

Characterization of recombinant cat albumin

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Summary

Background Indoor allergens derived from animals and mites often contribute to exacerbation of skin manifestations in atopic dermatitis (AD) patients.

Objective To produce and characterize recombinant cat albumin, a cross-reactive animal allergen.

Methods A complete cDNA coding for cat albumin was obtained by RT-PCR amplification from cat liver RNA. Recombinant cat albumin was expressed in *Escherichia coli* as hexahistidine-tagged protein, purified by nickel affinity chromatography and studied for IgE reactivity with sera from cat-allergic patients by ELISA and immunoblotting. Furthermore, CD203c expression of basophils from cat-allergic patients upon exposure to recombinant cat albumin was analysed.

Results Recombinant cat albumin, a cross-reactive animal allergen sharing most IgE epitopes with its natural counterpart, was produced in *E. coli*. It was recognized preferentially by IgE from AD patients and elicited IgE-dependent basophil activation in sensitized patients.

Conclusions Recombinant cat albumin may be used as a paradigmatic tool to analyse mechanisms of allergen-triggered exacerbation of AD, for diagnostic and, perhaps for therapeutic purposes.

Keywords atopic dermatitis, cat albumin, cat allergy, recombinant allergen

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Introduction

Atopic dermatitis (AD), recently also termed atopic eczema/dermatitis syndrome (AEDS), is a chronic, eczematous and itchy skin disease that affects up to 5% of the adult population and up to 10% of children [1–5]. In most cases, AD patients are sensitized against a broad spectrum of different allergen sources and frequently exhibit highly elevated levels of allergen-specific and total serum IgE antibodies. On the other hand, AD resembles several features of a cell-mediated, type IV-like immune reaction [6, 7]. Contact with exogenous allergens – besides other non-immunological factors (e.g. mechanical trauma, temperature, humidity, emotional stress, skin colonization with micro-organisms) – is a major cause for exacerbation of the disease [4]. It has also been demonstrated that the epicutaneous application of exogenous allergens to the skin of AD patients elicits and aggravates the eczematous skin lesions typical for AD [8–10].

Accordingly, a variety of allergens from exogenous sources, e.g. foods, pollens, mites, moulds, animal danders and yeast, and also endogenous antigens have been suggested as potential elicitors of skin exacerbation in AD [3, 11]. The most pronounced association between the severity of atopic

eczema and concentrations of allergen-specific IgE antibodies was, however, found for IgE antibodies against indoor allergens. The analysis of 2201 schoolchildren showed that high serum IgE levels to house dust mite (HDM) and cat dander allergens were strongly associated with the severity of atopic eczema [12]. While HDMs contain more than 14 known different allergenic proteins [13], only a few allergens have been characterized for cat dander [14]. Most of the cat-allergic patients are sensitized against the major cat allergen, Fel d 1 [15]. A considerably lower percentage of cat-allergic patients (i.e. 10–30%) are sensitized against cat cystatin and cat serum albumin [16–18]. Furthermore, it has been shown that albumin-sensitized patients frequently exhibit IgE cross-reactivity with albumins from various animals. Albumin has therefore been suggested to represent a highly cross-reactive allergenic structure in hair dander of a variety of animals [17–20].

In this study, we have isolated the complete cDNA for cat albumin by RT-PCR and expressed cat albumin as a recombinant allergen in *Escherichia coli*. Recombinant cat albumin was purified to homogeneity and characterized with respect to its IgE-binding capacity as well as by basophil activation assays to confirm biological activity. Our finding that recombinant cat albumin was recognized preferentially from cat-allergic patients suffering from severe manifestations of atopy (e.g. AD, allergic asthma) and to a much lower degree from patients suffering only from allergic rhinoconjunctivitis indicates that it may represent a useful marker for the diagnosis and monitoring of severe manifestations of atopy (e.g. AD).

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Materials and methods

Bacterial strains, plasmids, antibodies, patients' sera

Competent *E. coli* BL21 (DE3) and plasmid pET-17b were purchased from Novagen (Cambridge, UK). ¹²⁵I-labelled anti-human IgE antibodies were purchased from Pharmacia (Pharmacia Diagnostics, Uppsala, Sweden), a mouse monoclonal anti-hexahistidine antibody was obtained from Dianova (Hamburg, Germany) and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibodies were obtained from Pierce (Rockford, IL, USA). Monoclonal anti-human IgE antibodies were purchased from Pharmingen (San Diego, CA, USA) and a horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antiserum was obtained from Amersham (Little Chalfont, UK).

Patients with a positive case history of IgE-mediated allergy to cat ($n = 20$) were included in this study as they appeared in the clinic. Sera were obtained by venipuncture, frozen at -20°C and subsequently analysed for the presence of specific IgE antibodies. Serum from patient ES was obtained at three different time-points and designated ES1–ES3. The diagnosis of cat allergy was based on skin prick reactivity to cat dander extract (Allergopharma, Reinbek, Germany), and the presence of cat dander-specific serum IgE antibodies as determined by CAP-FEIA measurements (Pharmacia). Sensitization to other allergen sources was confirmed by skin prick testing. The diagnosis of AD in cat-allergic patients followed the criteria of Hanifin and Rajka [21]. Non-allergic persons ($n = 3$) were included in the serological and cellular analyses for control purposes.

PCR amplification, subcloning and expression of a cDNA coding for a hexahistidine-tagged cat albumin

Total RNA was isolated from cat liver as described [22]. A cDNA coding for cat albumin containing a C-terminal hexahistidine tag was obtained by reverse transcription using a GeneAmp RNA PCR-Core kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), and a subsequent PCR reaction using the following oligonucleotides designed according to the published sequence of cat albumin [23]: Cat albumin 5': 5' GGG CGA ATT CAT TAA TGG AAG CAC ATC AGA GTG AGA TTG C 3' (*Eco* RI: underlined; *Asn* I: italics); cat albumin 3': 5' GGG ATT GAA TTC TCA GTG GTG GTG GTG GTG GTG GGC TAA GGC AGC TTG AGC TGC AGC 3' (*Eco* RI: underlined; the hexahistidine-encoding sequence is printed in italics). The PCR product was digested with *Asn* I and *Eco* RI and subcloned into the *Nde* I/*Eco* RI cut pET-17b expression plasmid (Novagen). The double-stranded DNA sequence of the expression construct was determined by automated sequencing (MWG Biotech, Ebersberg, Germany). The McVector program (Kodak Rochester, NY, USA) was used to analyse the cat albumin DNA sequence, to establish the deduced amino acid sequence, to calculate pI, molecular weight, antigenic index and to search for sequence motifs. The DNA and the deduced amino acid sequence of cat albumin were compared with the sequences deposited in GenBank using the BLAST program.

Expression in E. coli and purification of recombinant cat albumin

E. coli BL21 (DE3) were transformed with the pET-17b construct and grown in an LB medium containing 100 mg/L ampicillin at 37°C to an optical density (600 nm) of 0.4. Protein expression was induced by the addition of isopropyl- β -D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and growing of cells for additional 4 h. *E. coli* cells were harvested by centrifugation at $5856 \times g$ (Sorvall RC5C, Sorvall, Wilmington, DE, USA) at 4°C for 10 min and lysed in buffer A (8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 8.0) and stirring at 4°C for 1 h. Insoluble material was removed by centrifugation at $20\,000 \times g$ at 4°C for 20 min. The supernatant was applied to a Ni-NTA Agarose column (Qiagen, Hilden, Germany); recombinant cat albumin was eluted with acidic buffer A (pH 4.5) and dialysed against double-distilled water. The concentration of the purified protein was determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as a standard. The purity was assessed by SDS-PAGE [24] and Coomassie staining [25].

Protein extracts, SDS-PAGE, immunoblotting and immunoblot inhibitions

Cat dander extract was prepared by aqueous extraction of cat dander as described [26]. Total *E. coli* extracts were obtained by boiling cells in sample buffer (0.0625 M Tris, 2.3% w/v SDS, 10% v/v glycerol, 0.025% w/v bromophenol blue, 5% v/v 2-mercaptoethanol) [24] for 5 min. Protein extracts or purified proteins were separated by SDS-PAGE [24] and blotted onto nitrocellulose [27]. Nitrocellulose-blotted proteins were exposed to 1:10 diluted sera from cat-allergic patients or from non-atopic control individuals. Bound IgE antibodies were visualized with ¹²⁵I-labelled anti-human IgE antibodies as described [28]. Furthermore, membranes containing recombinant cat albumin were incubated with a mouse monoclonal anti-hexahistidine antibody and developed with AP-conjugated anti-mouse antibodies using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/4-nitrobluete-trazoliumchloride (NBT) as a substrate [29].

Immunoblotting inhibition experiments were performed by pre-incubating sera from cat-allergic patients with 25 μg recombinant cat albumin for 12 h at 4°C . Thereafter, sera were tested for IgE binding to nitrocellulose-blotted cat dander extract [19].

ELISA experiments

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with each of the purified proteins (i.e. recombinant cat albumin, natural cat and dog albumin, Sigma, St Louis, MO, USA) at a concentration of 10 $\mu\text{g}/\text{mL}$ Sodium carbonate buffer pH 9.6 overnight at 4°C . On the next day, the plates were washed five times with Tris-buffered saline containing 0.5% v/v Tween 20 (TBST) and blocked with TBST containing 1% w/v human serum albumin at 37°C for 2.5 h. Patients' sera were diluted 1:5 in TBST and exposed to ELISA plate-bound proteins overnight. It was ensured that each serum was tested for IgE reactivity to each of the proteins on one plate. Plate-to-plate variabilities were

excluded by testing reference sera on each plate. Bound IgE antibodies were detected with a 1:500 diluted monoclonal anti-human IgE antibody (Pharmingen) and subsequently with a 1:1000 diluted HRP-labelled sheep anti-mouse IgG antiserum [30, 31]. All determinations were carried out as duplicates, and the results represent means with an error of less than 10%.

CD203c assay

Heparinized blood samples were taken from cat-albumin-allergic patients and, for control purposes, from a patient with grass pollen, but not cat allergy. Blood aliquots (100 µL) were incubated with recombinant allergens (0.1 µg/mL), anti-IgE antibody (1 µg/mL) or phosphate-buffered saline (PBS) for 15 min (37°C). After incubation, cells were washed in PBS/EDTA and then incubated with 10 µL of phycoerythrin (PE)-labelled CD203c mAb 97A6 for 15 min at room temperature [32]. Thereafter, the samples were subjected to erythrocyte lysis with 2 mL FACS™ Lysing solution (Becton Dickinson, San Diego, CA, USA). Cells were then washed, resuspended in PBS and analysed by two-colour flow cytometry on a FACScan (Becton Dickinson). Allergen-induced upregulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells, and expressed as stimulation index ($MFI_{stim} : MFI_{control}$) [32].

Results

Expression, purification and antibody reactivity of recombinant cat albumin

A complete cDNA coding for cat serum albumin was obtained by RT-PCR from cat liver RNA (Accession number: AJ487677). The recombinant cat albumin was produced as a C-terminally hexahistidine-tagged protein. A molecular weight of 66.863 Da and a pI of 5.41 were calculated based on the deduced amino acid sequence of recombinant hexahistidine-tagged cat albumin. It differed

from the previously published cat albumin sequence regarding nine bases in the nucleotide sequence of the coding region resulting in six amino acid exchanges [23]. Comparison of cat serum albumin with the albumin sequences showed high sequence identities with albumins from humans (81%), dog (86%), rhesus monkey (80%), pig (78%), cattle (77%), sheep (76%), horse (77%), rabbit (75%), rat (75%), mouse (73%) and chicken (45%). High surface probability and high antigenic index were predicted for conserved areas as well as for regions with high sequence variations among various albumins. Induction of bacteria containing the cat albumin-expressing plasmid with IPTG yielded a distinct accumulation of the recombinant protein in the cellular extract (Fig. 1: lanes 3). The recombinant protein was found predominantly in the insoluble inclusion body fraction and could be solubilized in urea (Fig. 1: lanes 4). The recombinant allergen was purified by nickel affinity chromatography (Fig. 1: lanes 5) reaching yields between 2 and 10 mg/L *E. coli* culture. After purification, the protein remained soluble in physiological buffers up to a concentration of 1 mg/mL and could be detected with a monoclonal anti-hexahistidine antibody as well as with serum IgE from the cat albumin-sensitized patient ES1 (Fig. 1: Anti-His-tag, IgE).

Recombinant cat albumin is predominantly recognized by cat-allergic AD patients

The IgE reactivity of recombinant cat albumin was analysed with sera from cat-allergic patients suffering from AD ($n = 11$) or only from allergic rhinoconjunctivitis (RC) ($n = 9$) (Table 1). The comparison of IgE reactivities with nitrocellulose-blotted cat dander extract (Fig. 2a) and recombinant cat albumin (Fig. 2b) revealed that all AD patients (lanes MM–TT) as well as all patients with rhinoconjunctivitis (lanes RS–MW) reacted with bands of 20 kDa and approximately 12 kDa corresponding to the major cat allergen, Fel d 1, whereas cat albumin (66 kDa) was predominantly recognized by the AD patients. Accordingly, we found that six out of 11 AD patients, but only one out of the nine RC patients, reacted with recombinant cat albumin (Fig. 2b). These results were confirmed by ELISA analyses

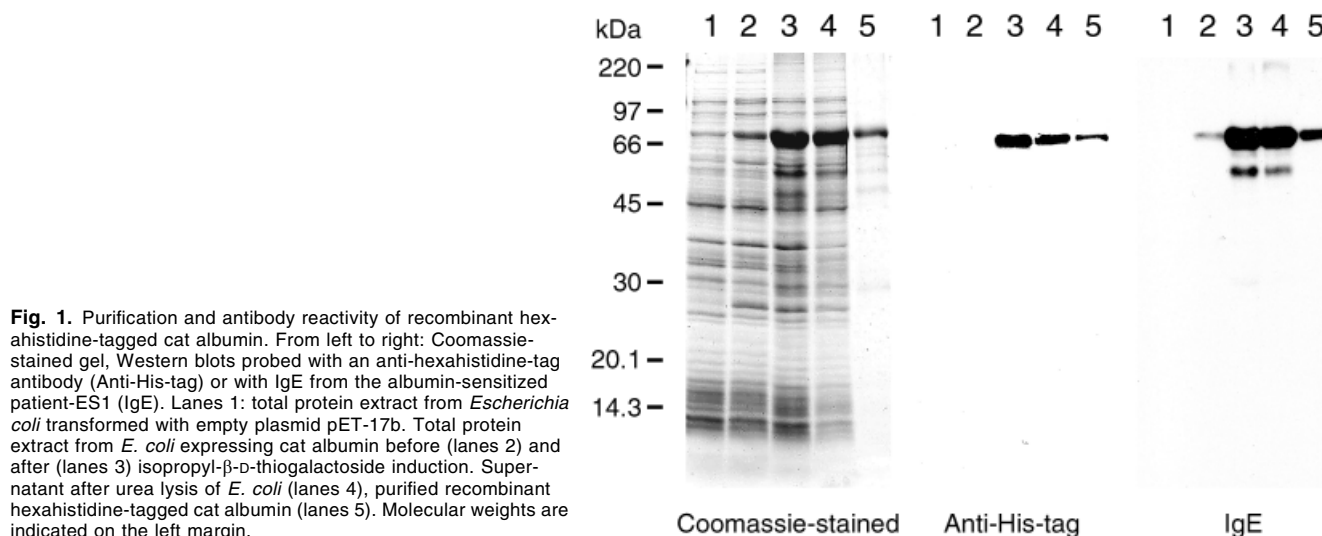


Table 1. Clinical characterization of cat-allergic patients

Patients							Sensitization to								
Initials	Sex	Age (years)	Clinical symptoms				Total IgE (kU/L)	Tree pollen	Grass pollen	Weeds	Cat dander	House dust mite	Moulds	Food	Venoms
MM	M	27	AD				>2000		+		+	+	+	cm, nu	
HZ	F	75	AD	AS	RH	CO	>2000	+	+		+	+			
ES	F	53	AD	AS	RH	CO	880		+		+	+		fi	
ED	F	35	AD	AS	RH	CO	>2000	+	+		+	+	+		
MD	M	49	AD	AS			>2000	+	+	+	+	+	+	nu, fr ,ve	
HH	F	31	AD				270	+		+	+			nu, fr, ve	
TS	M	27	AD		RH	CO	406		+	+	+	+			
HJ	F	30	AD		RH	CO	204	+			+	+		fr, ve	
SM	M	31	AD				>2000	+	+	+	+	+	+	cm, nu, fr, he, fi	
CP	M	45	AD	AS		CO	>2000	+	+	+	+	+			
TT	F	22	AD				>2000		+		+			cm, nu, fi, me	
RS	M	38			RH	CO	798	+	+	+	+				
EB	F	27			RH	CO	453	+	+		+			fr, sh	
CO	F	45			RH	CO	197	+	+		+			nu, fr	
ER	F	26			RH	CO	95	+	+	+	+			cm, nu, fr, ve	w
DJ	F	33			RH	CO	560	+	+		+	+		fr, ve	w
RM	M	31			RH	CO	293	+			+	+			
BH	F	29			RH	CO	129	+	+		+				b, w
EBi	F	51			RH	CO	66				+				
MW	M	27			RH	CO	178	+			+				

Symptoms, total IgE levels and sensitization to various allergen sources (plant pollen, cat dander, house dust mites, moulds, food and venoms) are displayed for 11 AD patients and nine allergic rhinoconjunctivitis patients. AD, atopic dermatitis; AS, asthma; RH, rhinitis; CO, conjunctivitis; cm, cow's milk; nu, nuts; fi, fish; fr, fruits; ve, vegetables; he, hens egg; sh, shrimp; me, meat; b, bee; w, wasp.

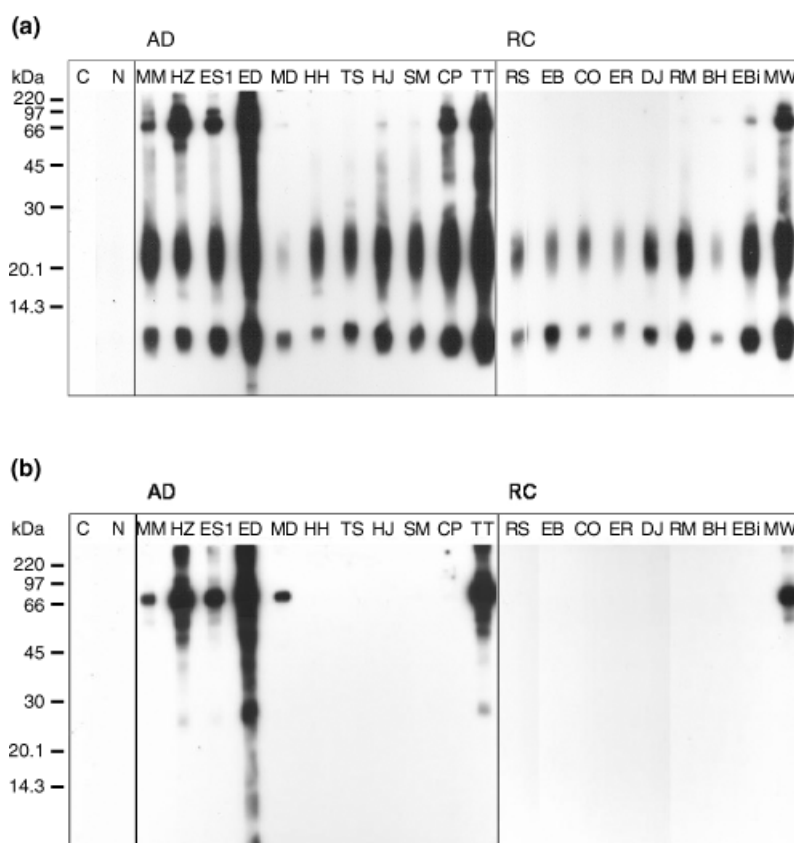


Fig. 2. IgE reactivity of patients suffering from atopic dermatitis (AD) or allergic rhinoconjunctivitis (RC) to cat dander extract and recombinant cat albumin. Sera from 11 AD patients and from nine patients suffering only from allergic RC and – for control purposes – buffer without serum (C) and serum from a non-allergic individual (N) were exposed to nitrocellulose-blotted cat dander extract (a) and recombinant cat albumin (b). Bound IgE antibodies were detected with ^{125}I -labelled anti-IgE and visualized by autoradiography. Molecular weights are displayed in kDa on the left margin.

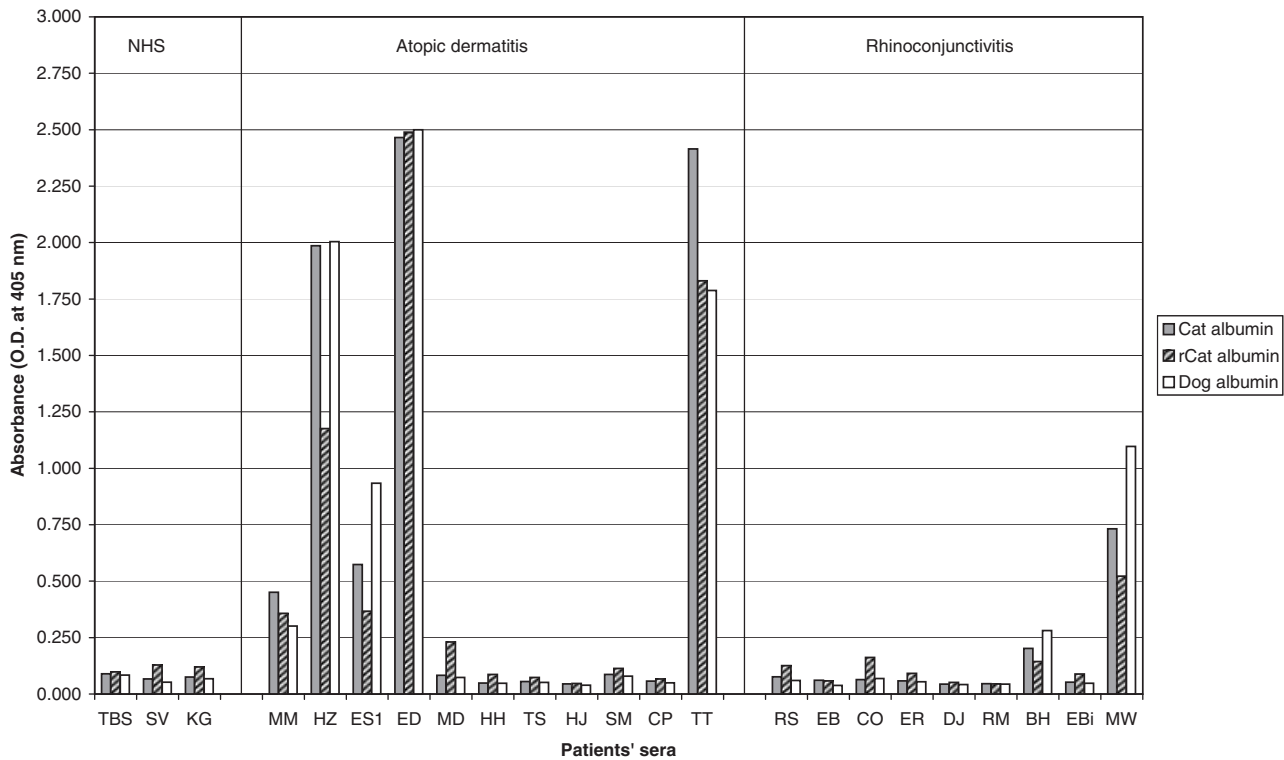


Fig. 3. IgE reactivity to ELISA plate-coated albumins. Sera from 20 cat-allergic patients (atopic dermatitis: MM–TT; rhinoconjunctivitis: RS–MW; non-allergic individuals: SV, KG), and buffer (Tris-buffered saline) were probed for IgE reactivity to natural cat (grey bars) and dog albumin (white bars) and recombinant cat albumin (hatched bars). The absorbance values at the y-axis correspond to the amount of bound IgE antibodies.

demonstrating that the very same sera reacted with ELISA plate-bound recombinant as well as with natural cat and dog albumin (Fig. 3). The IgE reactivities to bands in the range of 66 kDa by serum of AD patient HJ and CP and by serum of the RC patient EBi (Fig. 2a) were not directed against cat albumin because these sera failed to react to purified natural cat albumin in the ELISA (Fig. 3). Accordingly, sera HJ, CP and EBi did not recognize recombinant cat albumin either (Fig. 2b).

Recombinant cat albumin inhibits IgE binding to natural cat albumin and induces IgE-dependent, specific expression of CD203c on basophils

Pre-incubation of sera from four cat albumin-sensitized patients with recombinant cat albumin led to complete inhibition of IgE binding to nitrocellulose-blotted natural cat albumin at 66 kDa, whereas IgE binding to other cat allergens was not affected (Fig. 4: lanes +). Serum from a non-allergic individual and buffer without the addition of serum showed no reactivity (Fig. 4: lanes C, N).

The potential of recombinant cat albumin to activate in an IgE-dependent manner blood basophils from cat-allergic patients was studied by analysing the expression of CD203c, ecto-nucleotide pyrophosphatase/phosphodiesterase 3 (E-NPP3), an activation-induced antigen on basophils [32]. Fig. 5 shows the results from patients suffering from cat and grass pollen allergy. CD203c expression was up-regulated

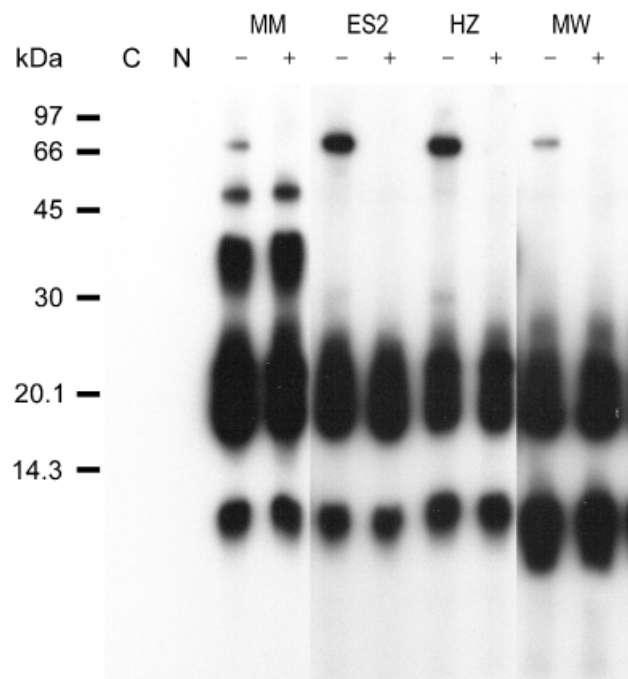


Fig. 4. Immunoblot inhibition experiment. Sera from four cat-allergic patients (MM–MW) were pre-incubated with phosphate-buffered saline (lanes –) or with recombinant cat albumin (lanes +) and tested for IgE-binding to nitrocellulose-blotted cat dander extract. C, buffer control; N, serum from a non-allergic individual. Molecular weights are indicated on the left margin.

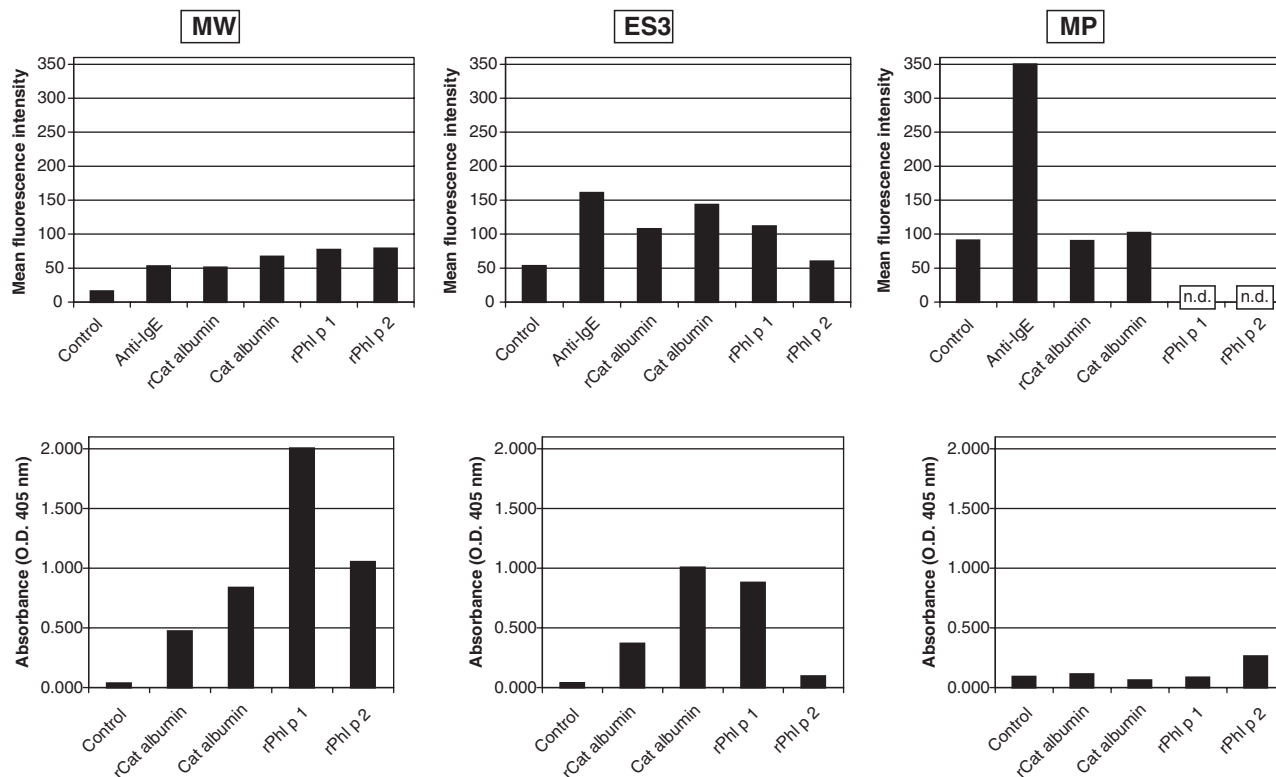


Fig. 5. Specific activation of basophils as determined by CD203c expression. The induction of CD203c expression after incubation of blood samples without (control) or with recombinant grass pollen allergens (rPhl p 1, rPhl p 2), recombinant and natural cat albumin, or anti-IgE antibodies is displayed as the mean fluorescence intensity on the y-axis for two patients with cat and grass pollen allergy (MW, ES3), and for one patient with grass pollen, but no cat allergy (MP). The corresponding IgE reactivity profile, as determined by ELISA, is shown in the lower part of the figure. Absorbance values at the y-axis correspond to the amount of bound IgE antibodies.

over baseline, i.e. without the addition of allergens (Fig. 5: control) only if patients had allergen-specific IgE antibodies (Fig. 5: lower part). Patient MW, who exhibited IgE reactivity to grass pollen allergens (rPhl p 1, rPhl p 2) and to recombinant as well as natural cat albumin, showed up-regulated CD203c expression when basophils were exposed to the very same allergens, and in response to anti-IgE mediated activation (Fig. 5: MW). Similar results were obtained for patient ES, who reacted with rPhl p 1, but not with rPhl p 2 and with natural as well as recombinant cat albumin (Fig. 5: ES3). The stronger basophil activation by allergens compared with the monoclonal anti-IgE antibody in patient MW may be due to the recognition of multiple IgE epitopes on the allergens. No up-regulation of CD203c expression over control levels (i.e. without addition of allergens) was observed when basophils from a grass pollen-allergic patient without cat allergy were exposed to cat albumin (Fig. 5: MP).

Discussion

It has been demonstrated that cat hair dander is an important allergen source involved in the induction of skin inflammation in AD patients and that the degree of sensitization to cat allergens is associated with the severity of the disease [12].

We have isolated a complete cDNA coding for cat albumin, which represents an important cross-reactive allergen for 10–30% of cat-allergic patients and expressed cat albumin in *E. coli* [17–20, 33].

Purified recombinant cat albumin inhibited IgE binding to natural cat albumin, demonstrating that the recombinant protein preparation contains most of the natural epitopes recognized by IgE antibodies from cat-allergic patients. The biological relevance of IgE recognition of recombinant cat albumin was demonstrated by its specific ability to activate basophils of sensitized patients. For this purpose, we have analysed the expression of CD203c, a novel activation marker, on basophils of allergic patients by flow cytometry.

One interesting and unexpected finding of this study, however, was the fact that predominantly patients suffering from AD, but to a much lesser extent, patients suffering only from allergic RC, exhibited IgE reactivity to recombinant cat albumin. These two groups of patients could not be discriminated on the basis of IgE reactivity to the major cat allergen, Fel d 1, which was recognized by all cat-allergic patients, regardless of whether they suffered from AD or RC. Several explanations may be considered for the preferential IgE recognition of albumin by AD patients. First, it is possible that IgE recognition of minor allergens in a given allergen source reflects only the high predisposition of patients suffering from more severe forms of atopy to mount IgE responses to otherwise harmless proteins. Second, it has been reported that preferentially AD patients mount IgE antibody responses to human proteins [34, 35], a phenomenon that may be explained as cross-reactivity between exogenous and endogenous proteins. In this context, it may be speculated that the preferential recognition of albumin, a highly conserved serum protein, may also reflect an increased

tendency of AD patients to mount IgE antibodies to structures that are similar to endogenous antigens. However, so far IgE reactivity of sera from cat albumin-allergic patients to immobilized human serum albumin could not be demonstrated, presumably because of the high levels of human albumin in serum that will complex autoreactive IgE as has been demonstrated for other IgE-reactive autoantigens [34, 35].

Although we have no definitive explanation why preferentially AD patients mount IgE responses to cat albumin, we believe that this finding could be of relevance for diagnostic applications.

The fact that recombinant cat albumin reacted equally well as natural cat albumin in *in vitro* serological experiments indicates that recombinant cat albumin may also be suited for *in vivo* testing (skin prick test, atopy patch test) providing the advantage that the recombinant protein can be easily produced in consistent quality and free of infectious agents or other contaminants resulting from the purification of animal tissues. The use of recombinant cat albumin for *in vitro* diagnostics may also have advantages because it is free of immunoglobulins and other allergenic components that may contaminate natural cat albumin preparations.

Should more extensive studies confirm the results obtained in our patients, recombinant cat albumin may be used as a diagnostic marker to predict the progression to severe manifestations of atopy and possibly to identify patients suffering from severe forms of atopy (e.g. AD and/or severe asthma), which may be less suitable for allergen-specific immunotherapy and hence may require other therapy forms.

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