

Detection of at least one high-molecular-mass, IgE-binding component of the dust mite *Lepidoglyphus destructor*

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We studied the allergen composition in an extract of the storage mite *Lepidoglyphus destructor*. Monoclonal antibodies (mAbs) were raised against *L. destructor* by a standard hybridoma technique. In the subsequent screening, we concentrated on mAbs fulfilling three criteria. First, in ELISA, mAbs were assessed against a panel of various mite species, and only those reacting exclusively with *L. destructor* extract were selected for further analyses. Secondly, mAbs were selected in immunoblotting according to whether or not a novel pattern of reactivity emerged in comparison with earlier results. Thirdly, by radioimmunoassay (RIA), we selected mAbs that recognized components which were also recognized by human IgE from sera RAST positive to *L. destructor*. This yielded an *L. destructor*-specific mAb (117F9) reacting with two previously unknown components of approximately 79 and 93 kDa, respectively. We also analyzed 80 sera for the presence of IgE binding to these components. These sera were divided into three groups according to their RAST specificity. Eighteen of the 30 sera (60%) that were RAST positive to *L. destructor* were also positive in RIA. Correlation was moderate between kU/l for *L. destructor* and the counts per minute values for the two components in RIA. The group of control sera lacking IgE antibodies against *L. destructor* displayed no positive results in RIA. However, 4/20 sera RAST positive to *Dermatophagoides pteronyssinus* but negative to *L. destructor* were scored as positive in RIA. We conclude that at least one of the high-mol.-mass components of *L. destructor* causes IgE-mediated sensitization.

S. Olsson, B. Härfast, S. G. O. Johansson, M. van Hage-Hamsten

Department of Clinical Immunology, Karolinska Hospital, Stockholm, Sweden

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Susanna Olsson
Department of Clinical Immunology
Karolinska Hospital
S-171 76 Stockholm
Sweden

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Dust mites can be divided into two categories. Those primarily found in homes are referred to as house-dust mites, and those primarily found in farming environments as storage mites. House-dust mites belonging to the genus *Dermatophagoides* have been given most attention as a cause of respiratory allergy worldwide (17, 19, 32). During the past years, we have studied mainly *Lepidoglyphus destructor*, which is the prevalent storage mite species causing allergy in northern Europe (6, 11, 26). Some storage mite species exist in house dust as well (14, 21). Indeed, mites are the most important source of allergens for farmers (6, 11, 23, 26), mite allergens by far exceeding those of pollen and animal danders as a cause of IgE-mediated sensitization (11, 28). We have earlier described allergen components of *L. destructor* reacting with IgE antibodies from sensitized subjects

(13). The major allergen was shown to have a mol. mass of approximately 15 kDa (12). This allergen has recently been defined with the aid of monoclonal antibodies (mAbs) (31). Using mAbs and immunoprecipitation, we have also previously identified a 39-kDa allergen of *L. destructor* (1). In the present study, we sought for further IgE-binding allergen components of *L. destructor*.

Material and methods

Mite extracts

Raw materials of six different mite species, *L. destructor*, *Acarus siro*, *Tyrophagus putrescentiae*, *Glycyphagus domesticus*, *Dermatophagoides pteronyssinus*, and *Euroglyphus maynei*, were purchased from Allergon AB (Ängelholm, Sweden). All extracts were

prepared by mixing them with 0.9% NaCl containing 0.02% NaN₃ at 1/10 or 1/20 w/v. The mixtures were stirred overnight at room temperature and were subsequently centrifuged at 6600 × *g* for 10 min. The supernatants were filtered through an 0.8-μm Miller-HA filter (Millipore, Bedford, IN, USA). The protein content was determined by BCA protein reagent assay (Pierce Chemical Co, Rockford, IL, USA) (*L. destructor* 5.8 mg/ml, *A. siro* 4.8 mg/ml, *T. putrescentiae* 4.0 mg/ml, *G. domesticus* 6.7 mg/ml, *D. pteronyssinus* 2.1 mg/ml, *E. maynei* 0.7 mg/ml). The *L. destructor* extract used for immunization of mice was treated with ammonium sulfate (3.3 M) for 30 min. The soluble fraction was further dialyzed for 30 h, and the protein content (0.7 mg/ml) was determined (BCA protein reagent assay).

Production of monoclonal antibodies

mAbs were produced according to the standard hybridoma technique (15). Briefly, female Balb/c mice were immunized four times intraperitoneally with 50 μg of ammonium-sulfate-treated *L. destructor* extract and mixed with the adjuvant Opti-Vant (1 mg/ml extract) (Transgenic Sciences, Inc, Worcester, MA, USA). Spleen cells were fused with the myeloma B-cell line SP2/0 (2:1) with polyethylenglycol to obtain hybridoma cells. Culture supernatants were tested for antibody reactivity against *L. destructor* extract by enzyme-linked immunosorbent assay (ELISA) (8).

Screening and characterization of mAbs

Microtiter plates (Nunc, Roskilde, Denmark) were coated with *L. destructor* extract (1:2500, 200 μl/well) in a carbonate buffer (0.1 M, pH 9.6) overnight at room temperature and subsequently incubated overnight with cell culture supernatants diluted in phosphate buffer (50 mM, pH 7.4) containing 0.2% BSA and 0.1% Tween (1:2, 100 μl/well) from wells with growing clones. Next, alkaline-phosphatase-conjugated rabbit antimouse immunoglobulin (Dakopatt, Glostrup, Denmark) was added (diluted in the phosphate buffer described above) (1:1000, 100 μl/well) and incubated for 3 h, followed by the addition of substrate *p*-nitrophenyl phosphatase disodium dissolved in a buffer containing 1 M diethanolamine (pH 9.8) (100 μl/well) (Sigma Diagnostics, St Louis, MO, USA). The absorbance at 405 nm was measured with an ELISA plate reader (SLT-Labinstruments, Austria). Unless otherwise stated, the same buffers as described above were used throughout this study for these purposes.

Cell culture supernatants with antibody reactivity were further tested in ELISA against six different mite species to study the specificity of their reaction.

Two different ELISAs were used to test the reactivity against the mite extracts in both coated and antibody-bound forms. The first ELISA was performed as described above, each extract being coated on microtiter plates (*L. destructor* 1:2500, *A. siro* 1:500, *T. putrescentiae* 1:500, *G. domesticus* 1:500, *D. pteronyssinus* 1:500, *E. maynei* 1:500). The second was a sandwich ELISA in which microtiter plates were first coated overnight at room temperature with a polyclonal rabbit antiserum against *L. destructor* (1:1000, 200 μl/well) raised in our laboratory. This antiserum also contains cross-reacting antibodies against the above mentioned mite species. Next, mite extracts (1:2500, 100 μl/well) were incubated for 6 h; subsequently, the selected mAb, 117F9, was added (1:2, 100 μl/well) and incubated overnight. Finally, the same conjugate and substrate were used as described above.

For determination of whether the mAb reacted with protein components rather than with carbohydrates, *L. destructor* extract was coated on microtiter plates (1:2500, 200 μl/well) and subsequently treated with three different concentrations of the proteolytic enzyme trypsin (0.0025–0.25%) for 1 h. Next the mAb was added (1:2), and the reaction was tested by ELISA. The effect of this treatment was assessed by comparison with untreated extract.

The subclass specificity of the mAb was determined by ELISA with specific reagents from Southern Biotechnology Associates, Birmingham, AL, USA. *L. destructor* extract was coated, and the mAb was added as described above. Subclass-specific conjugates were added (1:500, 100 μl/well) according to the manufacturer's instructions, and the substrate (*p*-nitrophenyl phosphatase disodium, Sigma) was finally added.

For determination of the specificity of the assays described above, exclusion of mAb and mite extract served as negative controls.

The mAb was purified on a Protein-A Sepharose CL-4B column according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). The mAbs were eluted with a buffer containing glycine at pH 2.5, and each fraction was neutralized with NaOH (0.1 M). The concentration of the purified mAbs was determined by nephelometry (Beckman Instruments, Inc, Fullerton, USA).

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed essentially as described by Johansson et al. (12). Proteins in *L. destructor* extract were separated with homogeneous polyacrylamid (7.5%) gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) with a Mini Protean II according to the operating instructions (Bio-Rad, Richmond, CA,

USA). Five Bio-Rad high-mol.-mass markers were used as standards: rabbit muscle myosin (200 000 Da), *Escherichia coli* B galactosidase (116 250 Da), rabbit muscle phosphorylase B (97 400 Da), bovine serum albumin (66 200 Da) and hen's egg white ovalbumin (45 000 Da). The *L. destructor* extract (120 µg/well) and the mol.-mass standard were boiled for 5 min in reducing sample buffer containing 10% SDS before they were applied to the gel. The electrophoresis was run for 45 min and was followed by a 1-h electrophoretic transfer of the separated proteins from the gel onto a nitrocellulose (NC) membrane (Trans-Blot, Bio-Rad). Nonspecific binding sites on the membrane were blocked by incubation with 3% gelatin for 30 min. For detection of mAb-binding proteins, the NC membrane was first incubated with the mAb (117F9 supernatant, 1:1000 in 1% gelatin) and subsequently incubated with 5 ml rabbit antimouse immunoglobulins (1:2000) (Dakopatts), followed by 5 ml goat antirabbit immunoglobulins conjugated with alkaline phosphatase (1:2000) (Bio-Rad). Finally, a substrate containing 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium was added, according to the instructions of the manufacturer (Bio-Rad), to visualize the proteins.

Patient sera

A modified RIA with 80 human sera was performed to determine whether or not mAb 117F9 detected IgE-binding components of *L. destructor*. The sera were divided into three groups by RAST reactivity to *L. destructor*. The first group included 30 sera RAST-positive to *L. destructor* (2.9–93 PRU/ml) (Ld group). The other two groups were controls, the former including 30 sera from nonatopic subjects who lacked IgE antibodies against *L. destructor* (non-atopic group), and the latter 20 sera positive in RAST to the house-dust mite *D. pteronyssinus* (0.4–15 PRU/ml) but negative to *L. destructor* (Dpt group).

Radioimmunoassay (RIA)

Microtiter plates (Nunc) were coated with an immunosorbent purified goat antimouse antibody (160 µg/ml, 200 µl/well) (Dakopatts, Glostrup, Denmark). In the first step, mAb 117F9 (10 µg/ml, 100 µl/well) was added, and in the second, *L. destructor* extract (100 µl/well). Thereafter, 100 µl undiluted human serum was added, followed by 50 µl ¹²⁵I-labeled rabbit antihuman IgE (Phadebase RAST anti-IgE tracer, Pharmacia, Uppsala, Sweden). The plates were thoroughly washed between each step, and all incubations lasted overnight at room temperature. Bound radiolabeled antibodies were eluted with citric buffer, pH 3.0, at 37°C for 30 min. The solution was then transferred to tubes and analyzed

in a gamma counter (1 min/tube). All sera were tested in the same experiment in duplicate, and the mean value was calculated and expressed as counts per minute (cpm). The cpm value given for each serum was corrected by subtracting the background value of approximately 25 cpm, obtained by replacing human serum with buffer. The limit for a positive cpm value (75 cpm) was set to exceed three times the background value. The assay gave no nonspecific uptake of labeled anti-IgE when tested with serum samples with IgE levels up to 2800 kU/l. To ascertain the specificity of the RIA, we replaced mAb 117F9 with a subclass-matched (IgG2a) irrelevant mAb, F1 (kindly provided by Dr I. J. Ansotegui, Department of Immunology, Karolinska Institute,

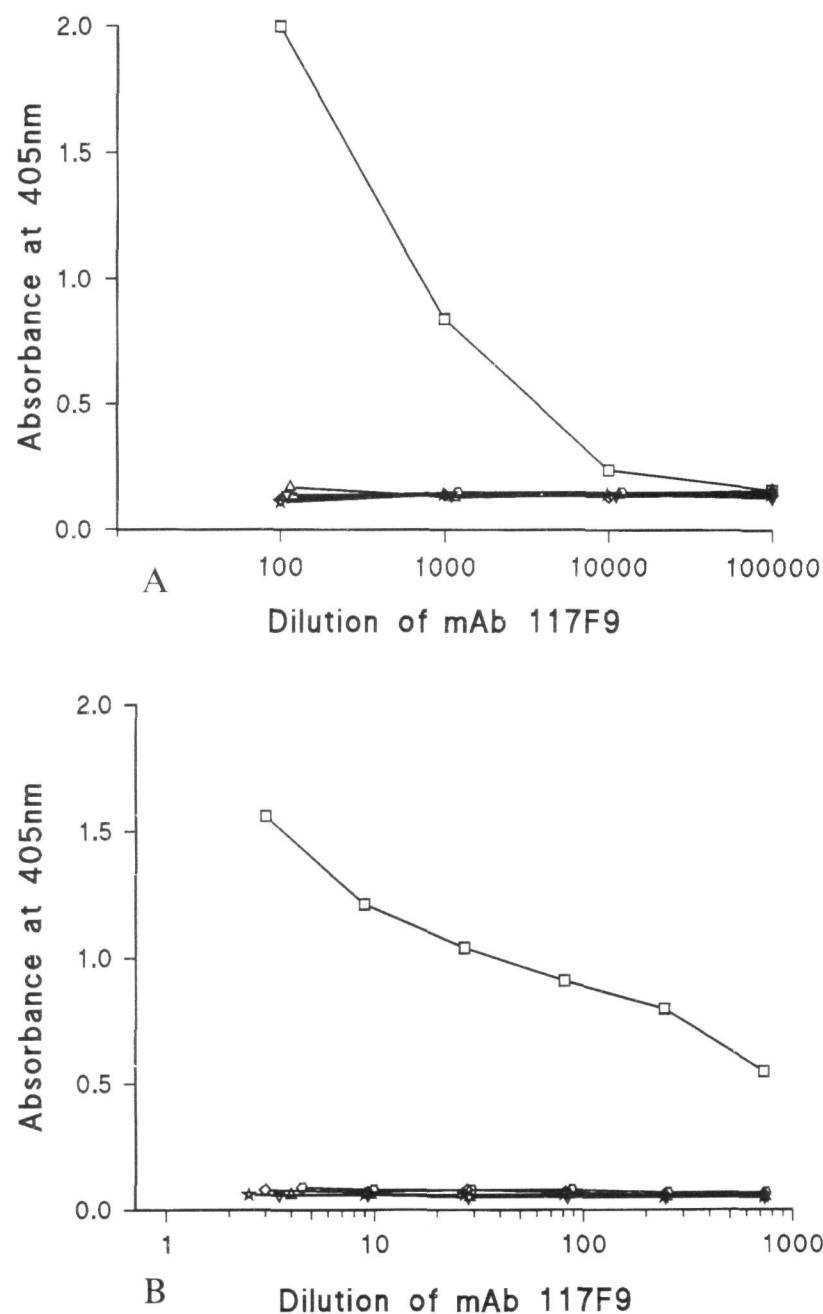


Fig. 1. Reaction of mAb 117F9 to six different mite species (*L. destructor* □, *D. pteronyssinus* ◇, *E. maynei* △, *A. siro* ▽, *T. putrescentiae* ★, *G. domesticus* ○). A, mite extracts coated on microtiter plates. B, microtiter plates coated with polyclonal rabbit antimite serum before addition of mite extract. In both assays, mAb 117F9 and ALP-conjugated rabbit antimouse immunoglobulin were added subsequently.

Stockholm, Sweden). Moreover, the *L. destructor* extract was excluded and replaced with *D. pteronyssinus* extract to ensure the specificity of the assay.

Results

Detection of an *L. destructor*-specific mAb

Using the hybridoma method, we generated several mAbs reacting with *L. destructor* extract. These were analyzed by ELISA against a panel of four different nonpyroglyphid and two pyroglyphid mite species. As shown by Fig. 1, we detected one mAb, 117F9, that exclusively reacted with *L. destructor* extract, and not with the five other mite species, in both coated (Fig. 1A) and antibody-bound (Fig. 1B) forms, indicating that the mAb is species-specific. When *L. destructor* extract was pretreated with various concentrations of trypsin, the reaction with the mAb was strongly affected, as compared with the reaction with untreated extract (Fig. 2), indicating that the allergen component involved in the reaction was of protein nature. Subclass determination revealed 117F9 to be a murine antibody of subclass IgG2a.

Electrophoresis and immunoblotting

By running SDS-PAGE under reducing conditions, followed by immunoblotting, we found that 117F9 reacted strongly with two high-mol.-mass bands of approximately 79 kDa and 93 kDa (Fig. 3.) When the Ld extract was run in SDS-PAGE and stained with Coomassie brilliant Blue, weak bands corresponding to the 79- and 93-kDa components were detected.

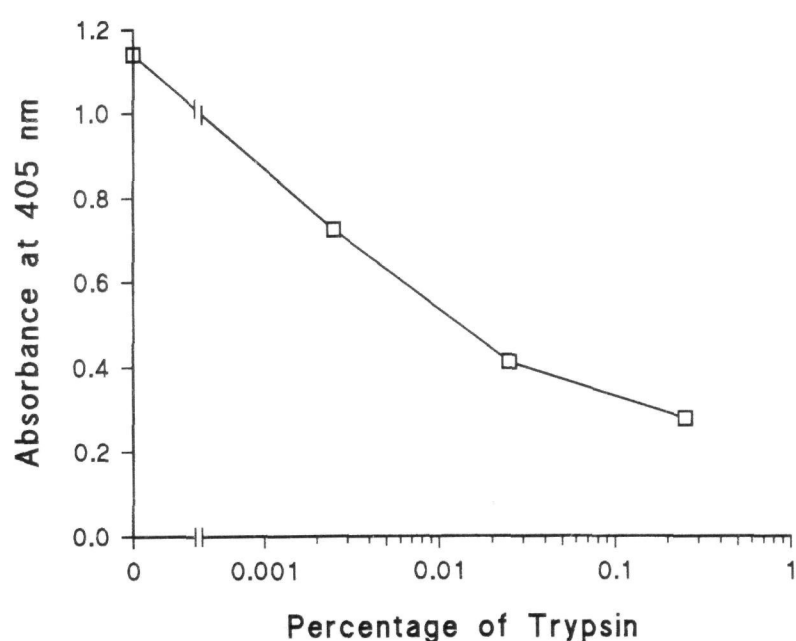


Fig. 2. Reaction of mAb 117F9 in ELISA to *L. destructor* extract pretreated with different concentrations of trypsin (0.0025–0.25%).

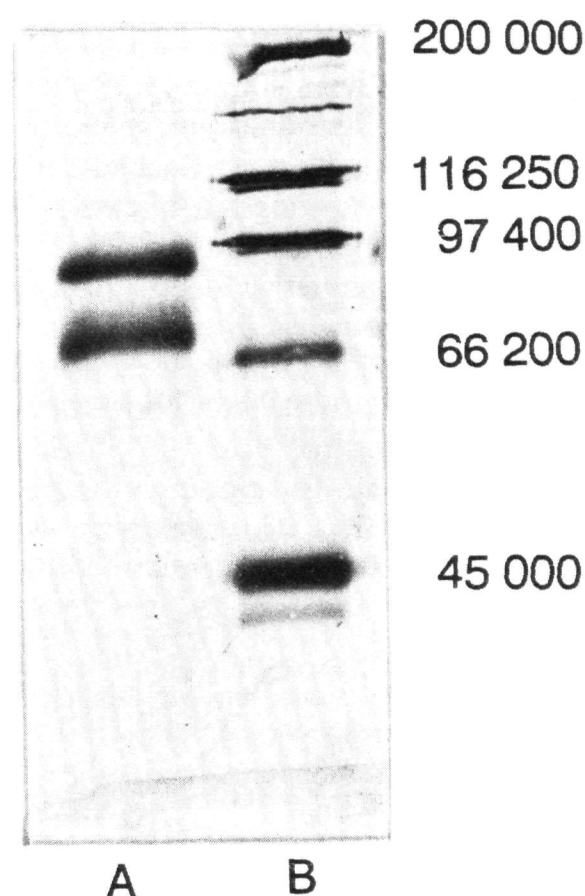


Fig. 3. SDS-PAGE and immunoblotting performed under reducing conditions of *L. destructor* extract. Lane A shows *L. destructor* extract detected with mAb 117F9; lane B shows high-range mol.-mass standard.

Radioimmunoassay

As illustrated in Fig. 4, 18 of the 30 sera (60%) with IgE antibodies against *L. destructor* were positive in the RIA to the two components ($n = 18$, geometric mean 160 cpm, range 75–1286 cpm; $n = 30$, geometric mean 88 cpm, range 5–1286 cpm). There was

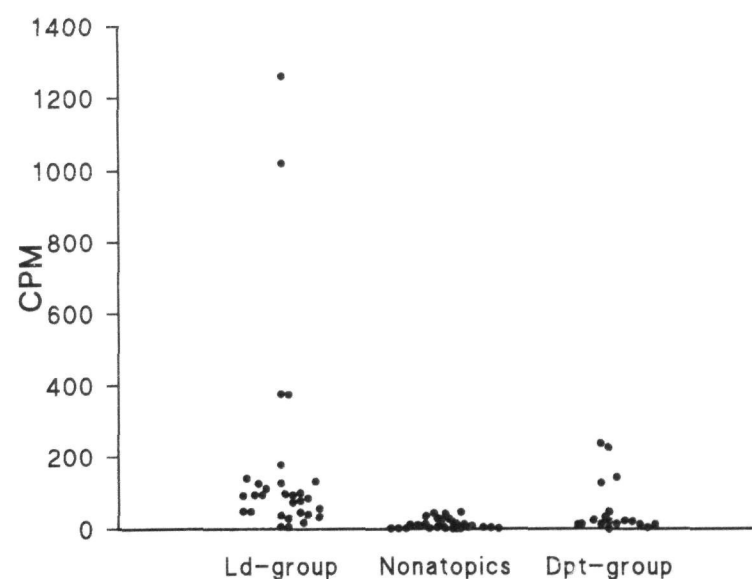


Fig. 4. Graph of cpm values obtained in RIA. Ld group: 30 sera positive in RAST for *L. destructor* (2.3–93 PRU/ml). Nonatopic group: 30 sera negative in RAST for *L. destructor*. Dpt group: 20 sera positive in RAST for *D. pteronyssinus* (0.4–15 PRU/ml). Limit for positive RIA value was set at 75 cpm.

moderate correlation between the RAST values and the cpm values obtained in RIA with the Spearman rank correlation coefficient ($r = 0.44$; $P < 0.05$). The 30 sera belonging to the nonatopic group all lacked an IgE response to the 79- and 93-kDa components (geometric mean 6 cpm, range 1–47 cpm). Four out of 20 sera in the Dpt group showed a value greater than 75 cpm ($n = 4$, geometric mean 176 cpm, range 126–237; $n = 20$, geometric mean 24 cpm, range 2–234 cpm).

In the group of *L. destructor*-allergic subjects, the subclass matched control mAb (F1) bound only 13%, as compared with the binding of mAb 117F9, which was set at 100%. The reactivity with both mAbs was very low with negative control sera ($< 3\%$).

Discussion

The past few years have seen great progress in the characterization of allergens. With mAbs and molecular biology several allergens have been defined and sequenced (4, 7, 24). There are, as yet, no recombinant allergens isolated from storage mites. However, great progress has been made also in this area of research. The major allergen of *L. destructor*, *Lep d* I, has been purified (31), and its N-terminal amino acid sequence determined (25). In previous immunoblotting studies, we have focused on allergen components of *L. destructor* with lower mol.-mass (12). In the present study, we report on two high-mol.-mass IgE-binding allergen components of *L. destructor* recognized by a mAb (117F9). The epitopes recognized by this mAb were affected by trypsin, indicating that they were of protein nature. mAb 117F9 was restricted to *L. destructor* and cross-reacted neither with three different storage mite species nor with two house-dust mite species when tested by ELISA. This contrasts to the reaction pattern obtained with another anti-*L. destructor* mAb, 42B6, previously described by us (1), which reacts with five different species of storage mites, but not with house-dust mites (10).

We have, as yet, not encountered mAbs reacting with both pyroglyphid and nonpyroglyphid species. However, the existence of such epitopes has been suggested by CRIE as well as RAST inhibition and affinity absorption experiments using human sera (2, 9, 16). Moreover, it has been shown that storage mites, like house-dust mites, each have distinct allergenic determinants (9, 12, 20, 27, 29).

We consider mAbs to be excellent tools for delineating cross-reactivity and detecting genus- and species-specific allergens. In immunoblotting, mAb 117F9 identified two components, one band of approximately 93 kDa and one of approximately 79 kDa. The relationship between these bands is pres-

ently not known. The 79-kDa band may be a degradation product of the 93-kDa band. The components are present in *L. destructor* extract, but probably in low concentrations because they are weakly visualized in Coomassie brilliant blue-stained SDS-PAGE. Obviously, based on the RIA, it was not possible to determine whether both of these components are IgE binding. Nevertheless, at least one could evoke an allergic response in persons exposed to *L. destructor*, because it showed IgE-binding capacity in the RIA. As many as 60% of our 30 *L. destructor*-positive sera reacted with these high-mol.-mass components, indicating that these allergens could be of clinical importance. In statistical analysis, a moderate degree of correlation was obtained between the RIA values and the RAST values in this group, possibly because in the RAST test the entire set of allergens in *L. destructor* is recognized, whereas in the RIA, with one mAb, only one allergenic epitope is recognized. Therefore, a strict correlation between RIA and RAST values from individual sera is not to be expected. Why four sera belonging to the Dpt group were positive in the RIA for the two components, despite being negative in RAST for *L. destructor*, is not altogether clear. It could stem from a low concentration of these components in the RAST assay. It is also possible that cross-reacting IgE antibodies were present. However, there was no correlation between RIA results and the amount of specific anti-Dpt-IgE in the Dpt group, and all 30 sera in the nonatopic control group were negative in the RIA to both the 79- and the 93-kDa components.

In a previous study, we used cryostat cut sections of *L. destructor* to localize the allergen component at 39 kDa recognized by mAb 42B6 (30). It was found to be present in the gastrointestinal tract and in the cytoskeleton. Future studies are needed to establish the localization and function of these high-mol.-mass components.

It is widely held that clinically relevant allergens have a mol.mass of less than approximately 70 kDa. However, regarding mite allergens, there have been reports of several allergens of *Dermatophagoides* spp. with mol.mass as high as 274 kDa (3, 5, 18, 22). It is possible that high-mol.-mass allergens are seldom reported because of the limitations in some methods used, which may result in underestimation of the number of these allergens. Thus, the field of high-mol.-mass allergens is an unexplored area which needs more consideration, with respect both to the number of new allergens and to their potency.

Finally, this study indicates that high-mol.-mass allergens should be used to obtain extracts of high quality for diagnostic purposes as well as for hypo-sensitization treatment.

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