High-level expression of immunoreactive recombinant cat allergen (Fel d 1): Targeting to antigen-presenting cells

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Background: Cat allergen Fel d 1 is a heterodimer encoded by 2 separate genes that has been difficult to produce as a fully immunoreactive molecule.
Objective: We sought to engineer recombinant (r) Fel d 1 with IgE and IgG antibody binding comparable with that of the natural allergen that could be targeted to antigen-presenting cells.
Methods: The rFel d 1 chains were coexpressed in baculovirus, either linked to the anti-CD64 antibody H22 (rFel d 1 H22$^+$) or alone (rFel d 1 H22$^-$). Binding of expressed allergens to mouse and human antibodies was compared with that of natural (n) Fel d 1 by means of enzyme immunoassay and antigen-binding and inhibition RIAs. Binding of rFel d 1 H22$^+$ to the CD64 receptor on leukocyte subpopulations and on the THP–1 cell line was analyzed by means of flow cytometry.
Results: The baculovirus-expressed allergens migrated with molecular weights of 49 kd (rFel d 1 H22$^+$) and 22 kd (rFel d 1 H22$^-$). The rFel d 1 inhibited IgG antibody binding to nFel d 1 by greater than 95% and showed identical dose-dependent inhibition curves. There was an excellent quantitative correlation between IgE and IgG antibody binding to rFel d 1 and nFel d 1 in sera from patients with cat allergy (IgE: n = 258, r = >0.72, P < .001). The rFel d 1 H22$^+$ bound to monocytes but not to lymphocytes or neutrophils, and binding of rFel d 1 H22$^+$ to THP-1 cells was inhibited by a soluble CD64 fusion protein.
Conclusions: Recombinant Fel d 1 chains have been successfully coexpressed as mature proteins with comparable immunoreactivities to nFel d 1. The rFel d 1 can be targeted to antigen-presenting cells through CD64. These constructs will facilitate structural studies of Fel d 1 and the development of improved allergy diagnostics and therapeutics. (J Allergy Clin Immunol 2002;110:757-62.)

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Fel d 1 is the major allergen produced by domestic cats and elicits IgE and IgG antibody responses in greater than 90% of persons with cat allergy. Although other cat allergens have been identified, including Fel d 2 (albumin) and Fel d 3 (cystatin), 60% to 90% of anti-cat IgE is directed against Fel d 1.[1-5] Fel d 1 has been used to monitor antibody responses during immunotherapy and to investigate the role of allergen sensitization in asthma.[4,6-12] Cat allergenic products used for diagnosis and treatment are standardized according to Fel d 1 content.[13,14] However, natural allergenic products contain other cat proteins derived from dander or pelt, many of which are nonallergenic. They might be contaminated with other allergens (eg, dust mites) and are a potential source of transmittable mammalian pathogens.[13] These problems can be circumvented by producing recombinant allergens that can be produced on demand, can be precisely formulated and standardized, and are free of irrelevant proteins.[4,15]

The production of recombinant (r) Fel d 1 has been challenging because Fel d 1 is a heterodimer composed of 2 protein chains produced by separate genes.[15-17] Fel d 1 chains 1 and 2, produced separately in *Escherichia coli*, had significantly reduced immunoreactivity compared with that of the natural allergen.[18,19] Denaturation and refolding of the *E coli*–expressed chains increased IgE antibody binding; however, the refolding process is not practical for producing large amounts of allergen.[4] Natural (n) Fel d 1 (chain 2) is glycosylated, whereas the *E coli*–expressed chains were not. Coexpression of both Fel d 1 chains in a eukaryotic expression system would provide the machinery for folding, processing, and glycosylation of the molecule similar to that of the natural allergen. Production of mature rFel d 1 is essential for studies to elucidate the structure and biologic function of the allergen. Fel d 1 has been used extensively for human T-cell studies, epitope mapping, and development of peptide-based vaccines.[16,20-22] Both T cell–proliferative responses and epitope-mapping studies have been inconsistent, suggesting that better targeting of allergen to antigen-presenting cells (APCs) would improve the magnitude and reproducibility of the response.

In this article we describe the engineering of fully immunoreactive rFel d 1 in the baculovirus expression

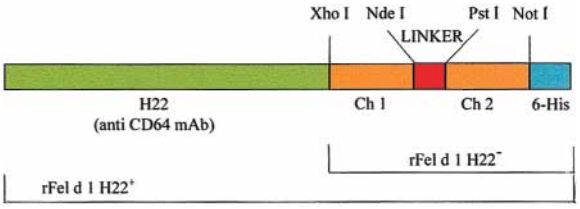

FIG 1. Linear map of rFel d 1 constructs, showing restrictions sites and the location of each Fel d 1 chain.

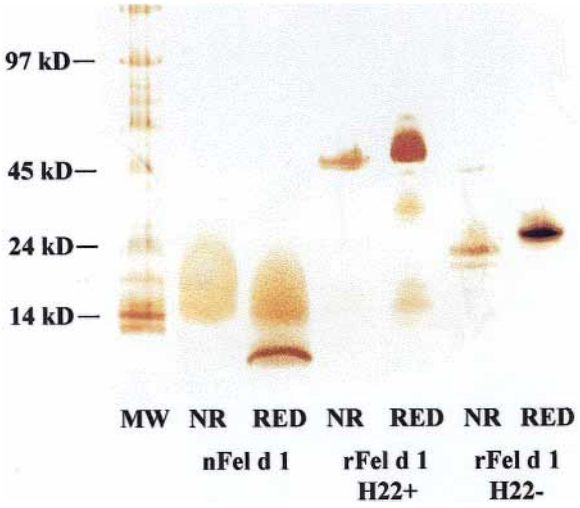

FIG 2. SDS-PAGE analysis of rFel d 1 constructs and nFel d 1 under nonreducing *(NR)* and reducing *(RED)* conditions (8%-25% gradient gel, silver stained). *MW,* Molecular weight.

Abbreviation used
APC: Antigen-presenting cell

system. Two constructs were expressed: linked Fel d 1 chains 1 and 2 (designated rFel d 1 H22⁻) and Fel d 1 chains linked to a single-chain variable-region antibody fragment of the anti-CD64 mAb H22 (designated rFel d 1 H22⁺). Both constructs showed comparable IgE and IgG antibody binding to nFel d 1. The anti-CD64 mAb (H22) binds to high-affinity FcγRI receptors on APCs, and there is evidence that targeting antigen to APCs by means of CD64 increases the efficacy of antigen presentation and enhances T-cell activation.[23-25] High-level expression of rFel d 1 will facilitate structural, biologic, and immunologic studies of this important cat allergen, as well as the development of better diagnostic reagents and new approaches to allergen immunotherapy.

METHODS

Expression of Fel d 1 in baculovirus-infected insect cells

Recombinant Fel d 1 H22⁺ construct. The sFv portion of the anti-CD64 mAb H22 was cloned from vector pJG225 (Medarex Inc, Annandale, NJ) into the *Bam*HI and *Xba*I sites of the baculovirus expression vector pAcSAG-LIC (PharMingen, San Diego, Calif). A 6× His tag was inserted at the end of the cloning site. Fel d 1 chain 1 cDNA was cloned into the baculovirus expression vector, whereas chain 2 cDNA was cloned into vector pCR2.1 by using the TA cloning kit (Invitrogen, Carlsbad, Calif).[18] Primers were designed to include 5′ *Xho*I and 3′ *Nde*I restriction sites on chain 1 and 5′ *Pst*I and 3′ *Not*I restriction sites on chain 2. Chains 1 and 2 were subcloned in succession by using a flexible oligonucleotide linker: 5′ TATG (GGT GGA GGA GGT TCT)$_{\times 3}$CTGCA3′ (with 5′ *Nde*I and 3′ *Pst*I sticky ends underlined).[26] A 4-part ligation subcloning into the *Xho*I and *Not*I sites of the baculovirus expression vector was performed. The final construct is shown in Fig 1.

Recombinant Fel d 1 H22⁻ construct. A similar baculovirus Fel d 1 chain 1 and 2 construct was designed without the sFv portion of mAb H22.

Recombinant virus, protein expression, and purification

Recombinant baculovirus was generated by cotransfecting 1 μg of linearized BaculoGold baculovirus DNA (PharMingen) and 2 μg of expression plasmid construct into 3×10^6 Sf9 cells. Supernatant containing recombinant virus was harvested after 4 days, amplified to approximately 10^9 pfu/mL in Sf9 cells, and stored at –80°C as a source of high-titer stock. Recombinant Fel d 1 protein was expressed by using 1 mL of virus to infect 70% to 80% confluent Sf9 cells. High-titer virus supernatant was harvested after 7 days and stored at 4°C for use in expression. High Five (Hi5) insect cells (Invitrogen) were grown to a density of 3 to 4×10^6/mL in a 2-L baffled flask containing 500 mL of Excel 401 media (JRH Biosciences, Lenexa, Kan). Cells were infected with 100 μL of high-titer Fel d 1 virus supernatant and incubated with shaking at 30°C for 72 hours. Cultures were centrifuged at 17,000*g* for 10 minutes, and supernatant containing rFel d 1 was filtered through a 0.22-μm filter and stored at 4°C.

Recombinant Fel d 1 was purified by means of nickel affinity chromatography, followed by anion-exchange HPLC. Allergen was analyzed in nonreducing sample buffer on silver-stained SDS-PAGE gels by using a PhastSystem (Amersham Biosciences, Piscataway, NJ).

Purification of nFel d 1

Natural Fel d 1 was purified from house dust extract by means of affinity chromatography with the anti-Fel d 1 mAb Fd1A and size exclusion HPLC, as previously described.[27,28]

Serum samples

Human serum samples for analyses of IgE and IgG antibodies against Fel d 1 were obtained from 3 sources: (1) a cohort of Japanese children with cat allergy and asthma or rhinitis (n = 158) that had previously been analyzed for IgE antibody to Fel d 1 and had Pharmacia CAP scores to cat of class 2 or greater[21]; (2) a cohort of adult patients allergic to cat (or other allergens, n = 75) kindly provided by Drs Mark Larché and Barry Kay (National Heart & Lung Institute, London, United Kingdom); and (3) sera from patients with cat allergy (n = 25) provided by Dr Peyton Eggleston (Johns Hopkins University, Baltimore, Md). Human subjects approval for collection of sera was obtained from each institutional review board.

Antigen-binding RIAs

Purified nFel d 1 and rFel d 1 (H22⁺ and H22⁻) were radiolabeled with iodine 125 and used in antigen-binding RIAs to measure IgE and IgG antibodies.[11] Binding of nFel d 1 and rFel d 1 to human IgE and IgG antibody was compared by means of linear regression with Microsoft Excel LINEST and least-squares analysis.

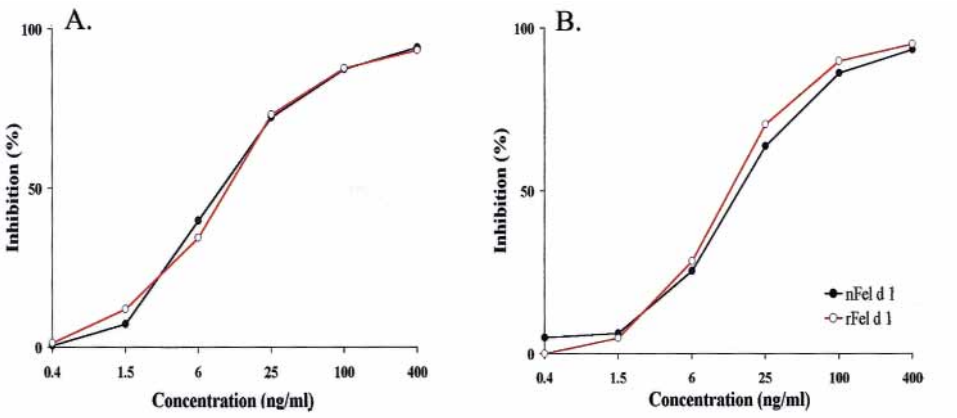

FIG 3. Inhibition dose-response curves comparing nFel d 1 and rFel d 1 (H22+). Binding of iodine 125–labeled nFel d 1 (2 ng, 110,000 cpm) to human IgG antibody in serum pools from either Japanese **(A)** or US **(B)** patients with cat allergy was inhibited with 0.4 to 400 ng/mL nFel d 1 *(filled circles)* or rFel d 1 *(open circles)*. Uninhibited binding was 16 to 20,000 cpm for the different serum pools, and the assay background was approximately 300 cpm.

Inhibition RIA

The ability of rFel d 1 to inhibit the binding of allergic sera to nFel d 1 was determined by using inhibition RIA. Serial 1:4 dilutions of unlabeled nFel d 1 (from 400 to 0.4 ng/mL) were incubated with pooled serum from 10 Japanese or 6 US patients with cat allergy. After a 2-hour incubation at room temperature, 110,000 cpm of iodine 125–labeled Fel d 1 H22+ was added to all tubes. Tubes were incubated for an additional 2 hours, precipitated overnight with goat anti-human IgG, washed, and counted.

Flow cytometric analysis

All procedures were performed at 4°C in PBS/0.2% sodium azide with 2 mg/mL BSA, as previously described.[29] Briefly, THP-1 cells (American Type Culture Collection, Rockville, Md) or human blood buffy coat cells were incubated for 1 hour with purified rFel d 1 H22+.[30] For the blocking studies, 100-fold excess soluble CD64-IgM fusion protein (Medarex Inc) was also added at this step.[31] After 3 washes, the cells were stained with anti-Fel d 1 mAb 2H4 (20 µg/mL) in the presence of 4 mg/mL human IgG. The cells were washed, stained with 17.5 µg/mL FITC-labeled goat $F(ab')_2$ anti-mouse Ig, and fixed with 1% methanol-free formalin. Populations of monocytes, lymphocytes, and neutrophils were gated by using forward- and side-scatter profiles, and cell fluorescence intensity was analyzed with a Becton Dickenson FACScan (Franklin Lakes, NJ).[30]

RESULTS

Expression of immunoreactive rFel d 1

Purified nFel d 1 migrated as a broad band at approximately 18 kd on SDS-PAGE (Fig 2). The rFel d 1 expressed in baculovirus was simultaneously expressed, folded, and secreted as a complete Fel d 1 molecule, and no additional refolding or processing was required. The rFel d 1 expressed with the sFv portion of anti-CD64 mAb (H22+) had a molecular weight of 49 kd on SDS-PAGE, and rFel d 1 expressed without H22 migrated at 22 kd (Fig 2).

Antibody binding to both forms of rFel d 1 was compared by using 4 mAbs directed against nonoverlapping epitopes on Fel d 1 in 2-site ELISA. Binding curves for rFel d 1 (either H22+ or H22−) were almost identical to those of nFel d 1 by using the mAbs 1G9 and 6F9 for allergen capture and the biotinylated mAb 3E4 for detection (data not shown). Inhibition experiments were used to compare the ability of nFel d 1 and rFel d 1 to compete for human IgG antibody binding by means of cross-inhibition in RIAs. Recombinant iodine 125–labeled rFel d 1 H22+ inhibited binding of nFel d 1 to anti-Fel d 1 IgG antibodies in 2 serum pools by greater than 95% and showed identical inhibition curves at concentrations of 0.4 to 400 ng/mL (Fig 3). These results showed that the rFel d 1 was antigenically indistinguishable from nFel d 1 by using polyclonal human IgG antibodies.

IgE and IgG antibodies to nFel d 1 and rFel d 1

Allergic sera (n = 258) from 3 different geographic locations were analyzed for IgE antibody binding to nFel d 1, rFel d 1 H22+, and rFel d 1 H22− by means of antigen-binding RIA with iodine 125–labeled allergens. Linear regression analysis showed a highly significant quantitative correlation between IgE antibody to both forms of rFel d 1 when compared with IgE antibody binding to nFel d 1 ($P < .001$, Fig 4). The specific activity of the radiolabeled rFel d 1 was higher than that of the natural allergen, and a subset of sera (approximately 10%) showed positive IgE antibody binding to rFel d 1 and weak or undetectable binding to nFel d 1. Because many of these sera were previously confirmed to be of CAP class 2 or greater to cat and IgE antibody levels to nFel d 1 were low (<25 U/mL), these results might indicate that the RIA with labeled rFel d 1 was more sensitive.[11] Only 5 (2%) sera showed higher IgE antibody binding to the natural allergen than to rFel d 1 (Fig 4).

IgG antibody binding to both forms of rFel d 1 and to nFel d 1 was compared in 73 sera from patients with cat allergy and positive skin test results and from nonallergic control subjects with negative skin test results from the United Kingdom. In keeping with the IgE antibody results and with the inhibition RIA data, there was a sig-

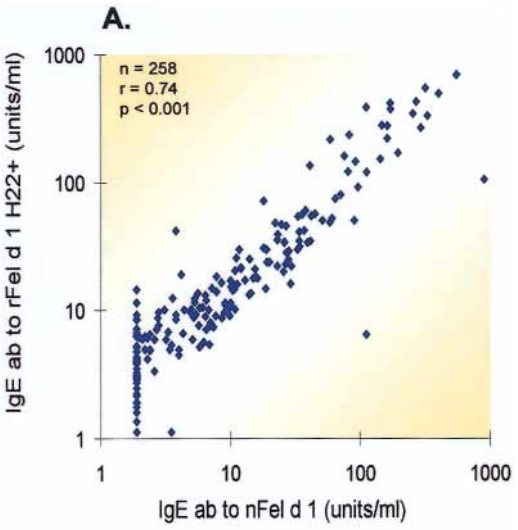

FIG 4. IgE antibody *(ab)* binding to rFel d 1 constructs. Linear regression analysis was used to compare IgE antibody binding to either rFel d 1 H22$^+$ **(A)** or rFel d 1 H22$^-$ **(B)** with binding to nFel d 1 in sera from 258 patients with cat allergy. Sera were incubated with iodine 125–labeled allergen in antigen-binding RIAs, and the results are expressed in arbitrary units relative to a standard serum pool.

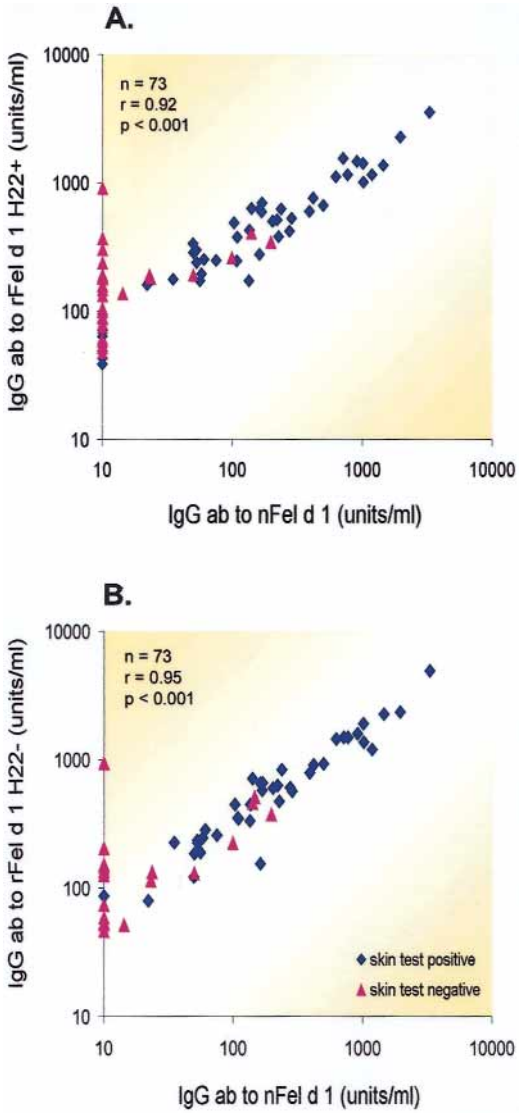

FIG 5. Comparison of IgG antibody *(ab)* binding to rFel d 1 constructs (**A,** H22$^+$; **B,** H22$^-$) and to nFel d 1 in patients with positive or negative skin test responses to cat allergen extract.

nificant quantitative correlation between IgG antibody to rFel d 1 H22$^+$ and H22$^-$ and nFel d 1 in these sera (r = 0.92, r = 0.95, and $P < .001$, respectively; Fig 5). We also observed that some sera, usually from patients with negative skin test results, had increased levels of IgG antibody to the rFel d 1 but low or undetectable levels of IgG antibody to nFel d 1. This effect was most marked for rFel d 1 H22$^+$, and 26 of 73 sera that were positive in the H22$^+$ assay were negative for IgG antibody to nFel d 1. When assayed for IgG antibody to rFel d 1 H22$^-$, 11 of the 26 sera had IgG antibodies to both rFel d 1 H22$^-$ and rFel d 1 H22$^+$ without IgG antibody to nFel d 1 (Fig 5). IgE antibody binding to nFel d 1 and rFel d 1 H22$^-$ for these 73 sera was also compared. There was a significant correlation in IgE antibody binding to rFel d 1 H22$^-$ and nFel d 1 (r = 0.88, $P < .001$), and 35 of 73 individuals had negative IgE results to both allergens. Three patients who had negative IgE results to nFel d 1 by means of RIA but positive results to rFel d 1 H22$^-$ were reported to have positive skin test results to cat allergen (Dr Mark Larché, written communication, February 1999).

Recombinant Fel d 1 H22$^+$ selectively targets CD64$^+$ APCs

Titration curves of buffy coat leukocytes prepared from whole blood, incubated with rFel d 1 H22$^+$, and stained with an anti-Fel d 1 mAb are shown in Fig 6, *A*. The rFel d 1 bound to the surface of monocytes and THP-

1 cells with high affinity and specificity because staining was saturated by 0.5 to 1 μg/mL rFel d 1 H22+, and no surface staining was seen with lymphocytes or neutrophils. Recombinant Fel d 1 H22− did not stain any of the cells (data not shown). To show that rFel d 1 H22+ specifically targeted Fel d 1 to CD64+ APCs, the CD64+ monocytic cell line THP-1 was exposed to 0.25 μg/mL rFel d 1 H22+ in the presence or absence of 25 μg/mL of the soluble CD64-IgM fusion protein. The cells were then stained for surface-bound rFel d 1. Incubating THP-1 cells with rFel d 1 H22+ resulted in the targeting of rFel d 1 to the cell surface, whereas the addition of the blocking CD64-IgM fusion protein reduced surface rFel d 1 staining to near-background levels (Fig 6, *B*).

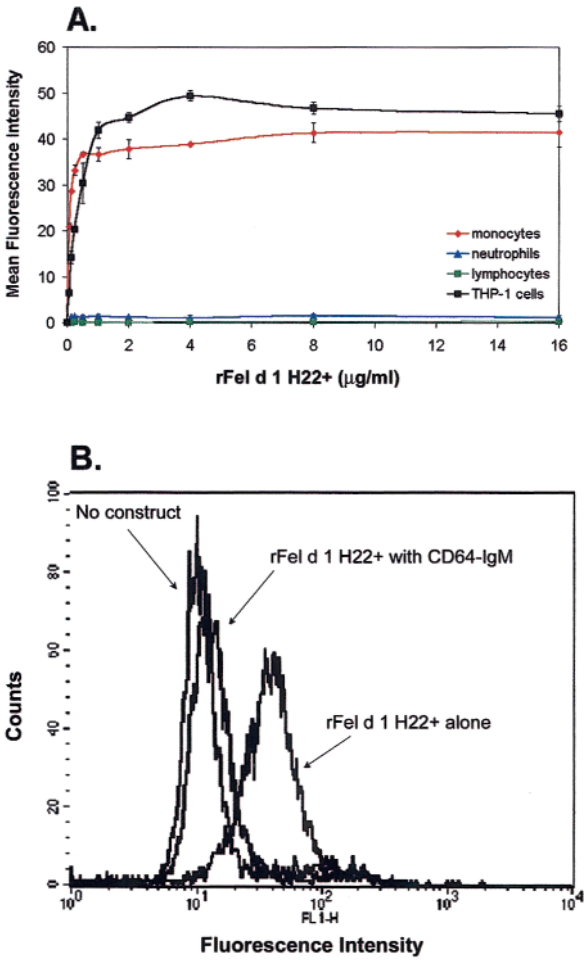

FIG 6. Targeting of rFel d 1 H22+ to APCs. **A,** Binding of anti-Fel d 1 mAb to monocytes *(shaded diamonds)*, THP-1 cells *(filled squares)*, neutrophils *(filled triangles)*, or lymphocytes *(shaded squares)* that had been incubated with different concentrations of rFel d 1 H22+ (background fluorescence was subtracted for each cell type) is shown. **B,** Binding of rFel d 1 H22+ to CD64 expressed on THP-1 cells is inhibited by soluble CD64-IgM fusion protein.

DISCUSSION

Chains 1 and 2 of Fel d 1 have been simultaneously expressed in a eukaryotic system, resulting in the successful production of a mature form of rFel d 1 having IgE reactivity comparable with that of nFel d 1. Recombinant Fel d 1 was purified directly from culture supernatant without the need for refolding or processing steps, as was required for *E coli*–expressed allergen.[4,16] Recombinant Fel d 1 was produced with and without an anti-CD64–targeting, single-chain, variable-region antibody fragment, and both forms showed excellent IgE antibody binding. The correlation between IgE antibody binding to rFel d 1 and nFel d 1 in vitro was as strong as has been observed for other recombinant allergens, including Der f 1, Der p 2, and Bla g 2,[32-34] and correlated with skin test reactivity to natural cat allergen extract.

The immunoreactivity of rFel d 1 was confirmed by means of IgG antibody analyses with murine mAb and human IgG antibody. The rFel d 1 inhibited IgG antibody binding to nFel d 1 by greater than 95%, and there was a good correlation between IgG antibody binding to both the H22+ and H22− forms of rFel d 1 and the natural allergen. There appeared to be some nonspecific IgG binding to rFel d 1 in a subset of sera, mainly from nonatopic control subjects, which showed IgG antibody to rFel d 1 but not to the natural allergen. This binding was more pronounced with the H22+ form of rFel d 1 and might be explained by the presence of IgG anticarbohydrate antibody or cross-reacting IgG antibody to a portion of the sFv region of the anti-CD64 mAb. Nonspecific IgG antibody binding to the H22− form of rFel d 1 was less common (11/73 [15%] sera). This could indicate that the carbohydrate moiety added to the molecule during secretion from the insect cell might be responsible for some IgG cross-reactivity. Glycosylation of rFel d 1 could be prevented by mutating the N-linked glycosylation site on chain 2. However, nFel d 1 is glycosylated, and although the carbohydrate side chains do not appear to be directly involved as antibody epitopes, they might be necessary for proper folding to create IgE-binding determinants.[4,35]

The rFel d 1 H22+ construct was designed to target the allergen to immunoregulatory cells (monocytes, macrophages, and dendritic cells) bearing the FcγRI receptor (CD64). Both the H22 moiety and Fel d 1 could be detected on human monocytes, suggesting that rFel d 1 H22+ could be used to investigate antigen processing, presentation, and T-cell responses to Fel d 1, as has been done for other antigens.[23-25,36] Targeting antigen to CD64 increases the efficiency with which dendritic cells sequester antigen and enhances their T-cell stimulatory potential.[23] We speculate that this form of targeting would improve the efficiency of allergen processing and lead to more reproducible allergen-specific T-cell responses. The recent observation that IFN-γ upregulates the expression of functional high-affinity FcγRI receptors on mast cells suggests that rFel d 1 H22+ could also be used to investigate non–IgE-mediated mechanisms leading to mast cell degranulation.[37]

In summary, we have engineered 2 rFel d 1 constructs that permit the expression of mature immunoreactive Fel d 1 without refolding and that facilitate targeting to APCs. The yield of rFel d 1 in baculovirus (4-6 mg/L) has been enhanced by expressing rFel d 1 H22$^-$ in *Pichia pastoris* (yield of 20-30 mg/L).[34] The rFel d 1 allergens have been used in miniaturized microchip arrays that allow IgE responses to multiple allergens to be analyzed in a single measurement.[38] These allergens will also provide essential tools for basic and clinical studies of the immune response to cat allergens, developing animal models of asthma and structural modifications of allergens that will allow new forms of immunotherapy to be developed.[15,39]

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