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Variable Content of Fel d 1 Variants in House Dust and Cat Extracts May Have an Impact on Allergen Measurement

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Abstract

Background: The major cat allergen, Fel d 1, is a tetrameric glycoprotein composed of 2 heterodimers. Polymorphisms in this allergen are well documented. Recent work shows that Fel d 1 samples can contain core fragments of variable immunoreactivity.
Objectives: Our objective was to compare Fel d 1 polymorphism in cat extracts and house dust, which is used as an indicator of allergen exposure and to understand how the combination of individual Fel d 1 variants can affect cat allergen measurement.
Methods: Natural Fel d 1 allergens were water-extracted from house dust and from the chest area and anal sacs of a cat. Recombinant Fel d 1 was provided commercially. The samples were analyzed by immunoblotting; variants were isolated using gel electrophoresis and tested using enzyme-linked immunosorbent assay.
Results: Four Fel d 1 variants of 40, 30, 19-21, and 14-16 kDa were consistently identified in Fel d 1 samples. Fel d 1 patterns found in house dust and the chest area wash were similar. Dimers were shown to be the major variant, while intact or truncated tetramers and core fragments were found in variable amounts. Intact and truncated dimers of Fel d 1 displayed similar antibody binding. Conversely, the intact tetramer—but not the core tetramer—was found to bind twice the antibody amount as the dimers and core fragments.
Conclusions: Despite a common pattern of Fel d 1 variants in cat extracts and house dust, variations in the tetramer-to-dimer ratio among samples may introduce major discordances in cat allergen measurements using immunoassays. Our findings indicate the need for further harmonization of allergen immunoassays.

Key words: Allergen measurement. Cat. Fel d 1. House dust. Immunoreactivity. Polymorphism. Recombinant Fel d 1. Variants.

Resumen

Introducción: Fel d 1, alérgeno mayoritario del gato, es una glicoproteína tetramérica compuesta de 2 heterodimeros. La existencia de polimorfismos de este alérgeno han sido bien documentados. Estudios recientes muestran que las muestras de Fel d 1 pueden contener polimorfismos de inmunorreactividad variable.
Objetivos: Nuestro objetivo fue comparar los polimorfismos de Fel d 1 en extractos de gato y de polvo de casa, utilizando ambos como indicadores de exposición alérgenica, y para investigar si la combinación de variables individuales de Fel d 1 afecta la determinación del alérgeno del gato.
Métodos: Los alérgenos naturales de Fel d 1 fueron extraídos en solución acuosa, tanto del polvo de casa, como de los sacos anales y del área torácica del gato. El Fel d 1 recombinante fue adquirido comercialmente. Las muestras fueron analizadas por inmunoblotting; las variantes fueron aisladas usando electroforesis en gel y ensayadas usando un enzimoinmunoensayo
Resultados: En los resultados obtenidos, se identificaron en las muestras de Fel d 1,cuatro variantes de Fel d 1 de 40, 30, 19-21, y 14-16 kDA. Los patrones de Fel d 1 encontrados en el polvo de casa y el área torácica del gato fueron similares.
Los dímeros eran la variante mayor, mientras que los tetrameros intactos y truncados de Fel d 1 se encontraron en cantidades variables y mostrando una unión similar a los anticuerpos. En cambio, el tetramero intacto (no el tetramero central) unió dos veces más anticuerpos que los dimeros y los fragmentos centrales.
Conclusiones: A pesar de encontrar un patrón común de variantes de Fel d 1 en extractos de gato y polvo de casa, las variaciones en la relación tetrámero/dímero entre las muestras puede introducir errores importantes en las mediciones de alérgeno de gato en inmunoensayos. Son necesarios estudios de estandarización de los inmunoensayos.

Palabras clave: Cuantificación de alérgeno. Gato. Fel d 1. Polvo de casa. Inmunorreactividad.

Introduction

Fel d 1 is the major cat allergen and an important cause of respiratory diseases such as asthma and allergic rhinitis. A member of the secretoglobin family [1], this allergen is a tetrameric 35-40–kDa glycoprotein composed of 2 polypeptide chains linked by 3 disulfide bonds, namely, chain 1 (70 amino acids) and chain 2 (90-92 amino acids), which also contains a heterogeneous triantennary N-glycan (N33) [2-4]. Fel d 1 is produced by the lachrymal, salivary, and sebaceous glands and secreted onto animal skin and fur; it is also present in large amounts in anal sacs [5-9]. Natural sources of Fel d 1 (house dust and salivary and epithelial extracts) were shown to contain tetramers (35 kDa), separate heterodimers (17 kDa), free chain 2 (11-15 kDa), and free chain 1 (4-5 kDa) on immunoblots [10]. Some authors have also described Fel d 1 polymorphisms with tissue-specific isoforms of chain 2 [11] and minor changes in chain 1 and 2 sequences [4,11]. We recently showed that Fel d 1 samples from various parts of a cat's body were composed of a mixture of forms of Fel d 1 with different molecular weights and altered immunoreactivity within a given cat population [12]. Thus, extensive polymorphism of Fel d 1 was observed in the primary source of Fel d 1, namely, the cat's body, to which patients are directly exposed during active interactions with their pet. However, in homes, Fel d 1 is mainly found at high levels in dust [13-15], to which the patients are passively but continuously exposed, even in the absence of cats [16]. The nature of Fel d 1 polymorphism has not yet been clearly established in dust samples.

The extensive polymorphism of Fel d 1 prevents accurate measurement of Fel d 1 in the environment and in extracts used in diagnosis and immunotherapy. This drawback is an important issue for patient safety and follow-up of therapy [17,18]. Consequently, several studies have suggested replacing natural allergen extracts by a recombinant preparation that is more homogeneous, may be easily and unlimitedly supplied, and for which production processes can be controlled [19,20]. Work is ongoing to determine whether such a preparation can be used for standardization of Fel d 1 [21,22].

We recently showed that a structural polymorphism of Fel d 1 was present at various sites of Fel d 1 production in cats. Interestingly, a single pattern of variants is observed among cats, and this pattern may vary both quantitatively and qualitatively at the production sites. The present study aimed to determine whether such a structural polymorphism existed in house dust and to elucidate how the combination of individual Fel d 1 variants affects measurement. The data obtained will be particularly useful, since measurement of Fel d 1 levels in house dust is used to monitor allergen exposure. Additionally, we characterized the polymorphism of recombinant Fel d 1 to assess whether the immunological behavior of this product reflected that of the natural allergens.

Materials and Methods

Fel d 1 Samples

The study was performed using 10 European cats from IRSEA (St Saturnin d'Apt, France). Chest area (CA) and anal sac (AS) samples were taken as described in Bienboire-Frosini et al [12]. House dust from 4 homes was collected with the DUSTREAM collector (INDOOR Biotechnologies) adapted to a vacuum cleaner by vacuuming the living room and the bedrooms, including the floors, upholstered furniture, bedspreads, and carpets, if present, for 10 to 15 minutes. All samples were weighed. Fel d 1 was water-extracted from cat samples and house dust (1 mL/0.1 g of dust) as previously described [12].

The 2 forms of purified recombinant (rec) Fel d 1 proteins (INDOOR Biotechnologies) were a glycosylated form of *Pichia pastoris* and a nonglycosylated form of a *P pastoris* clone N103Q [23].

Electrophoresis and Immunoblotting

Analytical electrophoresis and preparative electrophoresis were performed, respectively, using 16% and 14% Novex Tris-Glycine gels (Invitrogen) under weakly denaturing conditions, Novex Tris-Glycine Native Sample buffer without sodium dodecyl sulfate (SDS), and 0.1% SDS Novex Tris-Glycine Running buffer (Invitrogen). The SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used as a marker of molecular weight. Analytical electrophoresis was followed by transfer of proteins to a nitrocellulose membrane (25V, 100 mA for 1 hour), and immunodetection was performed using the WesternBreeze Chromogenic kit (Invitrogen), with saturation in 1% phosphate-buffered saline (PBS) Tween-bovine serum albumin for 1 hour. The monoclonal antibody (mAb) 6F9 was used at 1 μg/mL in this buffer. Pixel analysis of the different Fel d 1 bands on identical rectangular areas of immunoblots was carried out using ImageJ software.

Preparative electrophoresis was followed by protein staining using SimplyBlue Safe Stain (Invitrogen) to locate Fel d 1 components. Bands were cut at different molecular weights, crushed in 300 μL of phosphate buffer (50 mM), and incubated overnight with gentle shaking. After centrifugation at 10 000*g* for 10 minutes, supernatants were recovered and stored at –20°C.

Mass Spectrometry of Recombinant Allergens

Mass spectra were recorded on a Microflex II matrix-assisted laser desorption (MALDI)/ionization time-of-flight mass spectrometer (MS) (Bruker Daltonics) using Flex control software in a linear positive mode with pulsed ion extraction. A saturated solution of sinapinic acid matrix was prepared in 0.1% trifluoroacetic acid in water/acetonitrile (60:40, v/v). Aliquots of 0.7 μL rec Fel d 1 (55 μM) were immediately placed on a MALDI-MS stainless steel target plate with 0.7 μL of the matrix solution, and the spots were allowed to dry at room temperature before analysis. External mass calibration was carried out on the Protein Calibration Standard I (Bruker Daltonics) using the single-charged ions [M+H]+ at 5733.51 Da (insulin), 8564.76 Da (ubiquitin), 12 359.97 Da (cytochrom C), 16 951.30 Da (myoglobin), and the doubly-charged ion [M+2H]2+/2 at 8476.28 Da (myoglobin). Mass spectra were then treated with Gaussian smoothing.

Immunoassays

Enzyme-linked immunosorbent assay (ELISA) (INDOOR Biotechnologies) was used following the manufacturer's

instructions to quantify Fel d 1 in natural samples. Comparative binding assays were performed with samples containing 0 to 120 ng/mL of Fel d 1–related material using the anti–Fel d 1 mAbs 6F9 and 3E4 (INDOOR Biotechnologies), respectively, as a capture antibody (500 ng/well; coating overnight at 4°C in pH 9.6 carbonate-bicarbonate buffer) and a biotinylated tracer antibody (50 ng/well; incubation overnight at 4°C) on 96-well microtiter plates (VWR International). The Biotin Protein Labeling Kit (Roche) was used to house biotinylated tracer antibody, since the concentration of the biotinylated tracer from the commercial kit is unknown. Washing, blocking, and amplification were performed as described in the kit. For each sample, the recorded absorbances (OD 405 nm) at 4 concentrations from the linear part of the dose-dependent curves were plotted on the x and y axes of a graph to compare sample immunoreactivity in groups of 2 using linear regression.

Dilution range binding assays were performed as mentioned above, and dilutions were adapted to each sample in order to achieve the same absorbance values for all of them.

Two procedures were used to measure epitope expression of Fel d 1 isolated variants: *1)* direct ELISA with 6 ng/mL of individual Fel d 1 variant (coating overnight at 4°C in 50 mM PBS, pH 7.4) and 50 ng/well of biotinylated tracer antibody (incubation overnight at 4°C); and *2)* a sandwich ELISA designed as described above with 3 and 6 ng/mL of individual antigens. All incubation steps were carried out overnight.

Results

Diversity of Fel d 1 Molecular Forms in Various Allergen Sources

Figure 1 presents the Fel d 1 materials that were characterized in the house dust extracts. The materials were compared to rec Fel d 1 and samples from a typical cat (CA and AS), from which the Fel d 1 polymorphism had already been characterized [12]. Fel d 1 from AS was observed as variants of approximately 13-14 kDa and 30 kDa, while Fel d 1 from the CA exhibited 2 main components of approximately 19-21 kDa and 40 kDa. In accordance with our previous work [12], the 13-14–kDa variants were identified as core fragments of Fel d 1 dimers (18-22 kDa), and tetrameric Fel d 1 was identified as a 30-kDa variant in AS extracts and as a 40-kDa protein in CA washes [12]. All house dust samples displayed a large band of Fel d 1 molecules at approximately 18-20 kDa and a weak band at around 40 kDa. We also detected truncated Fel d 1 as a 16-kDa component in house dust extract when the gels were heavily loaded (data not shown), as observed for the CA sample [12]. Remarkably, all the samples shared a common pattern of 4 natural variants, namely, molecular forms weighing 40, 30, 20, and 13-14 kDa. The major component was consistently identified as the approximate 20-kDa Fel d 1 dimer in every house dust extract. Since these house dust samples were fully comparable to the CA sample, we only further analyzed the CA sample, as it was available in higher quantities. rec Fel d 1 was detected as polypeptides of approximately 18 kDa (triplet bands), 22 kDa, and 40 kDa, which were likely nonglycosylated, glycosylated, and dimer forms of recombinant Fel d 1, respectively, since this product was engineered as a single polypeptide including chains 1 and 2 fused by a peptide linker [23], as indicated by the rec Fel d 1 manufacturer.

Nondenaturing electrophoresis was performed on cat samples and recombinant preparations to compare the pattern obtained under weakly denaturing conditions: Fel d 1 was detected as 2 bands in CA and AS samples (as under weakly denaturing conditions) and as 4 bands in the rec Fel d 1 sample (data not shown).

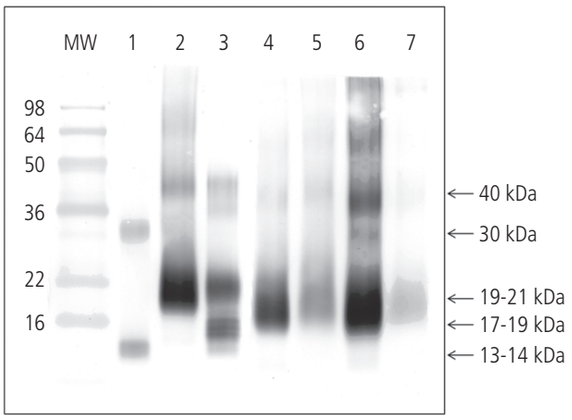

Figure 1. Immunoblot analysis of various house dust extracts compared to Fel d 1 (approximately 30 ng of allergen) from a recombinant origin or from a typical cat. Lane 1, anal sac sample; lane 2, chest area wash sample; lane 3, commercial recombinant Fel d 1 from *Pichia pastoris*; lanes 4-7, house dust extracts. Immunoblotting after electrophoresis under weakly denaturing conditions was carried out using the mAb 6F9, which can recognize all Fel d 1 variants, as previously described [12]. MW indicates molecular weight.

Immunoreactivity of Cat Samples With Different Compositions of Fel d 1 Variants

We compared the immunoreactivity of the Fel d 1 extracts with the standard supplied in the ELISA kit by INDOOR Biotechnologies (Figure 2). When the CA wash was compared with the standard (Figure 2A), we found a linear regression slope value of 0.9131, demonstrating satisfactory similarity between the 2 samples. In contrast, the AS extracts poorly resembled the standard (Figure 2B, slope value of 1.2883). Poor similarity was also observed between the CA wash and the AS extract (Figure 2C, slope value of 1.4122), even though both samples were from the same animal.

rec Fel d 1 was further compared to the standard and natural allergen. In Figure 2D, the slope value was 0.5781, indicating marked discordance between the standard and rec Fel d 1. In addition, natural Fel d 1 was different from rec Fel d 1, since the slope values of 0.6191 and 0.4371 were obtained for the CA wash (Figure 2E) and AS extracts (Figure 2F), respectively. All these data showed that antibody binding significantly varied between these preparations, the highest binding values being

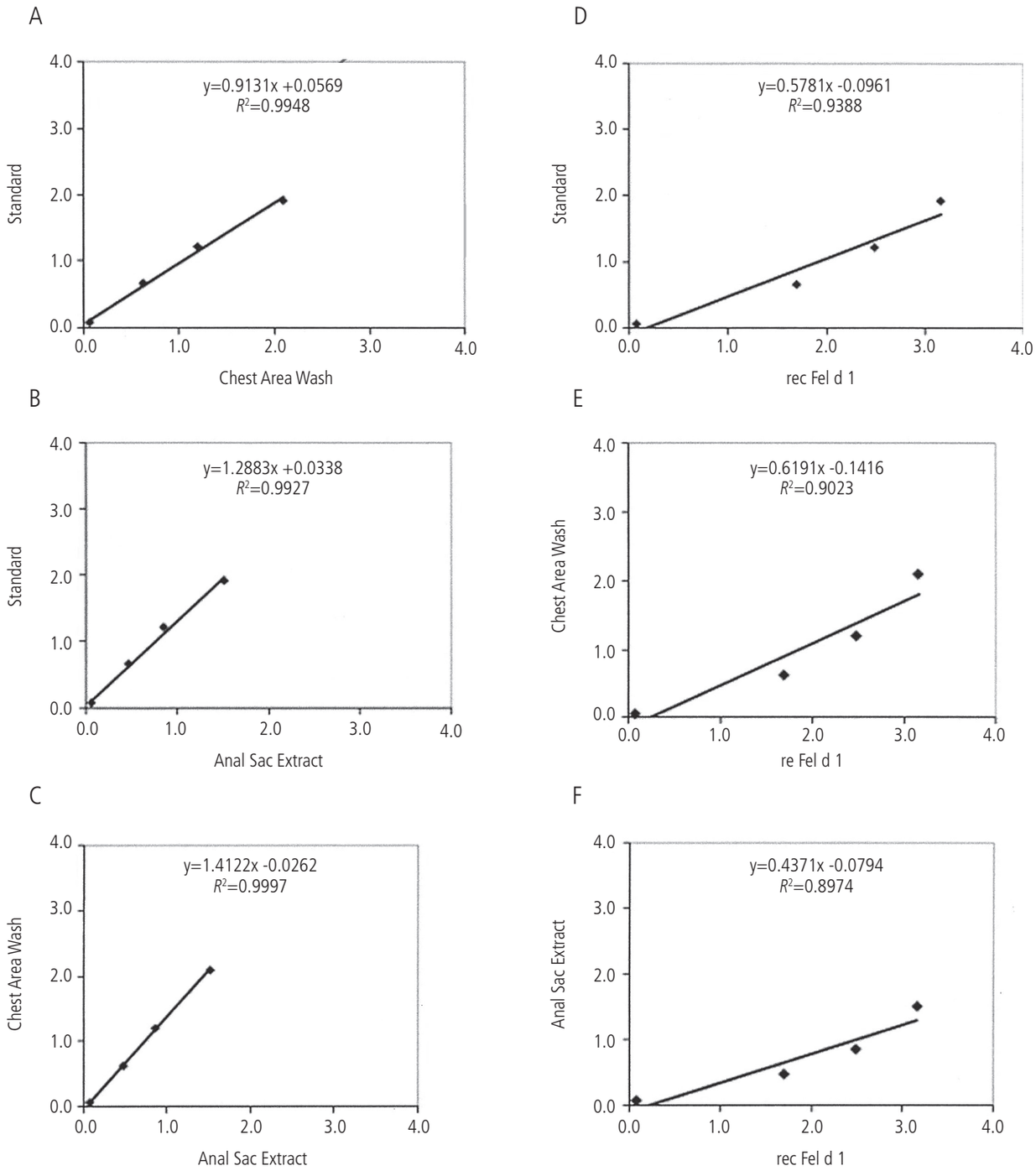

Figure 2. Comparative immunoreactivity of Fel d 1 samples in the 6F9/3E4 enzyme-linked immunosorbent assay. For each sample, OD 405 nm at 4 concentrations from the linear part of the dose-dependent curves (0, 1.875, 3.75, and 7.5 ng/mL of Fel d 1) are reported on the x and y axes. Linear regression was applied to compare the 2 extracts. When their antibody-binding characteristics are virtually identical, the slope value is close to 1. R^2 refers to the linearity of the absorbances at each concentration of the extracts and is ideally close to 1.

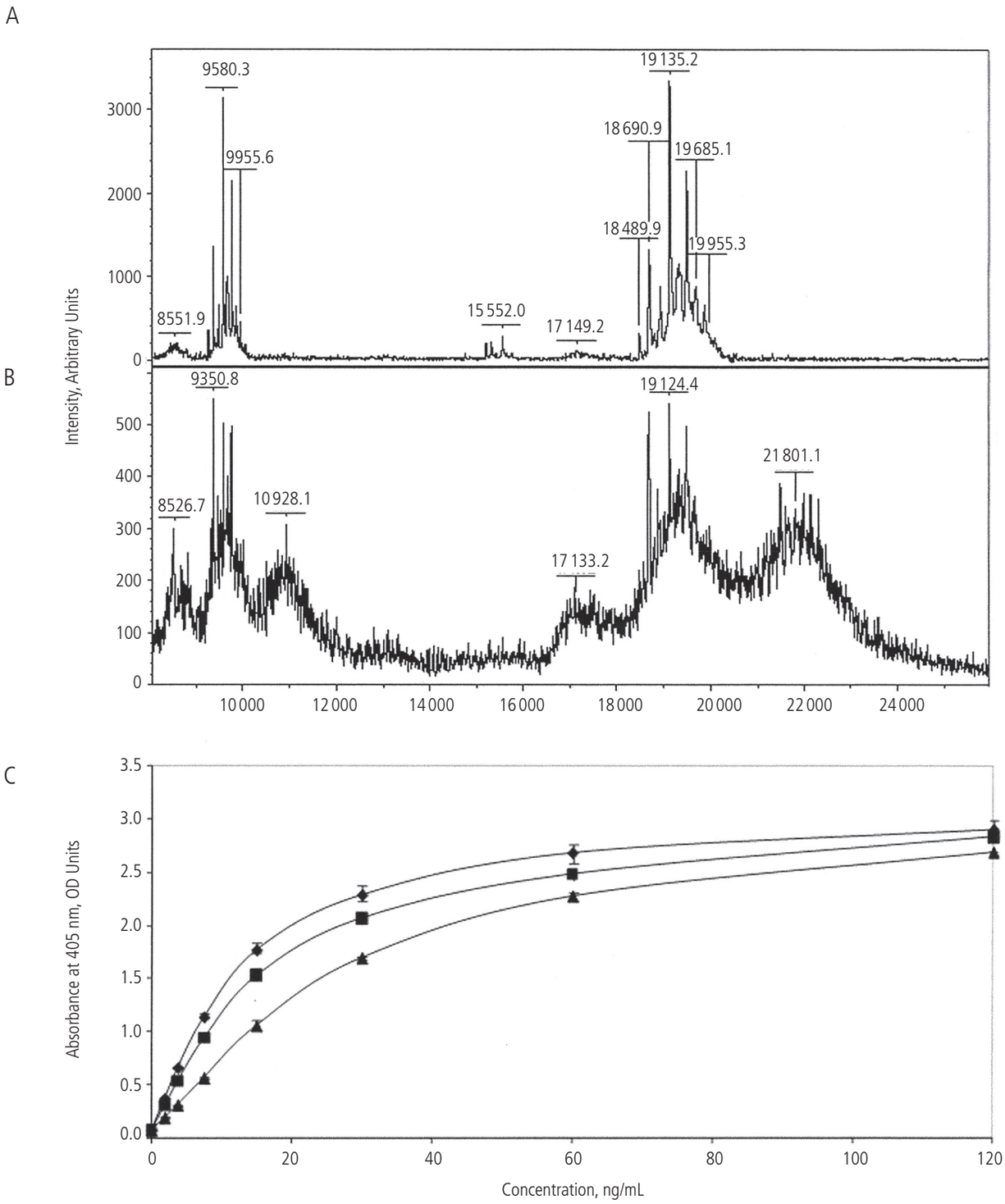

Figure 3. Analysis of recombinant Fel d 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectra of rec Fel d 1 produced in *Pichia pastoris*. A, Nonglycosylated form. B, Glycosylated form. C, Dose-dependent curves of the 6F9/3E4 format in enzyme-linked immunosorbent assay using glycosylated rec Fel d 1 (♦), nonglycosylated rec Fel d 1 (■), and natural Fel d 1 (pool of whole cat body washes) (▲). In panel A, the ions in the 9580-Da mass area correspond to the doubly-charged species, the single-charged ones being at around 19 135 Da.

observed with rec Fel d 1. Accordingly, all the samples were measured differently in a given immunoassay.

Characterization of Recombinant Fel d 1 Variants

The mass spectrum of the nonglycosylated variant appeared as a group of ions with peaks ranging from 18 490 to 19 875 Da (the most intense ion was observed at 19 135 Da). The mass spectrum of the glycosylated variant showed a more complex pattern of ions including a group with a mass-to-charge ratio (m/z) that was similar to the nonglycosylated variant (mean m/z at 19 124 Da), thus demonstrating the presence of the nonglycosylated species in this sample. Another group of ions showed masses 2600 Da heavier than those corresponding to the nonglycosylated form (mean m/z at 21 801, range 21 200-22 300), and which could be assigned to the glycosylated polypeptides. A third group of ions was observed at a mean m/z of 17 133 Da, about 1990 Da less than the nonglycosylated form, and was assigned to a truncated nonglycosylated form, as the rec Fel d 1 sequence includes a Pro-Asp bond in chain 1, which may spontaneously cleave in acidic conditions to generate a theoretical shortening of 1974 Da in the rec Fel d 1 chain 1 N-terminus. These data reinforce the assignment of the ion group at 17-19 kDa to the nonglycosylated form and the ion group at 20-22 kDa to the N-glycosylated form. When both glycosylated and nonglycosylated rec Fel d 1 were analyzed using ELISA and compared to natural Fel d 1 (Figure 3C), the recombinant allergens showed similar maximal binding capacity and almost similar half-maximal binding capacity, although the latter was superior to that of natural Fel d 1 (9.3 ng/mL and 12.0 ng/mL for glycosylated and nonglycosylated rec Fel d 1, respectively, compared with 19.4 ng/mL for natural Fel d 1).

Antibody Recognition of Purified Fel d 1 Variants

Fel d 1 variants were isolated by preparative gel electrophoresis, and their individual recognition by 6F9/3E4 mAbs (Figure 4) was analyzed to understand the differences in Fel d 1 measurements between the natural and recombinant sources. Serial dilutions of the isolated variants showed parallel curves for all Fel d 1 variants: the slopes of the linear part of all curves ranged from 0.750 to 0.855, with a mean (SD) of 0.801 (0.030) (Table 1), indicating that 6F9/3E4 antibody–binding characteristics are similar in all these individual variants, whether from natural or recombinant origins.

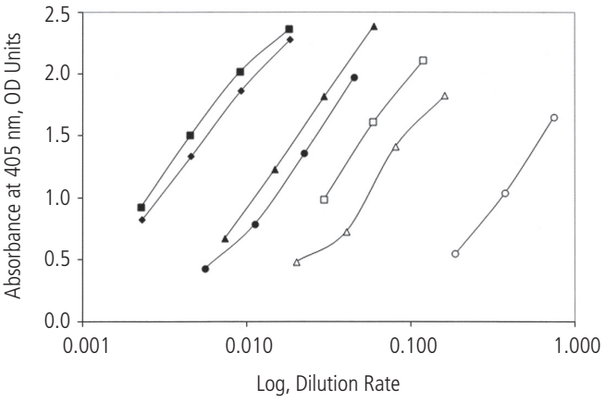

Figure 4. Binding of serially diluted isolated Fel d 1 variants from different sources to the 6F9/3E4 enzyme-linked immunosorbent assay at equilibrium. The dilution rate is presented on a logarithmic scale. ◆, 17-19–kDa band from rec Fel d 1; ■, 19-21–kDa band from rec Fel d 1; □, 40-kDa band from rec Fel d 1; ●, 20-kDa band from chest area wash; ○, 40-kDa band from chest area wash; ▲, 13-14–kDa band from anal sac extract; Δ, 30-kDa band from anal sac extract.

Estimation of Approximate Fel d 1 Tetramer/Dimer Ratio

Since the individual Fel d 1 variants displayed comparable antibody-binding characteristics, we were interested in estimating their variable ratio. Table 2 shows the relative estimated amount of tetramer and dimer in the samples used in the study. We observed a similar ratio for house dust extract and CA wash, with approximately 15 tetramers per 100 dimers. In AS, the estimated ratio was 36%, indicating that more tetramers were probably present in these samples. The highest estimated ratio was observed for rec Fel d 1, with approximately 40 tetramers per 100 dimers.

Table 1. Linear Regression Analysis of Serially Diluted Curves of Fel d 1 Individual Variants From Different Sources in the 6F9/3E4 Enzyme-Linked Immunoassay

Source	Approximate Band Size	Slope	Intercept	R^{2a}
rec Fel d 1	16	0.750	5.379	0.999
	22	0.785	5.720	0.998
	36-40	0.807	3.856	0.995
Fel d 1 from chest area	18-22	0.855	4.622	0.999
	36-40	0.796	1.869	0.995
Fel d 1 from anal sacs	14-16	0.824	4.717	0.999
	30	0.793	3.321	0.978

[a]R^2 is the correlation coefficient of the linear regression curve.

Table 2. Tetramer/Dimer Ratio of the Different Fel d 1 Bands From Immunoblots

Fel d 1 Sources	Mean (SD) Tetramer/Dimer Ratio, %
House dust extract	15 (8)
Chest area	14 (5)
Anal sacs	36 (17)
Recombinant Fel d 1	40 (12)

Immunoblots with 6F9 mAb under weakly denaturing conditions were scanned and treated in negative mode with ImageJ software. For every band, the mean pixel density values of a rectangular area (0.02 inches) were recorded and used to calculate the tetramer/dimer ratio.

Epitope Expression of Natural and Recombinant Variants of Fel d 1

Figure 5A shows the direct measurement of 6F9 and 3E4 epitope expression tested at the mean half-maximal effective concentration in 4 variants from the antigens with the most different immunoreactivities (AS and rec Fel d 1). Each of the 6F9 and 3E4 mAbs displayed a signal that varied between dimers and tetramers by a factor of 2, either from intact proteins (rec Fel d 1) or truncated proteins (AS Fel d 1). Hence, the number of epitopes was doubled for each mAb when Fel d 1 dimers clustered in tetramers. The extent of epitope expression proved to be different when these mAbs acted in a sandwich format (Figure 5B). Recognition of rec Fel d 1 molecular weight forms by the mAbs maintained the expected 1:2 ratio between dimers and tetramers, while the dimer and tetramer from AS exhibited a similar level of antibody binding. This showed that the 30-kDa tetrameric form was poorly immunoreactive: it

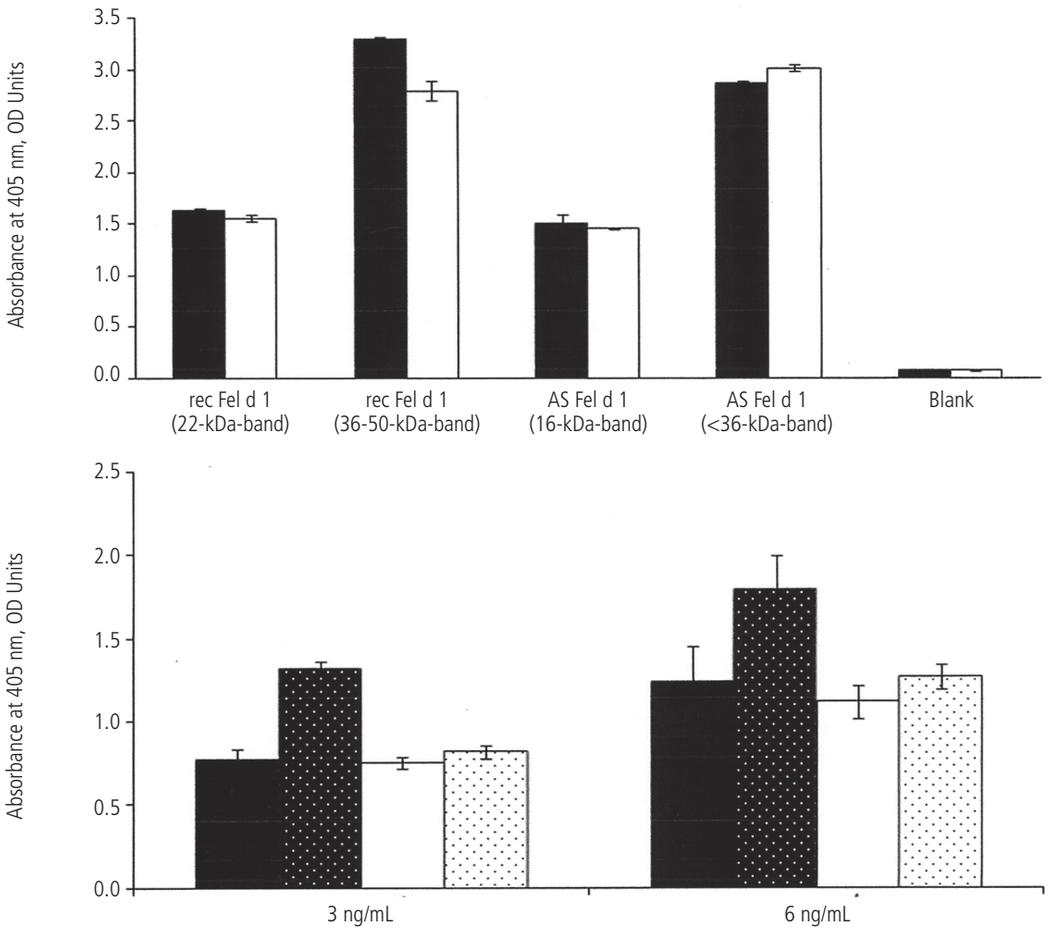

Figure 5. Measurement of 6F9 and 3E4 epitope expression in Fel d 1 individual molecular weight forms from recombinant and natural origins. A, Direct enzyme-linked immunosorbent assay using 6 ng/mL of each coated antigen variant. ■, 6F9 mAb; □, 3E4 mAb. B, 6F9/3E4 format sandwich enzyme-linked immunosorbent assay at 3 and 6 ng/mL of each antigen variant. ■, dimer rec Fel d 1 (19-21–kDa band); ▪, tetramer rec Fel d 1 (40-kDa band); □, dimer anal sac (14-16–kDa band); ⊡, tetramer anal sac (30-kDa band). Because of availability concerns, rec Fel d 1 was used as a reference molecule for Fel d 1 molecular forms of around 20 and 40 kDa, which were considered intact forms and are found in chest area and house dust extracts.

expressed the 6F9 epitope and was recognized by the capture antibody, although only a single 3E4 epitope was available for the tracer antibody. Furthermore, doubling the amount of Fel d 1 doubled the signal for all the variants except truncated tetramers, indicating that most variants were properly measured in the linear portion of the assay.

Discussion

We characterized Fel d 1 polymorphism as a common pattern of variants present in cat extracts as well as in house dust. This pattern is composed of a large array of components that are detected irregularly on immunoblots, namely, intact tetramers of 40 kDa, intact dimers of 20 kDa, truncated tetramers of 30 kDa, and truncated dimers weighing from 13-14 to 16-17 kDa. Intact and truncated Fel d 1 were previously detected in both dimeric and tetrameric forms from various body areas of several cats, notably by 6F9 and 3E4 mAbs [10,12]. However, antibody binding was clearly diminished in the Fel d 1–like material of the interdigital zones and anal sacs, indicating that core fragments may alter allergen assessment [12]. The present study aimed to demonstrate the structural basis of this effect and suggests that dimers and tetramers associate in a typical pattern at a highly variable ratio between extracts. Altogether, these findings clarify the molecular basis of the well-established polymorphism of Fel d 1, which could therefore be assigned to a variable combination of these 4 variants independently of allergen origin and animal. Additionally, the extent of epitope expression can differ between variants: in this study, 6F9 and 3E4 mAbs were able to measure all dimers and tetramers with an appropriate stoichiometry of 1:2 in a direct immunoassay but not in a sandwich format, where mAbs binding of a truncated tetramer is as effective as that of an intact or truncated dimer and half as effective as that of an intact tetramer. The ability of some mAbs to discriminate between different molecular forms in allergen measurement assays is not unprecedented in the literature [24-27]. Consequently, samples containing different mixtures of Fel d 1 variants can display specific immunoreactivity, thus affecting the accuracy of immunoassay, particularly since the measure will be quantified against a calibrator exhibiting quite a different variant pattern.

Our findings could also explain why rec Fel d 1 was more immunoreactive than a natural source of Fel d 1. This protein, which is produced in *P pastoris*, does not possess the glycosylation pattern of a mammalian protein. To specify this pattern and to clarify the differences in weight between the molecular forms of rec Fel d 1, we first analyzed nonglycosylated and glycosylated rec Fel d 1 (Figures 3A and 3B, respectively) using mass spectrometry and then ELISA to compare their immunoreactivity. Since the results indicated similar antibody recognition in both recombinant preparations, the high immunoreactivity of rec Fel d 1 does not originate from glycosylation. Consequently, the nonglycosylated mutant of rec Fel d 1 was not analyzed further.

As in previous studies demonstrating that N-glycan was not directly involved in antibody recognition [28,29], the recombinant preparation was found to contain a significant amount of nonglycosylated forms, which displayed full immunoreactivity. Indeed, epitope expression was fully comparable to that of the natural variants. However, the recombinant preparation was found to be hyperreactive to mouse mAbs. Vailes et al [23] also found that human sera showed a higher degree of IgE binding to rec Fel d 1 than to the natural allergen. We suggest that this hyperactivity in the recombinant preparation is likely to reflect higher tetramer content.

Finally, we also wanted to understand the clinical relevance of this immunological polymorphism in samples used to evaluate exposure to allergens such as house dust, of which the pattern of Fel d 1 variants was comparable to that of the CA sample: it was not the tetramer or a mixture of variants that remained indoors, but essentially the dimer that appeared to be the major structural form present in house dust. Interestingly, another study by members of our group found that the release of some Fel d 1 variants was influenced by cats' behavior [30], which could in turn result in a change in the composition of house dust.

Because it is impossible to assign a molecular weight to a band in native electrophoresis, and as native electrophoretic patterns were comparable to weakly denaturing electrophoretic patterns, we decided to perform pixel analysis on immunoblots from the latter. Our method of estimating the tetramer/dimer ratio following immunoblotting using densitometry might lack accuracy, particularly in light of the possible influence of the low quantity of SDS used under weakly denaturing conditions. Our results might be confirmed by a method such as size-exclusion chromatography, which could provide us with more accurate ratios, even though this technique has been shown to affect allergen conformation [31]. Here, we were only interested in estimating a preliminary ratio using a quick method to evaluate major differences between samples. Because we treated all samples under identical weakly denaturing conditions at a given allergen load, we assumed that the possible effect of SDS was the same across the samples and, taking into account the putative inaccuracy of the method, we were therefore able to compare the estimated tetramer/dimer ratios. These ratios revealed that house dust extracts, CA washes, AS extracts, and rec Fel d 1 displayed a variable amount of tetramers (approximately 15%-40%). This variable amount accounted for significant changes in antibody recognition of the whole extract. Indeed, the immunoreactivity of the 30-kDa tetramer variant was lower than that of the 40-kDa proteins and was measured in the same way as that of the 20-kDa or 13-14–kDa proteins, indicating a potential change in conformation that was sufficient to decrease antibody binding in a sandwich format. In ELISA measurements, this protein might be estimated at 50% of its molecular weight on a molar basis, since it is specifically assumed in immunoassays that a given antigen is estimated through the expression of a single epitope. Therefore, AS extracts appeared poorly reactive, because the truncated tetramer was not able to bind tracer antibody stoichiometrically, thus leading to a falsely low measurement.

Our findings show that measurements may suffer from artifactual bias, as they contain more or less traceable Fel d 1 material and are estimated against a standard material whose

composition is different. In recent years, harmonization of Fel d 1 and assays has been open to intense debate [18-20], and in this context, our study supports the view that cat extracts could not be standardized only by changing calibrators from a natural to a recombinant preparation at this stage. However, we favor the idea that molar calibration could be achieved, provided that antibodies ensure stoichiometric binding of allergen variants [32]. Therefore, the current status of Fel d 1 measurements warrants further investigation.

Data from this study were presented in abstract form (193 words) and poster form at the 4th International Symposium on Molecular Allergology in Munich, Germany, 29-31 October 2010.

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