

Monoclonal Antibodies against Der s 1, a Major Allergen of *Dermatophagoides siboney*

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Key Words

Monoclonal antibody · Mite allergens · ELISA

Abstract

Six stable clones secreting murine monoclonal antibodies (Mabs) against Der s 1 were obtained. The binding of Mabs showed cross-reactivity with *Dermatophagoides farinae*, as determined by enzyme-linked immunosorbent assay (ELISA). In a Western blot assay, antibodies reacted with a 24-kD protein considered to represent the major allergen Der s 1. The repertoire of antigenic sites on Der s 1 was studied using a panel of Mabs. Epitope specificity of the Mabs was determined by both competitive inhibition and sandwich ELISA assays. The results defined six different, non-overlapping and non-repeated antigenic sites on the allergen molecule. Der s 1 allergen from *Dermatophagoides siboney* extracts was purified by Mab affinity chromatography, this procedure gave 43% recovery of >90% pure allergen. The purified allergen had capacity to bind specific human IgE and demonstrated an allergenic activity of up to 77% of total *D. siboney* extract. An Mab-ELISA was developed using Mabs directed against different epitopes on Der s 1. This

assay could detect up to 1 ng/ml of Der s 1 and Der f 1 in allergen preparations.

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Introduction

Exposure to allergens from house dust mites of the *Dermatophagoides* genus, especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, is an important cause of immediate hypersensitivity [1, 2]. In Cuba, the existence of *Dermatophagoides siboney* was taxonomically described in 1982 [3], constituting up to 40% of the total dust mite population [4]. This mite species has also been reported to be present in Puerto Rico [5].

The high prevalence of sensitization to *D. siboney* found among asthmatics in Cuba indicates that this mite should be considered an important sensitizer [6]. *D. siboney* has been shown to cross-react with other house dust mite allergens, and a higher degree of cross-reactivity was reported between *D. farinae* and *D. siboney* than between *D. siboney* and other mite species [7].

Der s 1, a major allergen of *D. siboney*, was identified and extensively characterized by Ferrándiz [8] and Fer-

rándiz et al. [9]. Availability of Mabs to Der s 1 could provide more information about this protein, contributing to the understanding of the immunological aspects of this allergen.

In this study, we report the generation and preliminary characterization of a panel of Mabs against Der s 1. The results show that the Mabs defined six different epitopes on the allergen molecule. In addition, Mabs facilitated the development of a single-step procedure for immunopurification of Der s 1 from *D. siboney* extract and a Mab-ELISA useful for measuring Der s 1 and Der f 1 allergens.

Materials and Methods

Allergen Extracts

Extracts of *D. siboney* and *D. pteronyssinus* were obtained by extraction of whole mature cultures of mites grown at 25°C and 85% relative humidity on synthetic medium. Extraction was performed at 1:20 (w/v) in ammonium bicarbonate (0.125 M, pH 7.8), for 20 h at 4°C. After centrifugation, the supernatant was concentrated by ultrafiltration (cutoff 10 kD, Amicon, USA), aliquoted and freeze-dried. The aliquots were stored at -20°C. The ALK Laboratories, Denmark, supplied extract of *D. farinae*.

Serum Sources

Sera were obtained from 5 adult patients who had clinical symptoms of rhinitis, asthma, and/or atopic dermatitis and gave positive immediate skin tests to *D. siboney* extract (wheal > 3 mm).

Purified Antigen

Der s 1 was partially purified by preparative isoelectrofocusing (IEF) of a *D. siboney* mite extract culture in a vertical mini gel system (Hoeffer, Sigma). Briefly, 20 mg of *D. siboney* mite extract containing 12 mg of total protein were resuspended in 100 µl distilled water and then loaded onto one gel. The gel was 1.5 mm thick and contained 16% acrylamide mixture (30% (w/v) acrylamide, 1% (w/v) bisacrylamide and 5% ampholite (pH range 3–10)). Based on the similarity of several physicochemical and immunochemical properties reported for homologous major allergens Der s 1 and Der f 1 [9], it was supposed that Der s 1 might present a pI pattern similar to that reported for Der f 1 (4.7–7.2) [10]. Therefore, after focusing, the strip that corresponds to pI range 4–7 was cut along the entire gel into pieces and then extracted with PBS (pH 7.2). Separation of the eluted sample by SDS-PAGE showed the presence of a 24-kD band. This band was recognized by IgE antibodies in Western blot as previously described [8]. The content of Der s 1 was estimated by Mab-ELISA using two anti-Der f 1 Mabs (4C1/B8 and 6A8/B10) obtained by Chapman et al. [11]. These Mabs showed cross-reactivity with Der s 1 allergen [8, 9].

The diagnostic kit for quantitative measures of allergens Der f 1 and Der p 1 were supplied by INDOOR Biotechnologies (UK).

Immunization and Cell Fusion

Balb/c mice (10–12 weeks old) were immunized intraperitoneally with 15 µg of Der s 1 in complete Freund's adjuvant (Sigma, St.

Louis, Mo., USA) and boosted three times with an identical dose in incomplete Freund's adjuvant at intervals of 14 days. Three days before fusion, one mouse was boosted intraperitoneally with 10 µg of Der s 1 diluted in PBS. Spleen cells from the immunized mice were fused with non-secreting X63Ag8.653 myeloma cells in the presence of polyethylene glycol, MW 1500 (Sigma). Those hybridomas, found to be positive to Der s 1 antigen, were cloned by limiting dilution, and the selected clones were expanded. Ascitic fluids were purified by affinity chromatography using protein A-Sepharose CL 4B (Pharmacia).

Screening of Mabs by ELISA

Microtiter plates (MaxiSorp, Nunc) coated with 100 µl of Der s 1 (10 µg/ml) in phosphate carbonate-bicarbonate buffer 0.1 M were incubated for 2 h at 37°C. After washing, remaining binding sites were blocked by incubation for 1 h with PBS containing 1% BSA, then plates were washed and culture supernatants were added (100 µl/well). After extensive washing, peroxidase-labeled antibodies specific for mouse IgG (Sigma) were added 1/2,000. The reaction was developed with ortho-phenylenediamine (0.4 mg/ml) in 0.005 M citrate buffer containing 10 µl H₂O₂. The reaction was stopped by addition of 5 N HCl and the optical density was determined at 492 nm.

Determination of Mab Species Specificity

To define the specificity of Mabs, microtiter plates were either coated with *D. siboney*, *D. farinae*, *D. pteronyssinus* extracts (0.25 µg/well of Der 1 allergens) or Der s 1 (IEF; 1 µg/well) and blocked with PBS-BSA 1%, then plates were incubated with different dilutions of hybridoma culture supernatants. The ELISA was performed as described above.

Isotype Determination

The immunoglobulin isotype was determined using the Sigma Immuno Type Monoclonal Antibody Isotyping kit (Sigma).

Western Blotting Analysis

Antigen specificity of the Mabs against Der s 1 was tested by Western blotting. Protein was separated by SDS-PAGE under nonreduced conditions and transferred onto 0.45 µm nitrocellulose membrane and blocked with PBS-BSA 1% overnight at 4°C. The blots were incubated with purified Mabs (10 µg/ml), followed by incubation with biotin-conjugated goat anti-mouse IgG (Sigma), diluted 1/10,000. Bound biotin-labeled antibodies were revealed by addition of streptavidin-peroxidase conjugate (Sigma) diluted 1/2,000. The enzyme activity was determined by adding 0.7 mg/ml of diaminobenzidine (Sigma) in PBS.

Determination of Epitope Specificity of Mabs

In the competitive inhibition ELISA, microtiter plates were coated with Der s 1 (2.5 µg/ml) and blocked with 1% PBS-BSA, then plates were incubated with appropriate dilutions of biotinylated Mabs alone, or in the presence of either homologous or heterologous unlabeled Mabs used as inhibitors, at final concentrations ranging from 100 to 0.1 µg/ml. Bound biotin-labeled Mabs were revealed by addition of streptavidin-peroxidase conjugate (Sigma). The enzyme activity was detected as described above.

Mab H4/E7 specific to Der s 1, produced by our work group [9], and both Mab 6A8/B10 and Mab 4C1/B8 were used as positive controls [11].

In a double-antibody sandwich ELISA, microtiter plates were coated with the Mab1 (1 µg/well) and blocked with 1% PBS-BSA, then Der s 1 (100 ng/ml) was incubated with fixed Mab1. In preliminary experiments, this allergen concentration was able to saturate the available binding sites of the antibody. Subsequently, the second biotin-labeled Mab2 was added at appropriate dilution. Its binding to allergen was determined as described above.

Purification of Der s 1 Allergen from House Dust Extract

House dust extract (150 mg) containing 10.5 mg Der s 1 was resuspended in 150 ml of PBS and extracted overnight at 4°C. The suspension was centrifuged to remove debris. After centrifugation, samples of supernatant were applied on a column containing 5 ml wet gel of CNBr-activated Sepharose CL4B (Pharmacia) coupled with 20 mg of Mab. After extensive washing with PBS, the bound protein was eluted with 50% ethylene glycol in 0.005 M glycine (pH 10.0), dialyzed against PBS and then freeze-dried. The purified material was checked by Coomassie-stained SDS-PAGE, then the gel was subjected to densitometric analysis. Der s 1 concentration was estimated by ELISA [11].

Allergenic Activity of Purified Der s 1

The immunopurified protein was assayed for its ability to bind specific human IgE by an inhibition ELISA. The serum pool of human IgE diluted 1/10 was incubated overnight at 4°C with serial dilutions of either Der s 1 or *D. siboney* extract (from 5 to 0.1 µg Der s 1/ml). After incubation, the samples were added to a microtiter plate previously coated with *D. siboney* (0.25 µg/well) and the incubation continued for 2 h at 37°C with shaking. The amount of IgE antibodies bound to the solid phase was detected with a peroxidase-labeled Mab specific to human IgE (Biocen, Cuba). The enzyme activity was detected as described above.

Detection of Der s 1 Allergen in *D. siboney* Extracts Using an Mab-ELISA

A microtiter plate coated with Mab 5F7/H8 (0.5 µg/well) was incubated for 2 h at 37°C and blocked with 1% PBS-BSA for 1 h at 37°C. Control curves were performed with doubling dilutions of either *D. farinae* standard (2,500 ng Der f 1/ml; UVA 87/02) diluted from 1/100 to 1/1,600, partially purified Der s 1 (IEF; 0.1 mg/ml) from 1/1,000 to 1/32,000, affinity purified Der s 1 (1.7 mg/ml) from 1/20,000 to 1/624,000 or *D. pteronyssinus* extract (20 µg Der p 1/ml) from 1/1,000 to 1/16,000 used as non-related extract. The samples were applied to the wells and then incubated for 1.5 h at 37°C with shaking. The amount of allergen molecules bound to the capture antibody was detected with biotin-labeled 4E10/E10 Mab dilution 1/4,000. Bound biotin-labeled Mab was revealed as described above.

Results

Specificity of Mabs

Immunization of mice with Der s 1 partially purified from IEF followed by fusion experiments led to the production of six stable clones, secreting Mabs against Der s 1 named 5F7/H8, 8E3/E7, 1H2/H9, 4E10/E10, 3E10/C1 and 5E6/C12. Most of these Mabs were identified to be of

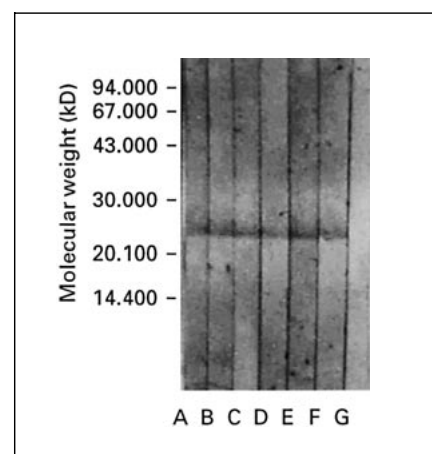


Fig. 1. Western blotting of Mabs to *D. siboney* extract proteins separated by SDS-PAGE. A = 5F7/H8; B = 8E3/E7; C = 1H2/H9; D = 4E10/E10; E = 3E10/C1; F = 5E6/C12; G = negative control. Locations of molecular weight markers are shown for reference.

the IgG1 isotype; except for 5F7/H8 Mab, which belonged to the IgG2b isotype. In order to determine the specificity of Mabs against allergens from different mite species, an ELISA assay was developed. Results are shown in table 1. The Mabs showed similar reactivity to *D. siboney* and *D. farinae* extracts. No reactivity to *D. pteronyssinus* extract was observed.

Western blotting demonstrated the specificity of the Mabs against the 24-kD protein (fig. 1).

Identification of Antigenic Epitopes on Der s 1

Epitope specificity of the Mabs was studied by two different ELISA assays. At first, a competitive inhibition ELISA was developed (table 2). The inhibition of the labeled Mabs exceeded that of unlabeled Mabs by 80%. In contrast, the inhibition values were <20% for the majority of the non-homologous inhibitions, except for a few combinations of Mabs in which a weak inhibition was observed (until 30% inhibition).

Group of Mabs 1H2/H9, 4E10/E10, 3E10/E10 and 5F7/H8, 6A8/B10 blocked their mutual binding presenting 83–92 and 94% of inhibition, respectively, suggesting that Mabs from each group recognized the same or closely related epitopes.

In order to confirm the previous results, another assay determining epitope specificity of the Mabs was developed using a double-antibody sandwich ELISA (table 3). The binding of labeled Mabs to Der s 1 was not relevant (<10%) when the allergen was presented by the homolo-

Table 1. Reactivity of hybridoma culture supernatants against extracts from mites of the genus *Dermatophagoides* and Der s 1

Mabs	<i>D. pteronyssinus</i>	<i>D. farinae</i>	<i>D. siboney</i>	Der s1
5F7/H8	0.099	1.073	0.802	1.364
8E3/D9	0.138	1.219	1.071	1.428
1H2/H9	0.217	1.173	1.078	1.197
4E10/E10	0.075	0.938	0.747	0.971
3E10/C1	0.159	0.822	0.485	1.123
5E6/C12	0.144	0.469	0.438	0.828
X63	0.177	0.174	0.068	0.099

Results of supernatant binding are presented as optical densities (280 nm). Nonspecific binding was determined with X63 mouse myeloma culture supernatant.

Table 2. Competitive inhibition of biotinylated Mabs to solid-phase Der s 1 by unlabeled Mabs

Inhibitor Mabs	Biotin-labeled Mabs						
	5F7/H8	8E3/E7	1H2/H9	4E10/E10	3E10/C1	5E6/C12	4C1/B8
5F7/H8	96	5	25	20	28	5	7
8E3/D9	15	100	18	10	19	7	18
1H2/H9	25	6	90	83	84	18	17
4E10/E10	21	6	83	97	88	23	13
3E10/C1	28	5	87	92	100	23	12
5E6/C12	10	11	20	11	20	100	19
H4/E7 ^a	9	5	8	5	6	6	32
6A8/B10	94	14	29	21	31	6	5
E2 ^b	5	9	10	6	8	11	7

Binding of biotinylated Mabs to immobilized Der s 1 (0.25 µg/well) was inhibited by unlabeled Mabs and the results are expressed as percent inhibition for each Mab tested.

^a Mab specific to Der s 1 [9].

^b Control for nonspecific inhibition of Mab E2 (anti-human IgE).

Table 3. Binding combination of various anti-Der s 1 Mabs in a double-antibody sandwich ELISA (%)^a

Fixed Mab	Biotin-labeled Mabs					
	5F7/H8	8E3/E7	1H2/H9	4E10/E10	3E10/C1	4C1/B8
5F7/H8	9	90	100	90	50	98
8E3/E7	83	7	100	80	58	92
1H2/H9	91	100	6	7	6	78
4E10/E10	100	96	8	7	6	80
3E10/C1	88	100	8	6	6	88
6A8/B10	8	99	100	81	50	92

^a Binding of biotinylated Mab2 to Der s 1 (100 µg/ml) presented by fixed Mab1 (1 µg/well) was expressed as a percentage of maximal response obtained for the pair which gave maximal optical density for a fixed Mab.

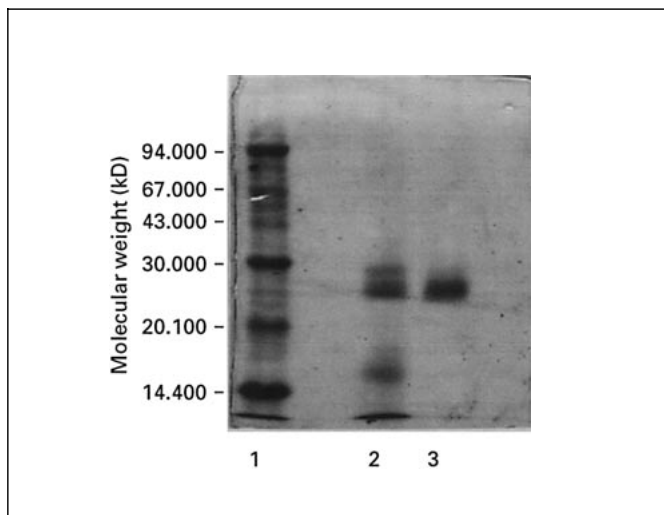


Fig. 2. SDS-PAGE analysis of monoclonal affinity-purified Der s 1. Samples were analyzed under non-reducing conditions. 1 = Molecular weight markers; 2 = *D. siboney* extract; 3 = single-step purified Der s 1.

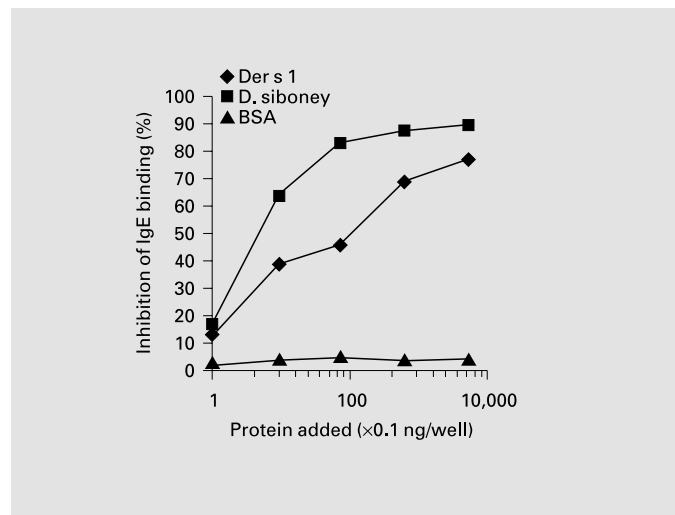


Fig. 3. Allergenic activity of purified Der s 1 determined by inhibition ELISA. *D. siboney* extract was fixed to the solid phase (0.25 µg Der s 1/well). *D. siboney* extract and BSA were used as positive and negative controls, respectively.

gous immobilized Mab, suggesting that the corresponding epitope is unique to the Der s 1 protein. Pairs of Mabs (4E10/E10, 1H2/H9, 3E10/C1 and 5F7/H8, 6A8/B10) inhibited their mutual binding to Der s 1, suggesting that these antibodies recognize the same or close epitopes. On the other hand, other combinations of Mabs were able to react simultaneously to allergen protein.

Purification of Der s 1 Allergen

Der s 1 was affinity purified from *D. siboney*, using 4E10/E10 Mab coupled to CNBr-activated Sepharose CL4B. The eluted sample gave a single band under non-reducing conditions on SDS-PAGE, with an apparent relative molecular weight of 24 kD (fig. 2) and showed >90% purity. This procedure yielded 5.1 mg of Der s 1 (49% recovery). Allergenic activity of the immunopurified protein was studied by an inhibition ELISA, in which the allergen inhibited the binding of human IgE to the whole *D. siboney* extract. Der s 1 inhibited up to 77% of the binding of human IgE to *D. siboney* extract (fig. 3).

Mab-ELISA

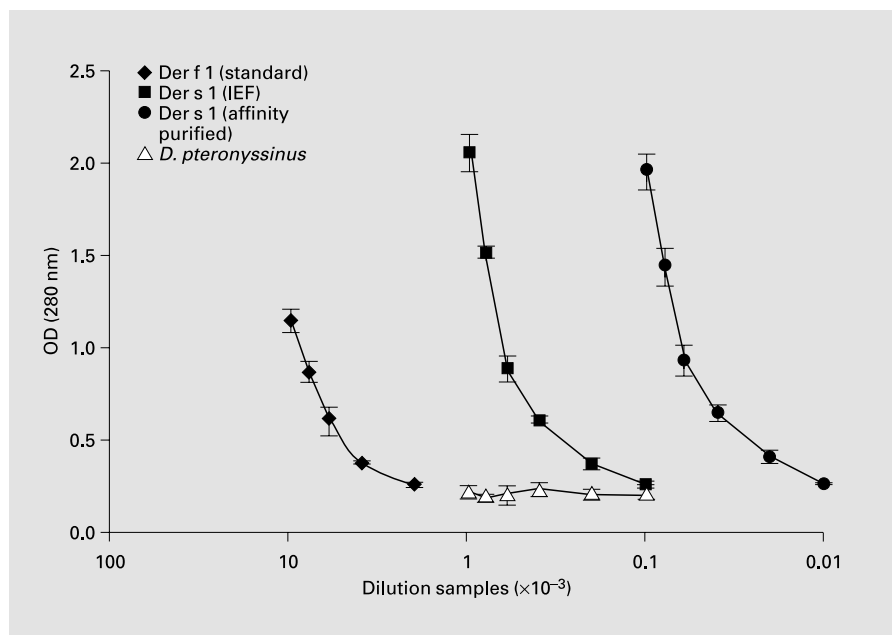
An Mab-based double antibody sandwich ELISA was developed to detect the Der s 1 allergen, using the combination of Mab 5F7/H8 as capture antibody and biotin-labeled Mab 4E10/E10 as probe antibody. Binding curves of different allergen samples were evaluated and parallel dilution curves were obtained (fig. 4). This ELISA was

capable to detect up to 1 ng/ml of Der s 1. No reactivity was observed when the extract of *D. pteronyssinus* was used as a negative control.

Discussion

In this report, the production and preliminary characterization of six Mabs against Der s 1, a major allergen of *D. siboney*, are described. Using an ELISA to determine species specificity of Mabs to *D. siboney*, *D. farinae* and *D. pteronyssinus* extracts, it was observed that antibodies are extensively crossreactive with *D. farinae*, but not with *D. pteronyssinus*. This finding indicates that there is a high degree of antigenic homology between allergens from these species, which is in concordance with previous reports about the closer relationship between individual allergens from *D. siboney* and *D. farinae* compared to *D. pteronyssinus* and with the taxonomic relationship between these species [7, 9, 12]. The antigen specificity of each Mab was confirmed by Western blotting and showed a specific recognition of a protein band of 24 kD (Der s 1). Antigenic sites on the allergen molecule were identified using two assays: a competitive inhibition ELISA and a double-antibody sandwich ELISA as a complementary assay, both being basically similar to that described by Le Mao et al. [13]. In these assays, serial combinations among all the Mabs were analyzed, with the exception of

Fig. 4. Dose-response curves for *D. farinae* standard (2,500 ng Der f 1/ml; UVA 87/02), Der s 1 partially purified (IEF; 0.1 mg/ml), Der s 1 affinity purified (1.7 mg/ml) and *D. pteronyssinus* extracts (20 µg Der p 1/ml) in an Mab-ELISA to detect either Der s 1 or Der p 1 major allergens. The confidence interval ($\alpha = 0.05$) was plotted for each point. Least-squares linear regression was performed to estimate slopes. Pairwise significance of differences between the slopes was determined by Student's *t* test demonstrating no differences between slopes ($p = 0.05$).



Mabs 5E6/C12 and H4/E7 which were excluded from the double-antibody sandwich ELISA because they had no capacity to bind the antigen in aqueous solution (data not shown). The results obtained in both assays allowed to define at least six different, non-overlapping and non-repeated antigenic site on Der s 1.

Between the group of Mabs 1H2/H9, 4E10/E10, 3E10/C1 and 5F7/H8, 6A8/B10 a weak inhibition in competitive inhibition ELISA was noted. On the other hand, they did not show any inhibition in the double-antibody sandwich ELISA: on the contrary, its combinations were able to react simultaneously with Der s 1. Results obtained in the competitive inhibition ELISA could be associated with structural changes in epitopes; binding of the first antibody (inhibitor) to the antigen may have resulted in a decreased accessibility to epitopes for the secondary antibody. Such conformational changes in epitopes have been reported previously [14].

Combinations of most of the Mabs at the double-antibody ELISA exceeded 80% binding to allergen, with the exception of labeled Mab 3E10/C1 which presented <60% binding capacity for a given concentration of allergen. A possible explanation for the diminished binding reactivity of Mab 3E10/C1 when used as a probe could be less affinity to the antigen or an effect of the biotinylation procedure on the binding site. Some relevant allergenic proteins have been immunopurified using Mab affinity chromatography [8, 15, 16]. In this work, the major aller-

gen Der s 1 could be successfully immunopurified from *D. siboney* in a single-step procedure using the Mab 4E10/E10, coupled to CNBr-activated Sepharose CL4B, obtaining 49% of recovery and a high-purity degree (>90%) of eluted protein. The purification process of large quantities of Der s 1 from extracts using conventional chromatography techniques is very difficult, the most crucial factors that affect this process are the starting concentration (<100 µg Der s 1/mg extract) and the extract composition. The availability of Mab 4E10/E10 for affinity chromatography improved the efficiency of allergen purification, obtaining milligram quantities of Der s 1, useful for important research application on allergy. The evaluation of the individual allergenic activity of Der s 1 confirmed that this protein is a major allergen of *D. siboney* extract.

An Mab-based double-antibody sandwich ELISA to measure Der s 1 was developed. In this assay, Mab 5F7/H8 was used as capturing antibody and biotin-labeled Mab 4E10/E10 as probe. The sensitivity achieved in this assay (1 ng/ml) suffices to estimate Der s 1 content in *D. siboney* extracts and the specificity enables the use of this assay system to determine absolute quantities of either Der s 1 or Der f 1 allergens.

The importance of *D. siboney* as sensitizing agent among asthmatics in Cuba [6] and the fact that *D. siboney* is common in dust from Cuban homes [4], whereas *D. farinae* has not been found in our country [17], make the Mab-ELISA a useful tool, since it could facilitate the bio-

logical standardization in vitro and test the stability of *D. siboney* allergenic extracts used in the diagnosis and treatment of allergy. Additionally, this Mab assay enables to study environmental mite allergens irrespective of the *Dermatophagoides* species being present.

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