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

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 molecule19,20 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-
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 oligo in the pPICZαA 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 method (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 Millingen et al.26 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 μM, representing a total amount of 292 mmol); 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 × 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 μg/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
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 levels in E. coli after 5 hours induction at 30°C was 43 μg/mL as determined by competitive RIA. In P. pastoris, 400 μg/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 efficiency was similar from yeast. SDS-PAGE (Fig. 1) of rFel d 1 under reducing conditions revealed one discrete band with an apparent molecular weight of approximately 4.5 kDa and a 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 ~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 (ELCPAVKRDV)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 (EEGVSEKRE). 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 ~56% of the protein) was determined to be ~18 kD, 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%
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 $\alpha$-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).

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 magnitude, suggesting a decrease in allergenicity.
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 >10^6-fold (Fig. 5B). Significant reduction (1000-fold) was observed for yeast-derived rFel d 1-mod (not shown).

**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 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 (■).
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 (□). Panels A–D each represent an individual patient.

**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 $\geq$1000 kDa. However, under the denaturating 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 denaturating 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. Modified rFel d 1 was shown to induce at least similar T-cell proliferation as its unmodified counterpart. Additionally, a role in immunotherapy of IgG as blocking antibodies is likely and we demonstrated that immunization of rabbits with modified rFel d 1 induced specific blocking antibodies is likely and we demonstrated that immunization of rabbits with modified rFel d 1 induced specific blocking antibodies.

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.

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