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 Fel d 1 (rFel 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 Fel d 1 and recombinant unmodified Fel d 1 were used as controls.

Results: rFel d 1 demonstrated similar IgE binding and biological activity as its natural counterpart. Upon modification, IgE-binding potency decreased to >1000-fold, which was translated into a $>10^6$-fold reduction in the biological 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

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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 olisaccharides,[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*, IgE binding was poor in natural Fel d 1 (nFel d 1),[13,14] but combining both chains, followed by a lengthy refolding protocol, a recombinant was obtained with similar IgE reactivity as the nFel 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, Ara h 2, and Ara h 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 allergen to be able to predict which amino acids should be mutated. Random mutagenesis might be an alternative but requires laborious screening protocols. Moreover, 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 March 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*.

MATERIALS AND METHODS

Cloning and Expression of Fel d 1

In one construct, both chains (α-chain and β-chain) were cloned together using a linker oligo[26] in the pPICZαA vector (Invitrogen, San Diego, CA). After transformation into *P. pastoris* GS115 ($His4^+$) and expression in a Bioflo 3000 benchtop fermentor, cells were harvested and the supernatant 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 at 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: first, hydrophobic interaction chromatography using a Phenyl-Sepharose column and second, 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 phosphate-buffered saline, concentrated (6-fold), and purity of the sample was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). nFel d 1 was affinity purified using monoclonal antibody (mAb) 4F7, as previously described.[1] Protein concentration was measured by the BCA method[27] (Pierce, Rockford, IL), as described by the manufacturer. For all experiments, *E. coli*-derived rFel d 1 was used unless indicated otherwise.

Murine mAbs

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 of 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 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 of aldehyde to 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 light scattering was performed using a 7.8 × 300 mm TSKgel $G2000_{SWXL}$ column (Tosoh Bioscience, Stuttgart, Germany) in a 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, intrinsic viscosity, 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 of 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, France).

Radioallergosorbent Test

For the 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 international units (IU)/milliliter, and for IgG, the results were expressed in percent bound activity.

ImmunoCAP Inhibition

ImmunoCAP (Phadia, Uppsala, Sweden) assay 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 percentage 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 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 μg Fel d 1.

The sandwich ELISA was developed by Chapman et al[33] (Indoor Biotechnologies, Warminster, United Kingdom). 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 biological 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 patients 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 μg/mL). Liberated histamine was measured by the fluorometric analysis 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 gentamicin (80 μg/mL; Duchefa, Haarlem, the Netherlands). Dilution series of allergen or allergoid (0.03–10 μg/mL) were added at day 0, followed by addition of ^{3}H-labelled thymidine at day 6, and harvesting 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-hour 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 the purification efficacy was similar from yeast. SDS-PAGE (Fig. 1) of nFel d 1 under reducing conditions revealed 1 discrete band with an apparent molecular weight (MW) 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 kDa and 23–28 kDa) were observed, whereas rFel d 1 from *E. coli* gave a single discrete band (MW, ~17 kDa). Glutaraldehyde modification of *E. coli*-derived rFel d 1 revealed 2 smears with an apparent MW of 12 to 20 kDa and 29 to 40 kDa, respectively.

N-terminal sequencing of the 17 kDa 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 (EIC-PAVKRDV),[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 (EEGVSLEKRE). 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 (VKMAETCPIF).[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

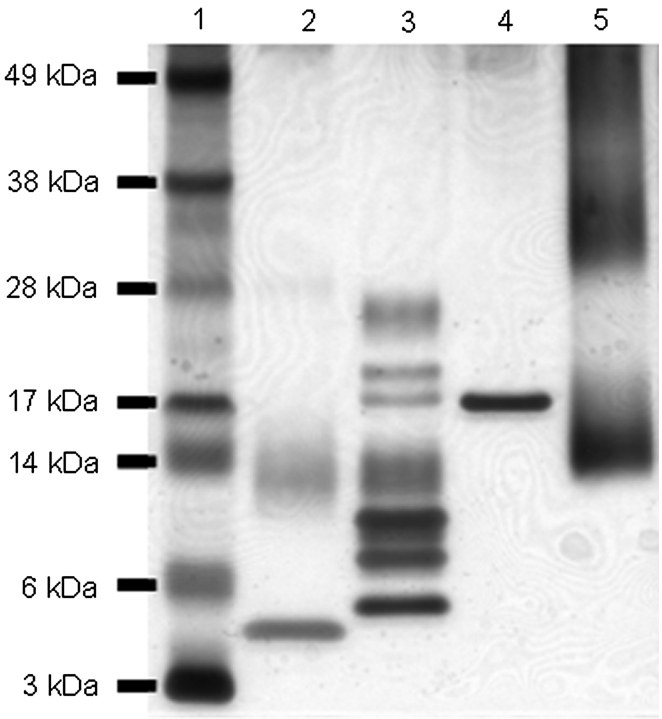

FIGURE 1. SDS-PAGE analysis of Fel d 1 preparations. SDS-PAGE was performed under reducing conditions and silver stained. Lane 1: blue plus marker (Invitrogen); lane 2: nFel d 1; lane 3: rFel d 1 (*P. pastoris*); lane 4: rFel d 1; and lane 5: glutaraldehyde-modified rFel d 1.

resulting in highly polydisperse mixtures of molecules (data not shown). These mixtures were separated by online high-performance size exclusion chromatography, and the values for 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 approximately 18 kDa, corresponding to monomeric Fel d 1. Small oligomers (MW, 48 kDa; R_H 3.3 nm) represented 22% and larger oligomers (MW >85 kD; R_H > 5 nm) 16% represented of protein. In addition, approximately 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 ($Rs = 0.9150$) [95% confidence interval, 0.8680–0.9458; $P < 0.0001$]. Overall, binding to rFel d 1 was slightly higher than to nFel d 1 (mean ratio, 1.3; 95% confidence interval, 0.85–1.75). Five patients were monosensitized to nFel d 1 (<2 IU/mL), but with low specific IgE titers, and 1 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 approximately 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 Fel d 1

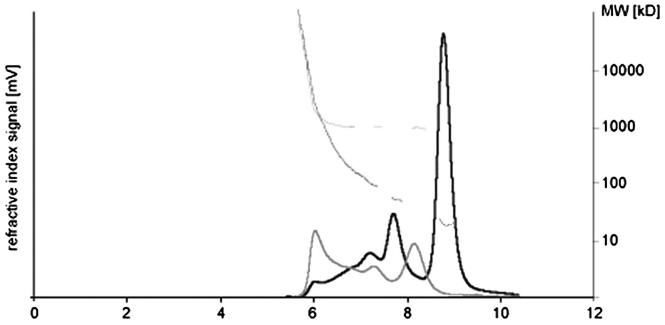

FIGURE 2. Determination of molecular size under physiological conditions. Refractive index signal and MW for native (black) and modified (gray) rFel d 1 determined by online high-performance size exclusion chromatography light scattering. Determined MW values of the eluting fractions (native: v_{ret}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

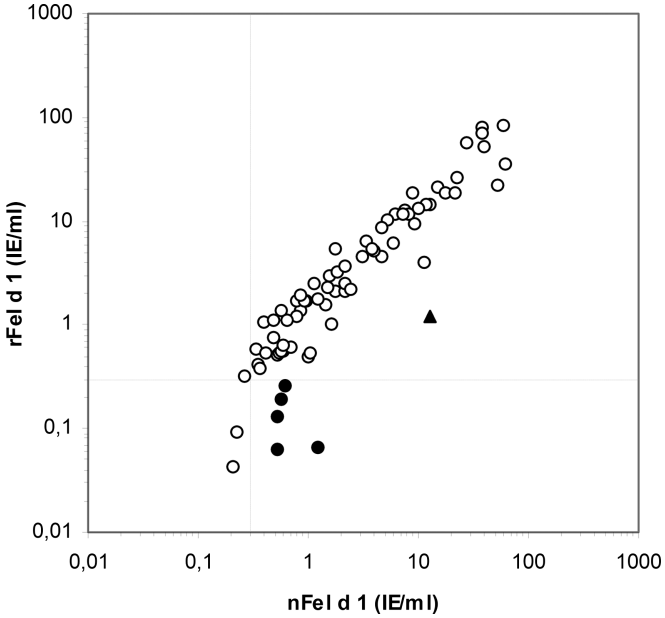

FIGURE 3. Comparison of IgE binding of natural versus recombinant Fel d 1. IgE responses to nFel d 1 (*x* axis) were compared with rFel d 1 (*y* axis) by RAST using sera of 76 cat-allergic patients. Results are expressed in IE/mL. Decreased reactivity toward rFel d 1 was observed for (5 of 76) sera. The filled circles are exclusively positive for nFel d 1, and the filled triangle was 5 times more reactive to nFel d1 than to rFel d 1.

and rFel d 1 with ImmunoCAP inhibition, using a serum pool of cat-sensitized subjects, demonstrated that both preparations have very similar inhibitory potencies (Fig. 5A). Additionally, the biological activity of both molecules assessed by BHR was comparable (Fig. 5B). Competitive RIA with polyclonal rabbit antiserum against nFel d 1 and ^{125}I-labelled 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 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 (~1300-fold) compared with 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-fold and ~5000-fold, respectively), it was highly significant in both cases. Similar results were obtained with yeast-derived rFel d 1 (not shown).

Modified rFel d 1: Reduced Biological Activity

The biological activity of rFel d 1-mod was assessed by the stripped BHR bioassay (n = 4). In the first experiment,

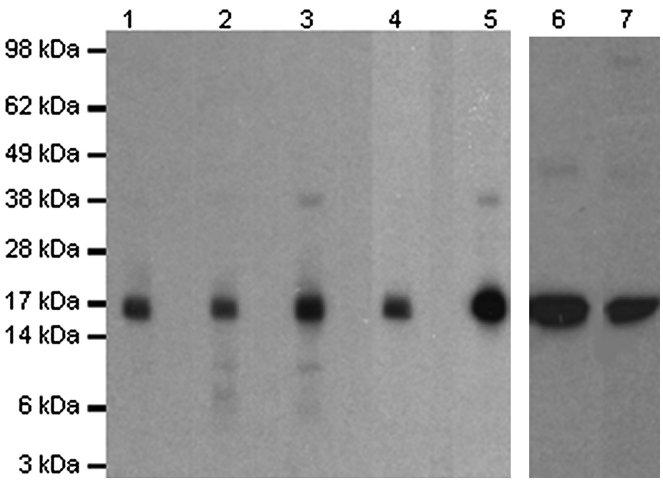

FIGURE 4. Immunoblotting of purified rFel d 1. Lanes 1 to 3 were detected with 3 Fel d 1-specific mAbs (4F7, 10F10, and 6B4, respectively); lanes 4 and 5 were detected by sera derived from 2 cat-allergic patients (serum 6 and 154); and lanes 6 and 7 were detected with rFel d 1-mod–specific rabbit polyclonal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

modified rFel d 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 biological activity of $>10^6$-fold (Fig. 5B). Significant reduction (1000-fold) was observed for yeast-derived rFel d 1-mod (not 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) than with the native molecule (5.6) ($P < 0.01$, Wilcoxon). These data demonstrated 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).

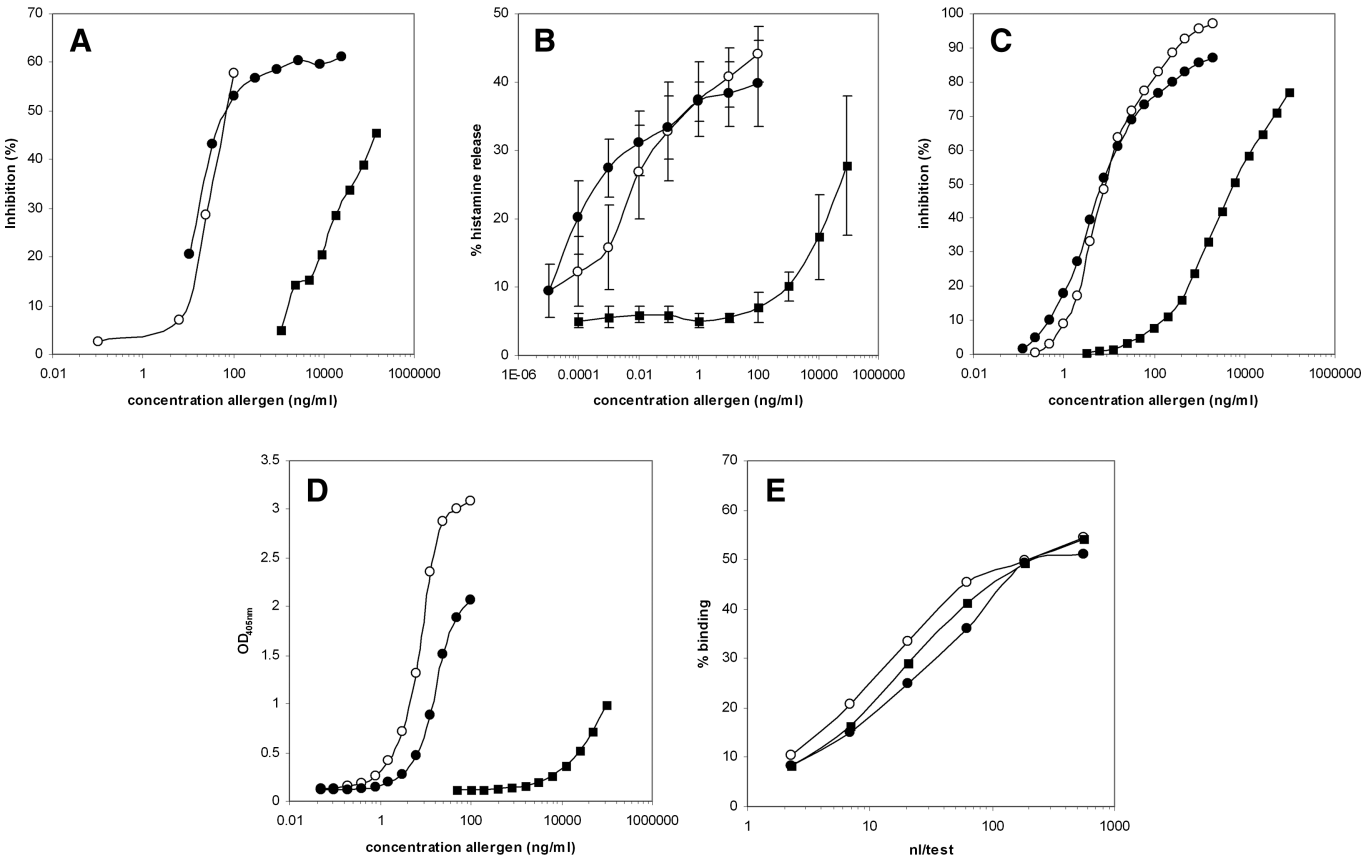

FIGURE 5. Immunological characterization of modified rFel d 1. A, ImmunoCAP inhibition. ImmunoCAP inhibition was performed with a cat epithelium and dander CAP (e1) and a serum pool of >100 cat-allergic patients. B, Stripped basophile histamine release assay. C, Competitive RIA. D, Fel d 1–specific sandwich ELISA. E, IgG RAST. Open circle represents nFel d 1, filled circle represents rFel d 1, and filed square represents rFel d 1-mod.

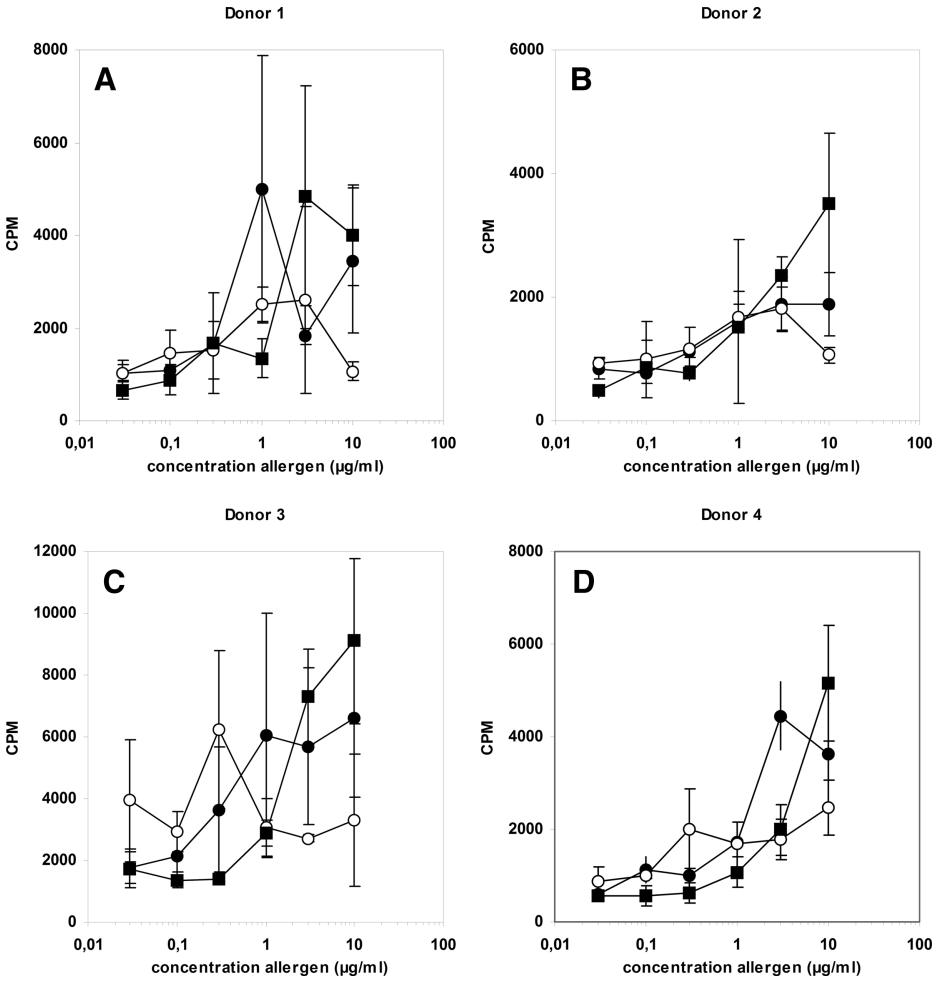

FIGURE 6. Allergen-specific proliferation of PBMCs. PBMCs from 4 cat-allergic patients were stimulated with dilutions of nFel d 1 (○), rFel d 1 (●) and rFel d 1-mod (■). Panels A-D each represent an individual patient.

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

DISCUSSION

Over the 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, the expression of both chains coupled by a linker sequence (this study). In our study, 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 2-chain heterodimer (confirmed by N-terminal sequencing of the resulting fragments). Both posttranslational modifications did not significantly affect the 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 approximately 1000-fold. This translated into a negligible biological 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 of rFel d 1 resulted in a MW ≥ 1000 kDa. However, under the denaturating conditions of SDS-PAGE, part of the rFel d 1-mod migrated at an apparent MW of 12 to 20 kDa and 29 to 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

also trap 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 the induction of regulatory T cells and IgG_4 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 IgG_4 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.

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