

Identification of IgE-binding proteins from *Lepidoglyphus destructor* and production of monoclonal antibodies to a major allergen

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1. Summary

The allergen composition of one of the most important storage mites, *Lepidoglyphus destructor*, has been studied by immunodetection after SDS-PAGE with individual patient sera. An allergenic polypeptide of 14 kDa was identified with 95% of the sera. This major allergen was isolated in the supernatant of 60% ammonium sulfate salt precipitation of the whole extract, which was subsequently used to immunize BALB/c mice so as to produce monoclonal antibodies. Four mAbs recognizing molecules with IgE-binding ability were obtained. The specificity of the mAbs was assayed against different allergenic extracts, and the molecules recognized by them were characterized by immunoblotting. Two mAbs (Le5B5 and Le9E4) were directed to the 14-kDa allergen; the other two to several proteins of lesser allergenic significance.

2. Introduction

The role of mites belonging to the Pyroglyphidae family as producers of house dust allergens has long been recognized since it was first reported by Voorhorst et al. [1]. However, the importance in human allergic pathology of different non-pyroglyphid

mites, so-called “storage mites”, had not been demonstrated until recently [2–4].

Although storage mites were initially considered only as a cause of occupational pulmonary disease in workers exposed to stored grain [2, 5, 6], it has been established that sensitization to these mites can occur in many cases as a co-factor to house dust allergy, particularly in patients living in houses with favorable conditions for the growth of these mites, such as a high relative humidity and the availability of cereal and flour products or other foodstuffs [7, 8]. However, this contribution to house dust allergy has usually and unjustly been overlooked in clinical practice, especially when taking into account the very limited cross-reactivity between storage mites and the *Dermatophagoides* genus [9, 10, 11]. On the other hand, little is known about the allergenic properties of “storage mites”; in fact, only few publications bearing on the identification and characterization of storage mites allergens have appeared [12, 13].

Here we present a study on the allergen composition of one of the predominant storage mites, the *Lepidoglyphus destructor* species. Also, we describe the production and characterization of mAbs against one of its most relevant allergens and other IgE-binding components from *L. destructor*.

3. Materials and Methods

3.1. Preparation of *L. destructor* extract (LDE)

L. destructor mite bodies (>99% purity) were purchased from Biopol Laboratory Inc. (Washing-

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ton, USA). Proteins were extracted with 50 mM ammonium bicarbonate after homogenization with a Potter-Elvehjem device and stirring for 1 h at room temperature. The soluble fraction was separated by centrifugation at $22\,000\times g$ for 1 h at 4 °C. Then, the supernatant was extensively dialyzed against distilled water using a membrane with mol. wt. cut-off of 10 000, filtered through a 0.2 μm pore size membrane, and freeze-dried. The lyophilized extract was stored at 4 °C until use. Protein content was estimated by the Folin method [14].

3.2. Fractionation of LDE

LDE was dissolved in water (60 ml) to a final protein concentration of 2 mg/ml. Solid ammonium sulfate was then gradually added, at 4 °C, with stirring, until a 60% concentration was reached. The precipitate was separated from the supernatant by centrifugation at $12\,100\times g$ for 30 min at 4 °C and resuspended in distilled water. Both supernatant and precipitate were extensively dialyzed against distilled water in a 10 000 mol. wt. cut-off dialysis tube and freeze-dried.

The fraction of proteins from LDE that remained soluble in the supernatant of 60% ammonium sulfate concentration is hereafter named 60SF.

3.3. Human sera

Human sera from 22 atopic patients were used throughout the experiments. These sera had serum levels of specific IgE to *L. destructor* of RAST class 3 and 4 (Pharmacia). No patient was under immunotherapy at the time of serum collection.

Control human sera were obtained from non-atopic individuals among healthy laboratory personnel.

3.4. Production of monoclonal antibodies

Female BALB/c mice were immunized with an intraperitoneal injection of 100 μg of 60SF in 100 μl of PBS, emulsified with 200 μl of Freund's complete adjuvant. Fifteen days later mice were boosted with an identical amount of antigen in Freund's incomplete adjuvant, and the same process was repeated 30 days later. Three days before fusion mice were injected intravenously with 60SF in PBS. Immune

splenocytes were fused with PX63 mouse myeloma cells as described [15]. After 2 weeks, hybridoma culture supernatants were harvested for screening.

Two different immunoassays were performed for selection of hybridoma. The first screening was carried out by an ELISA. Hybridoma culture supernatants were added to 60SF-coated PVC wells and incubated for 1 h at room temperature. After washing with 0.05% Tween 20 in PBS, wells were incubated with 100 μl of polyclonal goat anti-mouse Ig antiserum (GAM) conjugated with β -galactosidase for 1 h at room temperature. The enzymatic reaction was developed with *O*-nitrophenyl- β -D-galactopyranoside for 2 h at 37 °C, and stopped with 0.1 M sodium carbonate solution. The absorbance was measured at 405 and 620 nm in an automatic microplate reader.

Positive hybridomas producing antibodies to 60SF were subsequently screened for allergen binding-ability, in a four-step radioimmunoassay: PVC-wells were coated with a solution of GAM and, after saturation with 1% BSA, successively incubated for 1 h at 37 °C each with: (1) supernatant of hybridoma cells; (2) LDE at 1 mg/ml; (3) serum pool (diluted 1:2 in PBS) from *L. destructor*-sensitive patients, and (4) ^{125}I -anti-IgE antiserum (Pharmacia, Sweden). Four washing steps with 0.05% Tween 20 in PBS were performed between incubations. Selected hybridomas were cloned and subcloned by limiting dilution.

The isotype of the mAbs was determined by immunodiffusion using rabbit anti-mouse subclass antibodies (Nordic, Tilburg, The Netherlands). Monoclonal antibodies Le5B5 and Le9B12 were purified by precipitation with caprylic acid [16] starting from ascitic fluid produced in BALB/c mice.

3.5. Electrophoretic procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed with an 8–25% acrylamide gradient in a high-molarity Tris buffer system [17] under non-reducing conditions. The following standards were used as mol. wt. markers: phosphorylase b (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

Electrotransfer of separated polypeptides onto

nitrocellulose membranes was carried out by the method of Towbin [18]. For immunodetection of allergenic polypeptides, nitrocellulose sheets were further incubated with *L. destructor*-sensitive human serum and Hamlet[®] -¹²⁵I (Alergia e Inmunología Abelló S.A., Madrid), an iodinated mouse anti-human IgE monoclonal antibody [19].

Detection of mAb-binding polypeptides was carried out by sequential incubation with hybridoma culture supernatants and radiolabelled GAM.

4. Results

4.1. Allergen composition of LDE

To identify IgE-binding polypeptides in LDE, immunodetection experiments after SDS-PAGE with sera from patients allergic to *L. destructor* were carried out (Fig. 1). Three allergenic polypeptides with mol. wt. 57, 30 and 14 kDa reacted with more than 50% of the sera, and a third was detected with almost all the sera (21 out of 22) assayed.

To isolate the 14-kDa allergen, fractionation of LDE was performed by 60% ammonium sulfate salt precipitation. As can be seen in Fig. 2, most proteins from LDE (lane c) precipitated under these conditions (lane a); however, the 14-kDa polypeptide remained in the soluble fraction (60SF) and was only slightly contaminated by some other non-IgE-binding proteins (lane b). This partially purified allergen preparation was employed to immunize

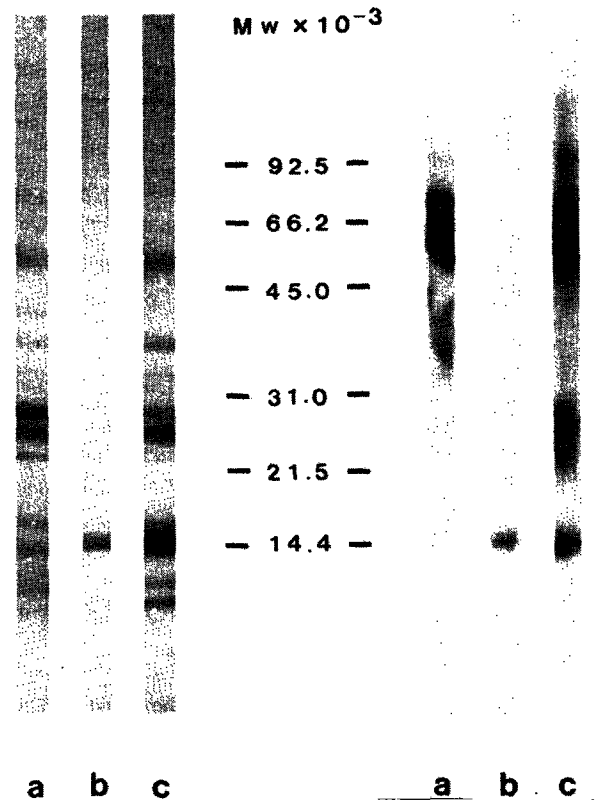


Fig. 2. Samples from 60% ammonium sulfate salt precipitation of LDE, carried out as described in Materials and Methods, were analyzed by SDS-PAGE and immunodetection. In lanes a and b are included, respectively, the precipitate and the soluble fraction (60SF) obtained from the starting material LDE (lanes c). Left: Coomassie Blue-stained patterns. Right: immunodetection with *L. destructor*-sensitive serum pool.

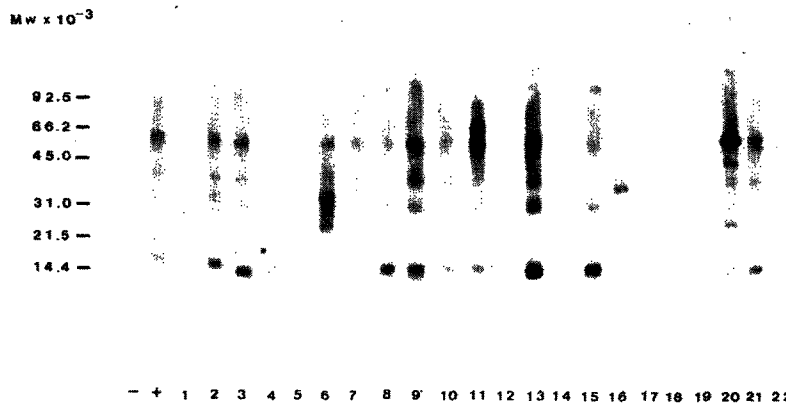


Fig. 1. Immunodetection of IgE-binding polypeptides in LDE with individual sera. LDE was subjected to SDS-PAGE under non-reducing conditions, and the separated polypeptides transferred to nitrocellulose membranes and incubated with sera from *L. destructor*-sensitive patients (1–22), a negative serum control (–), and a serum pool from allergic patients (+). Afterwards, nitrocellulose membranes were incubated with ¹²⁵I-anti-IgE and finally dried and autoradiographed.

TABLE 1

Binding of monoclonal antibodies to allergen molecules from LDE.

mAb	Isotype	Radioactivity bound (cpm) ^a	
		Specific serum	Negative serum
Le5B5	G1	12540	515
Le9E4	G1	11580	585
Le12B12	G3	2470	600
Le14D9	G3	2035	610
P3X63 ^b	-	575	515

^a Binding of ¹²⁵I-labeled polyclonal anti-human IgE to human specific IgE bound to allergen molecules from LDE selectively retained by mouse mAb complexed to GAM coated PVC wells.

^b Non-specific binding was determined with P3X63 mouse myeloma culture supernatants.

BALB/c mice in order to obtain monoclonal antibodies.

4.2. Selection and characterization of anti-*L. destructor* mAbs

Fifteen hybridoma culture supernatants with the ability to specifically bind 60SF LDE components were selected by an indirect ELISA. Then, hybridoma supernatants were assayed for capacity to bind allergen molecules from LDE using a 4-step radioimmunoassay (Table 1). Four out of the 15 mAbs tested were found to recognize *L. destructor* IgE-binding components and, in particular, Le5B5 and Le9E4 mAbs exhibited a highly positive signal.

TABLE 2

Specificity of monoclonal antibodies to LDE^a

mAb	LDE	<i>D. pteronyssinus</i>	<i>T. putrescentiae</i>	<i>F. domesticus</i>
Le5B5	1.790	0.056	0.032	0.027
Le9E4	1.400	0.047	0.033	0.029
Le12B12	1.600	0.061	0.040	0.020
Le14D9	1.800	0.059	0.031	0.042
P3X63 ^b	0.030	0.042	0.024	0.029

^a Results of an ELISA in which allergen extract-coated PVC wells were sequentially incubated with mouse hybridoma culture supernatants and GAM conjugated with β -galactosidase as described in Materials and Methods. ^b Non-specific binding was determined with P3X63 mouse myeloma culture supernatants.

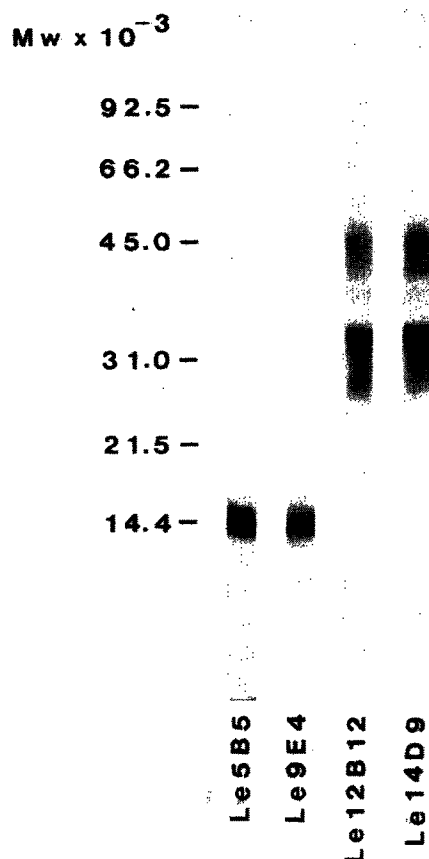


Fig. 3. Immunodetection of mAb-binding polypeptides. LDE was subjected to SDS-PAGE, and the separated polypeptides transferred to nitrocellulose membranes and incubated with anti-LDE mAbs. Afterwards, nitrocellulose strips were processed as described in Materials and Methods.

The specificity of these four monoclonal antibodies was assayed against a variety of allergenic extracts. Table 2 summarizes the results obtained for some extracts by an ELISA. It can be observed that the mAbs recognized LDE in a specific way since they were unable to recognize the other extracts.

4.3. Identification of allergen molecules recognized by anti-LDE mAbs

To determine the allergen molecules bearing the antigenic determinants recognized by the mAbs, immunodetection experiments after SDS-PAGE of LDE were carried out (Fig. 3). Both Le5B5 and Le9E4 mAbs bound to the same 14-kDa polypeptide, namely the main IgE-binding polypeptide. On the other hand, immunodetection with Le12B12 and Le14D9 yielded 3 strong broad bands centered around 43, 33 and 30 kDa, and 2 weak bands corresponding to 66 and 18 kDa.

Competition experiments between purified Le5B5 and Le9E4 mAbs showed that both of them defined the same epitope on the 14-kDa allergen (data not shown).

5. Discussion

We have obtained 4 mAbs to *L. destructor* allergens. The characterization of their specificities was carried out by taking together the results of immunoblotting after SDS-PAGE (Fig. 3) and the four-step RIA (Table 1), in which antibodies recognizing molecules with IgE-binding ability were detected. Two mAbs (Le5B5 and Le9E4) only recognized the most prominent LDE allergen (14 kDa) identified by immunodetection experiments with individual sera (Fig. 1), whereas the two other mAbs (Le12B12 and Le14D9) recognized several different molecules (Fig. 3). Nevertheless, the allergenic significance of these structures sharing the epitopes defined by Le12B12 and Le14D9 mAbs must not be very high according to the results shown in Fig. 1, except for the 30-kDa protein that might be ascribed to an IgE-binding component which is recognized by more than 50% of the sera; moreover, the IgE-binding level was found to be low with these mAbs in the 4-step RIA (Table 1). Apart from that, the molecules recognized by these mAbs must be very minor components of LDE since most of them were

not visualized in the Coomassie Blue-stained SDS-PAGE pattern, and even they were not detected in 60SF preparation used for immunization; however, they did elicit an immune response in mice.

The electrophoretic separation of polypeptides was carried out in the absence of a reducing agent throughout this work, in order to avoid the possibility of missing allergens with an IgE-binding ability dependent on disulfide bridges, as has been reported to occur, for instance, with Group I *Dermatophagoides* spp. allergens [20] and the major feline allergen *Fel d1* [21].

The differences in the electrophoretic conditions employed should be taken into account to explain the apparent divergences observed between our results on the identification of LDE allergens and previous findings reported by Johansson et al. [12], who demonstrated two polypeptides of 18 and 16 kDa to be the major allergen components of *L. destructor*. These discrepancies could be explained in analogy to apparent molecular mass variations (from 24 to 30 kDa) of Group I *Dermatophagoides* spp. allergens on SDS-PAGE, that have been shown to be caused by different conditions employed for electrophoresis [22].

On the other hand, we have observed an allergenic prevalence of several *L. destructor* components with higher molecular weights than those found by these authors [12]; more than 50% of the patients tested had IgE to the 57- and 30-kDa polypeptides. As a matter of speculation, these proteins either might have lost their IgE-binding ability upon reduction or they might be formed by complexing the 14-kDa allergen to itself or to other molecules by disulfide bridges, which would not dissociate under the experimental conditions used by us. However, the fact that Le5B5 and Le9E4 mAbs only recognized the 14-kDa allergen suggests that the higher-molecular-weight allergens consist of unrelated structures. The possibility of purifying the 14-kDa allergen by affinity chromatography, using Le5B5 or Le9E4 mAb, will allow us to solve this question by studying the inhibition of the IgE-binding to other molecules by the purified allergen.

The availability of these mAbs, the specificity of which was tested against a number of allergens that can accompany *L. destructor* in dust samples (Table 2), will also allow us to develop a quantification method that would be very valuable for the determi-

nation of environmental exposure to the major allergen of *L. destructor* as well as for the preparation of proper extracts for use in clinical practice. Finally, the mAbs obtained will facilitate further biochemical and immunochemical studies of this allergenic protein.

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