

Glutaraldehyde-Modified Recombinant Fel d 1: A Hypoallergen With Negligible Biological Activity but Retained Immunogenicity

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Background: Recombinant allergens are under investigation for replacing allergen extracts in immunotherapy. Site-directed mutagenesis has been suggested as a strategy to develop hypoallergenic molecules that will reduce the risk of side effects. For decades, chemically modified allergen extracts have been used for the same reason.

Aim: To evaluate whether glutaraldehyde modification is a good strategy to produce hypoallergenic recombinant allergens with retained immunogenicity.

Methods: Fel d 1 was cloned as a single construct linking both chains of the molecule and expressed in *Escherichia coli* and *Pichia pastoris*. After physicochemical purification, recombinant (r) Fel d 1 was chemically modified using glutaraldehyde. The effect of modification on immune reactivity was evaluated using radioallergosorbent test, CAP inhibition, competitive radioimmunoassay, enzyme-linked immunosorbent assay, basophil histamine release, and T-cell proliferation assays. Both natural and recombinant unmodified Fel d 1 were used as controls.

Results: rFel d 1 demonstrated similar IgE binding and biologic activity as its natural counterpart. Upon modification, IgE-binding potency decreased >1000-fold, translating into a >10⁶-fold reduction in biologic activity assessed by basophil histamine release. In contrast, the modified recombinant did not show a decreased but even a moderately increased capacity (1.5-fold) to stimulate proliferation of T cells ($P < 0.01$). Finally, it induced specific IgG antibodies in rabbits that recognized the unmodified allergen.

Conclusions: Chemical modification is a practical and highly effective approach for achieving hypoallergenicity of recombinant allergens with retained immunogenicity.

Key Words: allergoid, *Felis domesticus*, hypoallergen, immunotherapy, rFel d 1

(WAO Journal 2011; 4:113–120)

It is well established that Fel d 1 is responsible for most IgE reactivity in cat-allergic patients.^{1–6} Treatment of cat allergy by immunotherapy with epithelial extracts was demonstrated to be effective.^{7,8} From a regulatory perspective, treatment with mammalian extracts is not ideal because of the potential risk to transmit pathogens. The dominant role of Fel d 1 in cat allergy makes the development of a recombinant Fel d 1-based product an obvious choice. Fel d 1, a 38-kDa glycoprotein with N-linked oligosaccharides,⁹ was first cloned and expressed by Morgenstern et al.¹⁰ The allergen consists of two 19-kDa noncovalently linked heterodimers, each composed of a light α -chain and a heavy β -chain,¹⁰ encoded by 2 separate genes.¹¹ Three disulfide bridges connect the α -chain and β -chain in an antiparallel orientation.^{9,12} Expressed as individual chains in *Escherichia coli* (*E. coli*), IgE binding was poor,^{13,14} but combining both chains, followed by a lengthy refolding protocol, a recombinant was obtained with similar IgE reactivity as natural Fel d 1.^{1,14,15} More recently, Fel d 1 was also expressed as a single construct. In baculovirus both chains were connected in an antiparallel fashion by a linker sequence,^{16,17} whereas in *E. coli*, both chains were connected in a parallel orientation without linker sequence.¹⁸

One of the disadvantages of specific immunotherapy is the risk of anaphylactic reactions. By site-directed mutagenesis it is now possible to produce recombinant molecules with reduced IgE binding, so-called hypoallergens. This approach has been reported for several inhalant and food allergens. Mutating 6 amino acids of Bet v 1 gave a hypoallergenic but structurally unchanged molecule^{19,20} and similar results were reported for the peanut allergens Ara h 1–3.^{21,22} To our knowledge, this strategy has not yet been applied to Fel d 1. The disadvantage of this approach is that it requires considerable insight into the surface structure of an aller-

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gen to be able to predict which amino acids should be mutated. Random mutagenesis may be an alternative, but requires laborious screening protocols. Furthermore, reduction of IgE binding is sometimes limited and variable for individual patients.^{19,22}

Before the era of molecular biology, chemical modification by, for example, glutaraldehyde or formaldehyde was used as a way to reduce the IgE-binding potency of allergen extracts.²³ This was first described by Marsh et al.^{24,25} Here, we set out to evaluate the feasibility of chemical modification with glutaraldehyde of a recombinant allergen as an alternative to the production of a hypoallergenic mutant obtained by site-directed mutagenesis. To this end, we expressed Fel d 1 as a single construct in a parallel orientation, in *E. coli* and *Pichia pastoris* (*P. pastoris*).

MATERIALS AND METHODS

Cloning and Expression of Fel d 1

In one construct both chains (α - and β -chain) were cloned together using a linker oligo²⁶ in the pPICZ α A vector (Invitrogen, San Diego). After transformation into *P. pastoris* GS115 (His⁴⁺) and expression in a Bioflo 3000 bench-top fermentor, cells were harvested and the supernatant was stored at 4°C in 0.1% azide.

A second construct was created by directly linking the C-terminal residue of the β -chain with the N-terminal residue of the α -chain in the pET-19b vector. After transformation into *E. coli* BL21 (DE3) (Novagen Inc., Madison, WI) and expression, the cell pellet was frozen overnight at -20°C, thawed, and resuspended to one tenth of the original culture volume in 25 mM Tris/2 mM EDTA/pH 7.6. After sonication and pelleting of insoluble matter by centrifugation, the supernatant was saved for further processing of soluble rFel d 1.

Purification of Natural and Recombinant Fel d 1

rFel d 1 was purified in 2 steps: (1) hydrophobic interaction chromatography, using a phenyl-sepharose column; (2) ion-exchange chromatography, using a MonoQ 5/50 GL column (both GE Healthcare Biosciences AB, Uppsala, Sweden). The rFel d 1-containing fractions were pooled, dialyzed against PBS, and concentrated (sixfold), and purity of the sample was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Natural Fel d 1 (nFel d 1) was affinity-purified using monoclonal antibody (mAb) 4F7 as previously described.¹ Protein concentration was measured by the BCA method²⁷ (Pierce, Rockford, IL) as described by the manufacturer.

For all experiments *E. coli*-derived rFel d 1 was used unless indicated otherwise.

Murine Monoclonal Antibodies

MAb 4F7 to nFel d 1 was obtained as described by de Groot et al.⁶ MAbs 6B4 and 10F10 against reduced/alkylated nFel d 1 were obtained as described by van Milligen et al.²⁸ MAb 6B4 had been shown to be specific for the light chain and 10F10 for the heavy chain.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed as described previously.²⁹

Glutaraldehyde Modification of rFel d 1

Glutaraldehyde modification was done similarly for both recombinant preparations. Purified rFel d 1 (5 mL of 1.055 mg/mL from *E. coli*) was dialyzed on a 1-kDa Amicon membrane with 100 mM sodium carbonate (pH 9.0). The residue was collected and diluted in the same buffer to 21 mL, resulting in a protein concentration of 0.250 mg/mL (13.9 μ M, representing a total amount of 292 nmol); 1.37 mL of 25% glutaraldehyde (Merck, Darmstadt, Germany) was slowly added (final amount, 3.26 mmol). As rFel d 1 contains 12 lysine residues and glutaraldehyde has 2 aldehyde groups, the molar ratio aldehyde/rFel d 1 is 1860. The mixture was gently stirred overnight at room temperature; 6.54 mmol of glycine was added in a time span of 30 minutes to quench remaining free aldehyde groups. The mixture was stirred for 1 hour and dialyzed on a 10-kDa Amicon membrane. Protein was not measured in the final product because reliable results cannot be obtained for highly aggregated structures. For further analysis it was assumed that the modification procedure did not result in significant losses. A correction was applied to account for dilution of the sample.

Online High-Performance Size-Exclusion Chromatography Light Scattering

Online high-performance size-exclusion chromatography (HPSEC) light scattering was performed using a 7.8 \times 300 mm TSKgel G2000_{SWXL} column (Tosoh Bioscience, Stuttgart, Germany) in single-column mode on a HP1100 analytical chromatography system (Hewlett-Packard, San Jose, CA) equipped with a built-in UV detector and a sequential refractive index (RI), intrinsic viscosity (IV), and right-angle light-scattering detection (TDA 302, Viscotek Corp., Houston, TX) as previously described.³⁰

Rabbit Antisera

Two female New Zealand white rabbits were immunized and boosted 5 times at 4-week intervals with 100 μ g/mL glutaraldehyde-modified rFel d 1 (rFel d 1-mod) from *P. pastoris*. For each immunization, 1 mL of rFel d 1-mod was mixed with 1 mL of Montanide ISA-50 (Seppic, Paris).

Radioallergosorbent Test

For measurement of specific IgE or IgG against nFel d 1, rFel d 1, and rFel d 1-mod, a radioallergosorbent test (RAST) was performed as described previously.³¹ For IgE, the results were expressed as IU/mL, and for IgG, the results were expressed in % bound activity.

ImmunoCAP Inhibition

ImmunoCAP (Phadia, Uppsala, Sweden) was performed according to the manufacturer's instructions. For CAP-inhibition, a serum pool of cat-allergic patients ($n > 100$) was preincubated at room temperature for 1 hour with serial dilutions of inhibitor, before addition to cat ImmunoCAP (e1). The following inhibitors were used: nFel d 1, rFel

d 1, and rFel d 1-mod. Results were expressed as percentages of inhibition.

Competitive Radioimmunoassay

To quantify Fel d 1 in different samples, a competitive radioimmunoassay (RIA) or sandwich enzyme-linked immunosorbent assay (ELISA) were used. The competitive RIA was performed essentially as described elsewhere.³² The Fel d 1 concentration was calculated by using the standard curve of a house dust extract with known Fel d 1 content (14 U/mL). One unit was considered to be equivalent to 1 μ g of Fel d 1.

The sandwich ELISA was developed by Chapman et al³³ (Indoor Biotechnologies, Warminster, UK). Serial sample dilutions were assayed. Reference Fel d 1 standard (9.7 U/mL) was used at serial dilutions from 20 to 0.04 mU/mL. Plates were read in an ELISA microplate reader at 405 nm.

Stripped Basophil Histamine Release Bioassay

To assess biologic activity of Fel d 1, the stripped basophil histamine release (BHR) bioassay was performed. White blood cells were isolated from whole blood of a nonallergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere.^{34,35} Subsequently, cells were resensitized with the patient's serum ($n = 4$). Histamine release was performed with nFel d 1, rFel d 1, and rFel d 1-mod (of 0.01 pg/mL to 100 μ g/mL). Liberated histamine was measured by the fluorometric analysis essentially as described by Siraganian.³⁶

Allergen-Specific Proliferation of Freshly Isolated Peripheral Blood Mononuclear Cells

T-cell reactivity was assessed by measuring proliferation of peripheral blood mononuclear cells (PBMCs) from cat-allergic donors to nFel d 1, rFel d 1, and rFel d 1-mod. PBMCs were plated out at 2×10^5 cells per well in IMDM (Bio-Whittaker, Walkersville, MD) supplemented with 5% normal human serum (Bio-Whittaker) and gentamycin (80 μ g/mL; Duchefa, Haarlem, The Netherlands). Dilution series of allergen or allergoid (0.03–10 μ g/mL) were added at day 0, followed by addition of ³H-labeled thymidine at day 6 and harvesting of the radioactively labeled cells at day 7 to measure the Ag-induced proliferation.

RESULTS

Production, Purification, and Modification of rFel d 1

Constructs encompassing both chains of Fel d 1, either directly linked together (*E. coli*) or using a linker sequence (*P. pastoris*), were successfully developed. Expression level in *E. coli* after 5 hours induction at 30°C was 43 μ g/mL as determined by competitive RIA. In *P. pastoris*, 400 μ g/mL was reached. rFel d 1 was purified by hydrophobic interaction chromatography and ion-exchange chromatography, giving a final yield of 92% from *E. coli* and purification efficacy was similar from yeast. SDS-PAGE (Fig. 1) of nFel d 1 under reducing conditions revealed one discrete band with an apparent molecular weight of approximately 4.5 kDa and a

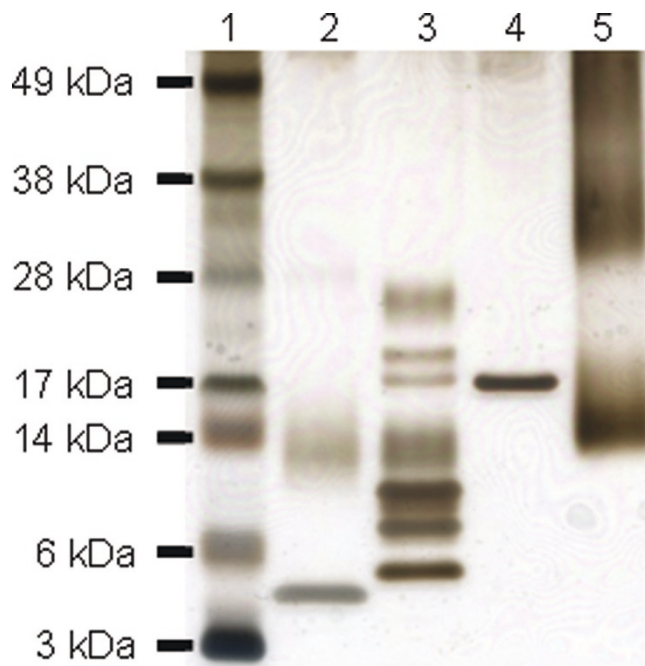


FIGURE 1. SDS-PAGE analysis of Fel d 1 preparations. SDS-PAGE was performed under reducing conditions and silver-stained. Lane 1, see blue plus marker (Invitrogen); lane 2, nFel d 1; lane 3, rFel d 1 (*P. pastoris*); lane 4, rFel d 1; lane 5, glutaraldehyde-modified rFel d 1.

smear between 10 and 16 kDa. For rFel d 1 from *P. pastoris*, 5 bands (5, 7, 10, 17, and 20 kDa) and 2 smears (12–15 and 23–28 kDa) were observed, whereas rFel d 1 from *E. coli* gave a single discrete band (molecular weight \sim 17 kDa). Glutaraldehyde modification of *E. coli*-derived rFel d 1 revealed 2 smears with an apparent molecular weight of 12–20 and 29–40 kDa, respectively.

N-Terminal sequencing of the 17 and 20 kDa bands from rFel d 1 (*P. pastoris*) demonstrated that the lower band is rFel d 1 with the correct N-terminal sequence (EICPAVKRDV)¹⁰ and the upper band is rFel d 1 with 9 residual amino acids originating from the signal peptide, upstream of the *Kex 2* site (EEGVSLKRE). N-Terminal sequencing of the *E. coli*-derived 17-kDa band showed that the first 10 amino acids were identical to the N-terminal amino acid sequence (β -chain) of Fel d 1 (VK-MAETCPIF).¹⁰

Native and chemically modified rFel d 1 were characterized regarding molecular size using small-angle x-ray scattering and dynamic light-scattering methodologies, both resulting in highly polydisperse mixtures of molecules (data not shown). These mixtures were separated by HPSEC and the values for molecular weight (MW) and hydrodynamic radius (R_H) of eluting fractions were determined by online light scattering and viscosimetry (Fig. 2). The main fraction of native Fel d 1 (representing \sim 56% of the protein) was determined to be \sim 18kD, corresponding to monomeric Fel d 1. Small oligomers (MW 48 kD; R_H 3.3 nm) represented 22% and larger oligomers (MW $>$ 85 kD; R_H $>$ 5 nm) 16%

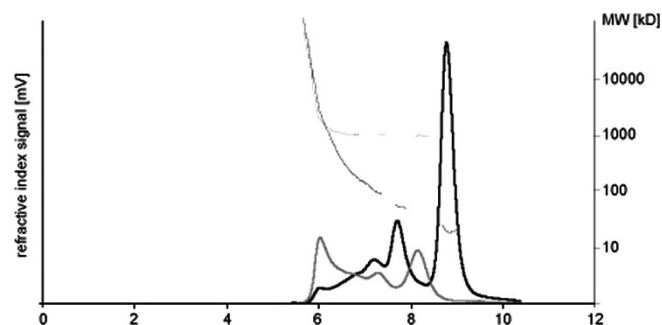


FIGURE 2. Determination of molecular size under physiologic conditions. Refractive index signal (bold lines) and MW for native (black) and modified (gray) rFel d 1 determined from online HPSEC light scattering. Determined MW values of the eluting fractions (native: $v_{\text{ret}} = 5.6\text{--}7.3$, $7.6\text{--}7.8$, and $8.6\text{--}8.9$ mL; modified: $v_{\text{ret}} = 5.6\text{--}6.8$, $7.2\text{--}7.4$, and $8.0\text{--}8.3$ mL) are shown in thin dashed lines.

of protein. In addition, ~6% of highly aggregated protein accounting for strong light-scattering intensity were detected. Chemical modification of rFel d 1 caused formation of polymers with a MW of ≥ 1 MDa. Three different major fractions were found spanning large regions of the chromatogram.

Comparison of Immune Reactivity of Natural and Recombinant Fel d 1

The IgE-binding characteristics of rFel d 1 were assessed by RAST, immunoblot, and CAP-inhibition. Sera of cat-sensitized patients ($n = 76$) were tested in a RAST for specific IgE antibodies against nFel d 1 and rFel d 1 (Fig. 3). By Spearman rank correlation, IgE responses to nFel d 1 and rFel d 1 were shown to closely correlate ($R_s = 0.9150$ [95% confidence interval (CI): 0.8680 to 0.9458]; $P < 0.0001$). Overall, binding to rFel d 1 was slightly higher than that to nFel d 1 (mean ratio 1.3; 95% CI: 0.85–1.75). Five patients were monosensitized to nFel d 1 (<2 IU/mL), but with low specific IgE titers and one was >5 times more reactive to nFel d 1 than to the recombinant. On immunoblot IgE reactivity to rFel d 1 was detected at 16 kDa, in some cases accompanied by a faint band at ~38 kDa, presumably representing the rFel d 1 dimer (Fig. 4). MAb 4F7 raised to purified nFel d 1 and both chain-specific mAbs bound to the same 16-kDa band. MAb 6B4 (specific for the light α -chain) also detected the 38-kDa band, confirming the Fel d 1 nature of this band. Comparing IgE-binding potencies of natural and rFel d 1 with ImmunoCAP inhibition, using a serum pool of cat-sensitized patients, demonstrated both preparations have very similar inhibitory potencies (Fig. 5A). Additionally, the biologic activity of both molecules assessed by BHR was comparable (Fig. 5B). Competitive RIA with polyclonal rabbit antiserum against nFel d 1 and ^{125}I -labeled nFel d 1 (Fig. 5C) further confirmed the similarity. Only the sandwich ELISA based on 2 mAbs showed preference for nFel d 1 by a factor of 6 (Fig. 5D). Overall, rFel d 1 is a good mimic of its natural counterpart. Comparable results were obtained with yeast-derived rFel d 1 (not shown).

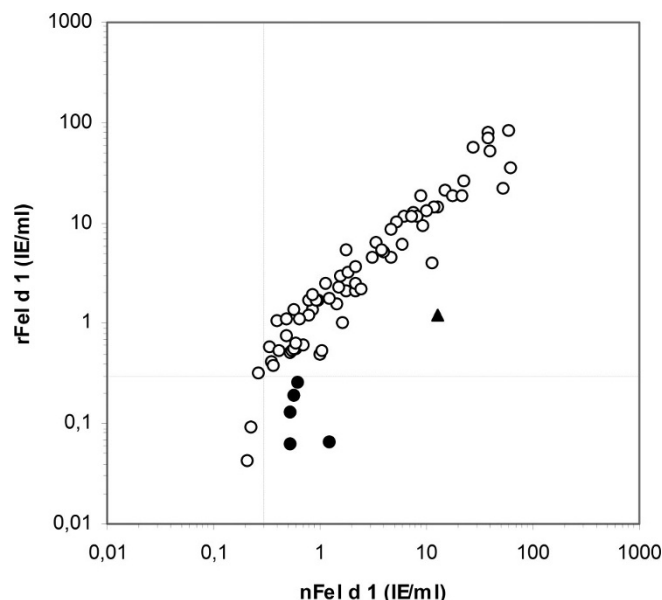


FIGURE 3. Comparison of IgE binding of natural versus recombinant Fel d 1. IgE responses to nFel d 1 (x-axis) were compared with rFel d 1 (y-axis) by RAST using sera of 76 cat-allergic patients. Results are expressed in IE/mL. Decreased reactivity toward rFel d 1 was observed for (5 of 76) sera. The filled circles are exclusively positive for nFel d 1 and the filled triangle was 5 times more reactive to nFel d 1 than to rFel d 1.

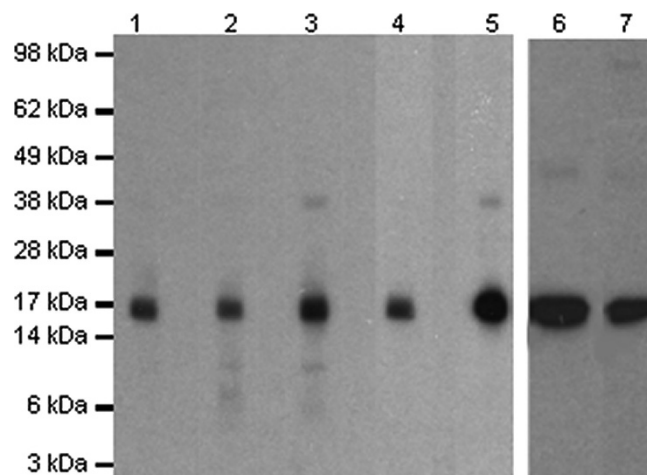


FIGURE 4. Immunoblotting of purified rFel d 1. Lanes 1–3 were detected with 3 Fel d 1-specific mAbs (4F7, 10F10, and 6B4, respectively); lanes 4 and 5 were recognized by sera derived from 2 cat-allergic patients (sera 6 and 154); and lanes 6 and 7 were detected with rFel d 1-mod-specific rabbit-polyclonal antibody (rabbit 140662 and 140831, respectively).

Modified rFel d 1: Reduced IgE and IgG Antibody Binding

Glutaraldehyde-modified rFel d 1 was evaluated by CAP-inhibition, competitive RIA and ELISA. The IgE-binding potency in CAP-inhibition was reduced by >3 orders of

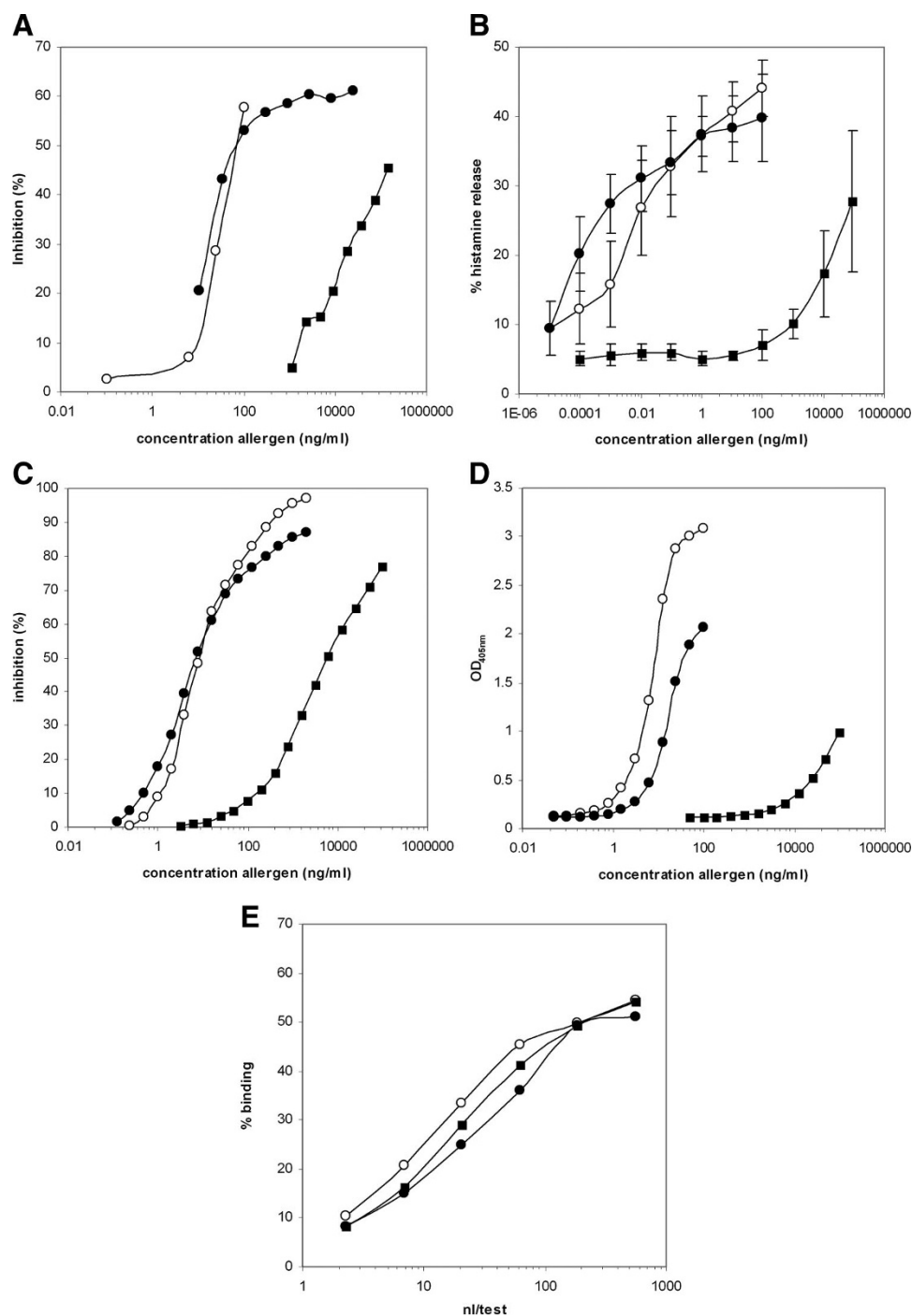


FIGURE 5. Immunologic characterization of modified rFel d 1. A, ImmunoCAP inhibition. ImmunoCAP inhibition was performed with a cat epithelium and dander CAP (e1) and a serum pool of >100 cat-allergic patients. B, Stripped basophil histamine release assay. C, Competitive RIA. D, Fel d 1-specific sandwich ELISA. E, IgG RAST. Symbols used: nFel d 1 (○), rFel d 1 (●), and rFel d 1-mod (■).

magnitude (~1300-fold) compared with that of the unmodified recombinant allergen (Fig. 5A). Although the magnitude of reduction observed in competitive RIA (Fig. 5C) and ELISA (Fig. 5D) was not identical (~500- and ~5000-fold, respectively), it was in both cases highly significant. Similar results were obtained with yeast-derived rFel d 1 (not shown).

Modified rFel d 1: Reduced Biologic Activity

The biologic activity of rFel d 1-mod was assessed by the stripped basophil histamine release bioassay ($n = 4$). In a

first experiment, modified rFel d demonstrated >1000-fold reduced reactivity compared with unmodified rFel d 1, but an accurate assessment was not possible because the native allergen was not diluted far enough (not shown). In a follow-up experiment, for 2 of the 4 original sera, native allergen was diluted up to 0.01 pg/mL, allowing a more accurate calculation. rFel d 1-mod showed a decrease in biologic activity of >10⁶-fold (Fig. 5B). Significant reduction (1000-fold) was observed for yeast-derived rFel d 1-mod (not

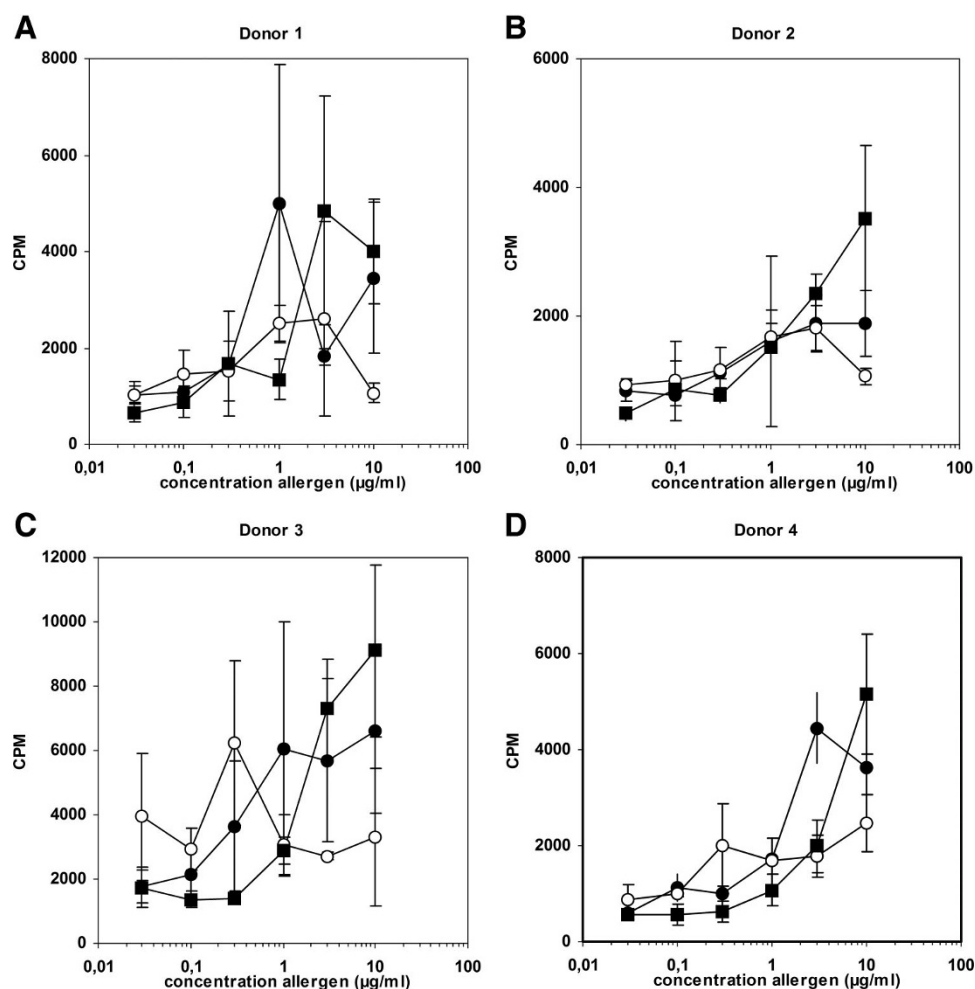


FIGURE 6. Allergen-specific proliferation of PBMCs. PBMCs from 4 cat-allergic patients were stimulated with dilutions of nFel d 1 (○), rFel d 1 (●), and rFel d 1-mod (■). Panels A–D each represent an individual patient.

shown). The stripped cells (negative control) showed a release of <4% (not shown).

Modified rFel d 1: Retained T-Cell Proliferation

PBMCs from 4 cat-allergic patients were stimulated with dilutions of nFel d 1, rFel d 1, and rFel d 1-mod (Fig. 6). The mean stimulation index at 10 µg/mL was significantly higher upon stimulation with modified allergen (8.4) compared with the native molecule (5.6) (p (Wilcoxon) < 0.01). These data demonstrate that rFel d 1-mod had retained its capacity to trigger cat allergen-specific T-cells. Similar observations were made for the yeast-derived recombinant molecule (not shown).

Immunogenicity of Modified rFel d 1

To assess the capability of rFel d 1-mod to induce IgG antibodies recognizing the natural allergen, 2 rabbits were immunized with the modified allergen from yeast. The resulting rabbit antisera were tested in RAST and immunoblot experiments for IgG reactivity with nFel d 1, rFel d 1, and rFel d 1-mod. IgG antibodies induced upon immunization with rFel d 1-mod bound in a similar fashion to nFel d 1, rFel d 1, and rFel d 1-mod (Fig. 5E). Both

antisera also clearly detected unmodified rFel d 1 on immunoblot (Fig. 4).

DISCUSSION

In past years, several strategies have been evaluated in our laboratory to express rFel d 1, starting with separate expression of both chains of the molecule in *P. pastoris* (unpublished data), and subsequently, expression of both chains coupled by a linker sequence (this study). In our hands, this approach resulted in a rFel d 1 preparation with good immune reactivity but an undesirable degree of molecular heterogeneity caused by hyperglycosylation of a fraction of the molecules at the N-linked glycosylation site in the β -chain of Fel d 1. Additionally, some instability of the linker sequence resulted in partial cleavage of the two-chain heterodimer (confirmed by N-terminal sequencing of the resulting fragments). Both post-translational modifications did not significantly affect immune reactivity but the observed heterogeneity is less favorable from a production standpoint. A mutant lacking the N-linked glycosylation site showed decreased heterogeneity, but was still partially cleaved (not shown). Therefore, we chose to express rFel

d 1 as a heterodimer of both chains without the instable linker in *E.coli* resulting in a stable nonglycosylated homogeneous preparation.

Glutaraldehyde modification of this molecule resulted in a truly hypoallergenic molecule with a reduction in IgE-binding potency of around 1000-fold. This translated into a negligible biologic activity (10^6 -fold reduction). To our knowledge this is unmatched by any hypoallergenic strategy using site-directed mutagenesis.

The size distribution in solution demonstrated that modification resulted in a molecular weight ≥ 1000 kDa. However, under the denaturing conditions of SDS-PAGE, part of the rFel d 1-mod migrated at an apparent molecular weight of 12–20 and 29–40 kDa, indicating that not all aggregation observed in solution was caused by covalent interactions. This suggests that glutaraldehyde modification results in polymerized and aggregated molecules of high MW that may trap also monomers and low MW oligomers, which can be released under reducing and denaturing conditions.

Although the mechanism of allergen-specific immunotherapy is still a matter of debate, it is clear that successful therapy is accompanied by induction of regulatory T-cells and IgG₄ antibodies.³⁷ It is generally accepted that a novel immunotherapy product should have the potency to trigger allergen-specific T-cells. Modified rFel d 1 was shown to induce at least similar T-cell proliferation as its unmodified counterpart. Additionally, a role in immunotherapy of IgG₄ as blocking antibodies is likely and we demonstrated that immunization of rabbits with modified rFel d 1 induced specific IgG antibodies that were able to bind to native Fel d 1.

In summary, it was convincingly demonstrated that chemical modification of a recombinant allergen is an easy and highly effective way to achieve hypoallergenicity. This approach will allow safe administration of higher doses of allergen to achieve better efficacy.

REFERENCES

- van Ree R, van Leeuwen WA, Bulder I, Bond J, Aalberse RC. Purified natural and recombinant Fel d 1 and cat albumin in vitro diagnostics for cat allergy. *J Allergy Clin Immunol*. 1999;104:1223–1230.
- Kleine-Tebbe J, Kleine-Tebbe A, Jeep S, Schou C, Lowenstein H, Kunkel G. Role of the major allergen (Fel d 1) in patients sensitized to cat allergens. *Int Arch Allergy Immunol*. 1993;100:256–262.
- Ohman JL Jr, Lowell FC, Bloch KJ. Allergens of mammalian origin. III. Properties of a major feline allergen. *J Immunol*. 1974;113:1668–1677.
- Lowenstein H, Lind P, Weeke B. Identification and clinical significance of allergenic molecules of cat origin. Part of the DAS 76 Study. *Allergy*. 1985;40:430–441.
- Duffort O, Carreira J, Lombardero M. Monoclonal antibodies against Fel d 1 and other clinically relevant cat allergens. *Immunol Lett*. 1988;17:71–77.
- de Groot H, van Swieten P, van Leeuwen J, Lind P, Aalberse RC. Monoclonal antibodies to the major feline allergen Fel d 1. I. Serologic and biologic activity of affinity-purified Fel d 1 and of Fel d 1-depleted extract. *J Allergy Clin Immunol*. 1988;82:778–786.
- Ewbank PA, Murray J, Sanders K, Curran-Everett D, Dreskin S, Nelson HS. A double-blind, placebo-controlled immunotherapy dose-response study with standardized cat extract. *J Allergy Clin Immunol*. 2003;111:155–161.
- Varney VA, Edwards J, Tabbah K, Brewster H, Mavroleon G, Frew AJ. Clinical efficacy of specific immunotherapy to cat dander: a double-blind placebo-controlled trial. *Clin Exp Allergy*. 1997;27:860–867.
- Kristensen AK, Schou C, Roepstorff P. Determination of isoforms, N-linked glycan structure and disulfide bond linkages of the major cat allergen Fel d 1 by a mass spectrometric approach. *Biol Chem*. 1997;378:899–908.
- Morgenstern JP, Griffith JJ, Brauer AW, Rogers BL, Bond JF, Chapman MD, Kuo MC. Amino acid sequence of Fel dI, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci U S A*. 1991;88:9690–9694.
- Griffith JJ, Craig S, Pollock J, Yu XB, Morgenstern JP, Rogers BL. Expression and genomic structure of the genes encoding FdI, the major allergen from the domestic cat. *Gene*. 1992;113:263–268.
- Kaiser L, Gronlund H, Sandalova T, Ljunggren HG, van Hage-Hamsten M, Achour A, Schneider G. The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobulin family. *J Biol Chem*. 2003;278:37730–37735.
- Rogers BL, Morgenstern JP, Garman RD, Bond JF, Kuo MC. Recombinant Fel dI: Expression, purification, IgE binding and reaction with cat-allergic human T cells. *Mol Immunol*. 1993;30:559–568.
- Slunt JB, Rogers BL, Chapman MD. IgE antibodies to recombinant forms of Fel d I: dichotomy between fluid-phase and solid-phase binding studies. *J Allergy Clin Immunol*. 1995;95:1221–1228.
- Keating KM, Segal DB, Craig SJ, Nault AK, Semensi V, et al. Enhanced immunoreactivity and preferential heterodimer formation of reassociated Fel d I recombinant chains. *Mol Immunol*. 1995;32:287–293.
- Ichikawa K, Wen W, Wu Z, Vailes LD, Guyre P, Chapman MD. Fully immunoreactive recombinant cat allergen, Fel d 1, expressed in baculovirus [abstract]. *J Allergy Clin Immunol*. 1999;103:S184.
- Vailes LD, Sun AW, Ichikawa K, Wu Z, Sulahian TH, Chapman MD, Guyre PM. High-level expression of immunoreactive recombinant cat allergen (fel d 1): Targeting to antigen-presenting cells. *J Allergy Clin Immunol*. 2002;110:757–762.
- Kaiser L, Gronlund H, Sandalova T, Ljunggren HG, Schneider G, van Hage-Hamsten M, Achour A. Production, crystallization and preliminary crystallographic study of the major cat allergen Fel d 1. *Acta Crystallogr D Biol Crystallogr*. 2003;59:1103–1105.
- Ferreira F, Ebner C, Kramer B, Casari G, Briza P, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J*. 1998;12:231–242.
- Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, et al. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol*. 2003;325:123–133.
- Rabjohn P, West CM, Connaughton C, Sampson HA, Helm RM, Burks AW, Bannon GA. Modification of peanut allergen Ara h 3: effects on IgE binding and T cell stimulation. *Int Arch Allergy Immunol*. 2002;128:15–23.
- Bannon GA, Cockrell G, Connaughton C, West CM, Helm R, et al. Engineering, characterization and in vitro efficacy of the major peanut allergens for use in immunotherapy. *Int Arch Allergy Immunol*. 2001;124:70–72.
- Akdis CA, Blaser K. Regulation of specific immune responses by chemical and structural modifications of allergens. *Int Arch Allergy Immunol*. 2000;121:261–269.
- Norman PS, Lichtenstein LM, Marsh DG. Studies on allergoids from naturally occurring allergens. IV. Efficacy and safety of long-term allergoid treatment of ragweed hay fever. *J Allergy Clin Immunol*. 1981;68:460–470.
- Norman PS, Lichtenstein LM, Kagey-Sobotka A, Marsh DG. Controlled evaluation of allergoid in the immunotherapy of ragweed hay fever. *J Allergy Clin Immunol*. 1982;70:248–260.
- De Jonge J, Brissinck J, Heirman C, Demanet C, Leo O, Moser M, Thielemans K. Production and characterization of bispecific single-chain antibody fragments. *Mol Immunol*. 1995;32:1405–1412.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150:76–85.
- van Milligen FJ, van Swieten P, Aalberse RC. 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. *Int Arch Allergy Immunol*. 1992;99:63–73.
- Bolhaar ST, Ree R, Bruijnzeel-Koomen CA, Knulst AC, Zuidmeer L. Allergy to jackfruit: a novel example of Bet v 1-related food allergy. *Allergy*. 2004;59:1187–1192.

30. Carnes J, Himly M, Gallego M, Iraola V, Robinson DS, Fernández-Caldas E, Briza P. Detection of allergen composition and in vivo immunogenicity of depigmented allergoids of *Betula alba*. *Clin Exp Allergy*. 2009;39:426–434.
31. Aalberse RC, Koshte V, Clemens JG. Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol*. 1981;68:356–364.
32. van Ree R, van Leeuwen WA, van den BM, Weller HH, Aalberse RC. IgE and IgG cross-reactivity among Lol p I and Lol p II/III. Identification of the C-termini of Lol p I, II, and III as cross-reactive structures. *Allergy*. 1994;49:254–261.
33. Chapman MD, Aalberse RC, Brown MJ, Platts-Mills TA. Monoclonal antibodies to the major feline allergen Fel d I. II. Single step affinity purification of Fel d I, N-terminal sequence analysis, and development of a sensitive two-site immunoassay to assess Fel d I exposure. *J Immunol*. 1988;140:812–818.
34. Kleine Budde I, de Heer PG, van der Zee JS, Aalberse RC. The stripped basophil histamine release bioassay as a tool for the detection of allergen-specific IgE in serum. *Int Arch Allergy Immunol*. 2001;126:277–285.
35. Knol EF, Kuijpers TW, Mul FP, Roos D. Stimulation of human basophils results in homotypic aggregation. A response independent of degranulation. *J Immunol*. 1993;151:4926–4933.
36. Siraganian RP. Refinements in the automated fluorometric histamine analysis system. *J Immunol Methods*. 1975;7:283–290.
37. Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allergy Clin Immunol*. 2009;123:735–746.