine release assays. Deamidation of rFel d 1 may, however, have influenced the initial stimulation of some T-cell lines, modifying T-cell-specific epitopes (34, 36). On the other hand, comparable results were obtained in many cases when the lines were tested with the two Fel d 1 preparations.

Few groups have explored receptor-specific interactions between allergens and cells that are involved with allergen uptake (37). γ-Carboxylation was first discovered in proteins of the blood coagulation cascade (38), and it has recently been shown to facilitate the interaction of proteins with membrane receptors (39). Because this modification was not previously detected in nFel d 1 (20), the potential immunostimulatory role of γ-carboxylation in addition to deamidation(s) found in the subpopulation of rFel d 1 needs to be further investigated.

Numerous allergens are glycoproteins; however, the role of glycosylation versus allergenicity is poorly understood. Furthermore, the studies of glycosylation have mainly been focused on IgE or IgG binding and/or histamine release responses (22–23, 40). In the present study, the rFel d 1 β-chain was expressed with and without N-glycosylation (Table II, F-7/F-9). Furthermore, the glycosylation analysis suggests that the N-glycosylation profile of the rFel d 1 is different from nFel d 1. Natural cat allergen was shown to carry terminal sialic acids as well as terminal fucose residues, whereas they were not detected when T. ni expressed the rFel d 1. In addition, rFel d 1 was shown to carry core α-L3-fucosylation, which is not found present in nFel d 1 (20). Core α-L3-fucosylation is commonly found in plant and bee venom allergens (40). Previously studies have suggested that glycosylation is playing important structural and immunobiological roles in nFel d 1 (20, 23). In the present study different glycosylation patterns between natural and recombinant Fel d 1 did not show a significant effect for IgE-related immunological assays in vitro. However, recent findings by Cobb (41) show that some carbohydrates may facilitate important immunological responses through T-cell activation. Therefore, further studies are needed to elucidate the immunoregulatory role of specific carbohydrate structures in allergy.

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REFERENCES
1. Schou, C. (1996) Allergy and Allergic Diseases, 1st Ed., pp. 900–902, Blackwell Sciences, London.
2. Prussin, C., and Metcalfe, D. D. (2003) J. Allergy Clin. Immunol. 111, S486–S494.
3. Till, S. J., Francis, J. N., Nouri-Aria, K., and Durham, S. R. (2004) J. Allergy Clin. Immunol. 113, 1025–1034.
4. Lilja, G., Sundin, B., Graff-Lonnevig, V., Hedlin, G., Heilborn, H., Norrlind, K., Pegelow, K.-O., and Løwenstein, H. (1989) J. Allergy Clin. Immunol. 83, 37–44.
5. Ohman, J. L., Lowell, F. C., and Bloch, K. J. (1973) J. Allergy Clin. Immunol. 52, 231–241.
6. Leiternmann, K., and Ohman, J. L. (1984) J. Allergy Clin. Immunol. 73, 147–153.
7. Løwenstein, H., Lind, P., and Weeke, B. (1985) Allergy 40, 430–441.
8. Hilger, C., Grigioni, F., and Hentges, F. (1996) Gene Anal. 169, 285–296.
9. Ichikawa, K., Vailes, L. D., Pomes, A., and Chapman, M. D. (2001) Clin. Exp. Allergy 31, 1502–1504.
Expression of rFel d 1 as a Heterodimer

van Ree, R., van Leeuwen, A., Builer, I., Bond, J., and Aalberse, R. C. (1999) J. Clin. Allergy Immunol. 104, 1223–1230

Kaiser, L., Gronlund, H., Sandalova, T., Ljunggren, H.-G., van Hage-Hamsten, M., Achour, A., and Schneider, G. (2003) J. Biol. Chem. 278, 37730–37735

Reichert, J. C., and Paquette, C. (2003) Curr. Opin. Mol. Ther. 5, 139–147

Dwek, R. A., Butters, T. D., Platt, F. M., and Zitzmann, N. (2002) Nat. Rev. Drug Discov. 1, 65–75

O’Reilly, D. R., Miller, L. K., and Luckow, V. A. (1994) Baculovirus Expression Vectors: A Laboratory Manual, pp. 135–136, Oxford University Press, New York

Gobson, J., Northoff, E., Mirgorodskaya, E., Ekman, R., and Roepstorff, P. (1999) J Mass Spectrom. 34, 105–116

Pettersen, A. B., Gutmann, P., Mulvang-Gronager, P., Morkeberg, R., Bogestrand, S., Linneberg, A., and Johansen, N. (2004) Clin. Biochem. 37, 882–892

Wurtzen, P. A., Bufe, A., Wissenbach, M., Madsen, H. O., Ipsen, H., Arved, J., and van Neerven, J. J. (2003) Clin. Exp. Allergy. 29, 1614–1625

Hsu, T.-A., Takahasi, N., Teukamoto, Y., Kato, K., Shimada, I., Katsushii, M., Whiteley, E. M., Fan, J.-Q., Lee, Y.-C., and Betenbaugh, M. J. (1997) J. Biol. Chem. 272, 39062–39073

Weintraub, S. J., abd Mansor, S. R. (2004) Mech. Ageing Dev. 125, 255–257

Arentz-Hansen, H., McAdam, S. N., Molberg, O., Fleckenstein, B., Lundin, K. E., Jorgensen, T. J., Jung, G., Roepstorff, P., and Solild, L. M. (2002) Gastroenterology. 123, 803–809

Reefer, A. J., Carneiro, R. M., Custis, N. J., Platts-Mills, T. A. E., Sung, S.-S. J., Hammer, J., and Woodfolk, J. A. (2004) J. Immunol. 172, 2769–2772

Chen, C. L., Lee, C. T., Liu, Y. C., Wang, J. Y., Lei, H. Y., and Yu, C. R. (2003) J. Immunol. 170, 528–536

Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2730–2733

Walker, C. S., Shetty, R. P., Clark, K., Kuzko, S. G., Letsou, A., Olivera, B. M., and Bandypadhyay, P. K. (2001) J. Biol. Chem. 276, 7769–7774

Foetisch, K., Westphal, S., Lauer, I., Betzke, M., Altman, F., Kolarich, D., Scheurer, S., and Viehle, S. (2003) J Allergy Clin. Immunol. 111, 889–896

Cobl, B. A., Wang, Q., Txianabos, A. O., and Kasper, D. L. (2004) Cell 117, 677–687
Molecular Characterization of Major Cat Allergen Fel d 1: EXPRESSION OF HETERODIMER BY USE OF A BACULOVIRUS EXPRESSION SYSTEM
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