LETTER TO THE EDITOR

IgE reactivity to house dust mite allergen components in sensitized asymptomatic subjects: a role for Der p 20

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To the Editor,

Whether the presence of allergen specific IgE translates into clinically significant symptoms and signs of allergy depends on the interplay of multiple factors. These include genetics, the ratio of total versus allergen-specific IgE, epitope-specificity and clonality of IgE (1) and IgE sialylation (2). The balance of T regulatory cells (Treg) and Th1/Th2 cells, the polymorphisms of the high affinity receptor for IgE (Fc-epsilon RI) and other factors regulating the activation of Fc epsilon RI-bearing cells may also play relevant roles. In this scenario, component-resolved diagnosis (CRD) may help clinicians in distinguishing between symptomatic and asymptomatic sensitized patients, particularly in the case of house dust mite allergy (3). In patients with house dust mite sensitization, different patterns of reactivity to mite allergen components have been reported (4) and polysensitization to multiple house dust mite (HDM) allergens have been variably correlated with the allergic phenotype (5).

Here, we analysed IgE reactivity to HDM allergens available to the clinical lab (Der p 1, Der p 2, Der p 10, Der p 23) in a cohort of patients with a positive prick test to HDM, who did not report symptoms of respiratory allergic rhinitis triggered by environmental house dust. HDM-sensitized patients who experienced allergic rhinitis and/or conjunctivitis and/or asthma when environmentally exposed to house dust served as controls. In one patient IgE did not react to any of these components and IgE to Der p 20 was the only sensitizing allergen. Reactivity to Der p 20 was compared in symptomatic versus asymptomatic subjects.

MATERIALS AND METHODS

This is a retrospective, observational study based on electronic records of patients in the outpatient Allergology clinic of the San Raffaele Hospital, Milan, filed between January 2018 and December 2019 during routine clinical activity. All patients included in the analysis performed prick test with a standard panel of inhalant allergens according to GA2LEN recommendations (6). Reagents for prick testing were from ALK Abello (Horsholm, DK). Answers to questions about circumstances when patients observed symptoms of rhinitis (indicated as sneezing, rhinorrea and/or nasal occlusion), if any, were recorded during the collection of the clinical history. Evidence of correlation between symptoms and positive skin prick test (SPT) to tested inhalant allergens was methodically registered.

Key words: allergy; component resolved diagnosis; allergen sensitization; house dust mite proteome

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Sixty-two subjects with a positive SPT to *D. pteronyssinus* and/or *D. farinae* extract were preliminarily identified. They were assigned to the symptomatic or asymptomatic group based on their positive or negative answer to specific questions about the elicitation of nasal symptoms (sneezing, rhinorrea or nasal occlusion), ocular symptoms (itchy eye, red eyes, lachrymation) and/or asthma symptoms (cough, dyspnoea, wheezing) in the presence of house dust in an indoor environment.

The following exclusion criteria were applied: urticaria, atopic dermatitis, allergen-specific immunotherapy in the previous 5 years, co-sensitization to perennial and/or indoor allergens other than HDM (animal dander, Alternaria, pellitory). Seventeen patients were excluded due to allergen-specific immunotherapy in the previous 5 years (7 patients) or sensitization to perennial and/or indoor sensitizing allergens other than HDM (10 patients). Based on these criteria, thirty patients were provisionally allocated to the symptomatic group and 15 to the asymptomatic group, respectively. These subjects were re-contacted by telephone to obtain their consent to participate anonymously to this study. In case this was granted, patients were attributed consecutive number identification codes and asked to confirm the answers they gave to questions related to the elicitation of symptoms in the presence of house dust within indoor environment. Twelve patients who could be re-contacted and confirmed the correlation of symptoms with exposure to house dust were allocated to the symptomatic group. Eleven subjects with positive skin prick test to house dust mite who denied any relevant symptom in the presence of house dust were allocated to the asymptomatic group. Specific IgE to Der p 1, Der p 2, Der p 10 and Der p 23 (ImmunoCAP, Thermo Fisher) in sera were measured from all patients included in the study, according to the manufacturer's instruction. Sera were either available from stored frozen samples, or from blood freshly redrawn. The flow chart of study subjects is presented in Fig. 1.

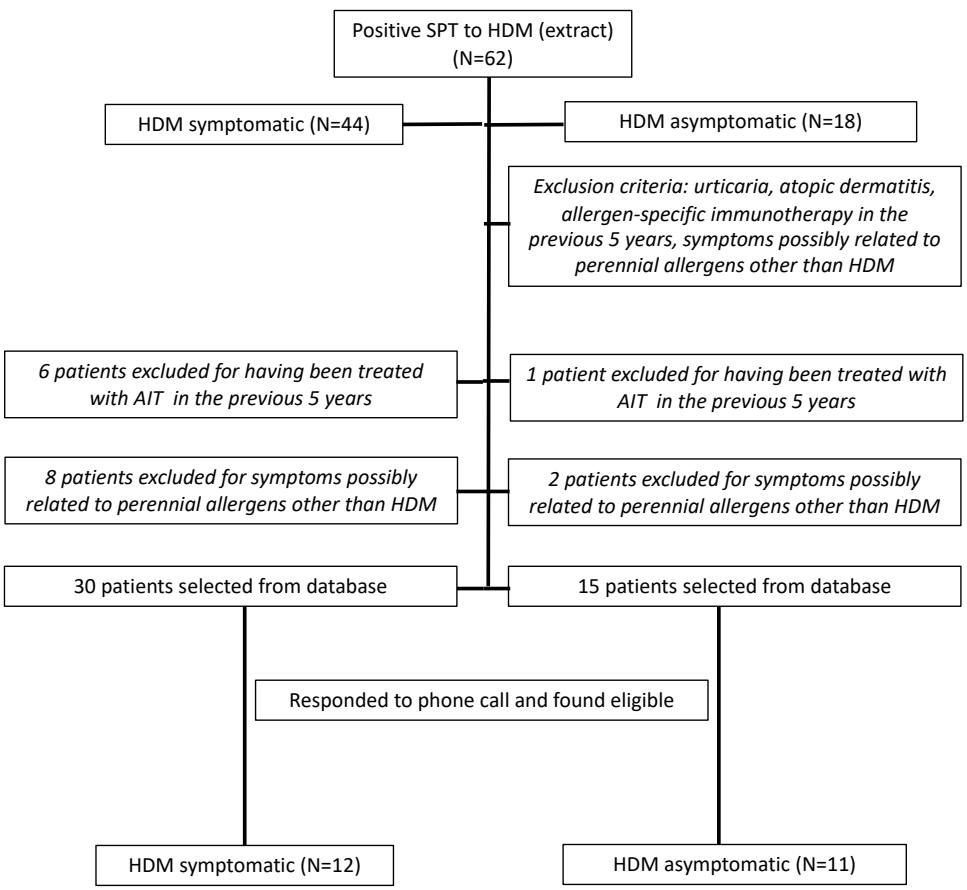

Fig. 1. *Flow chart of patient inclusion. HDM, house dust mite; SPT, skin prick test; AIT, Allergen-specific Immunotherapy*

Informed consent was obtained from all patients, according to the study protocols [BIO-IMMUNO-ALL CE 05.12.2002, rev. CE 02/12/04 (privacy) Vers. 04/10/2005], which were approved by the Ethics Committee of the IRCCS-San Raffaele Scientific Institute. Stored frozen samples were stocked in the San Raffaele institutional biobank (Biorep, Milan, Italy) until use.

Allergen extract for proteome analysis

Dermatophagoides pteronyssinus raw extract was purchased from Allergon/Thermo Fisher Scientific (Angelhom, Sweden) and used for the identification of IgE-reacting proteins as representative species of the mite allergen source.

Two-dimensional electrophoresis (2DE)

Serum from one patient (patient code: 310) was used as a probe for the evaluation of IgE reactivity to the mite proteome. For 2DE analysis, control serum was obtained from another patient allergic to HDM (patient code: 301). 2DE was performed as described in a previous study (7). In brief, 200 µg of total protein extracts were applied to 7-cm IPG strips (pH 3-10 NL; GE-Healthcare) by in-gel rehydration. Focusing was performed with an IPGphor system (GE-Healthcare) (50 mA/strip maximum; gradient voltage, max 5,000 V; 25 kVh total). Strips were reduced and alkylated by sequential incubation in equilibration buffer (50mmol/L Tris-HCl, pH 8.8; 6mol/L urea; 30% glycerol; 2% sodium dodecyl sulphate) containing 2% dithiothreitol or 2.5% iodoacetamide, respectively. Then, strips were transferred onto a 4-20% gradient or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE] for the second-dimension separation. Preparative 2DE gels for protein spot excision (see below) were generated by loading 700 µg of total protein.

Western blot (WB) analysis

Total protein mite extracts or histidine-tag purified recombinant allergen Der p 20 (8) resolved by SDS-PAGE were transferred onto nitrocellulose membranes and incubated for 12 h at 4°C with patients' sera diluted 1/250 in tris-buffered saline containing 1% tween-20. Immunoreactivity was revealed by incubation with polyclonal goat anti-human IgE, conjugated with horseradish peroxidase (Sigma), followed by electro-chemo-luminescence (GE-Healthcare) reaction and film exposure. Images were acquired using a G:BOX Chemi XX6 System (Syngene, Cambridge, UK) and 2-DE protein patterns were analysed using Progenesis P240 software (NonLinear Dynamics, Newcastle, United Kingdom). Relative mass was estimated by comparison with reference markers (Precision, Bio-Rad) and isoelectric point values were assigned to the detected spots according to GE-Healthcare guidelines.

Protein identification by mass spectrometry analysis

Preparative gel was stained with Coomassie colloidal blue, images were acquired at high resolution and analysed as described above. Spots of interest were excised from the gel, reduced, alkylated, and in-gel digested with bovine trypsin (Roche Diagnostics Corp). Liquid chromatography electron spray ionization tandem mass spectrometry [LC-ESI-MS/MS] analysis was performed as previously described (7). In brief, 5 µL of digested sample were injected into a capillary chromatographic system (Agilent 1100 Series) equipped with a Nano Pump (Agilent) and peptide separation occurred in an RP C18 nanocolumn. The eluting peptides were ionized by a nano-ESI online source and analysed on an API QStar PULSAR (AB-Sciex, Toronto, Ontario, Canada) mass spectrometer. Full-scan mass spectra (range m/z 350-1,600) were collected, and for each spectrum, the 2 most intense doubly- and triply-charged ion peaks were selected for fragmentation. Tandem MS data files from chromatographic runs were combined and converted to MASCOT files using MASCOT.dll 2.2.07 through Analyst QS 1.1 (AB Sciex). All MS/MS samples were analysed using the MASCOT engine to search the Swiss Prot, Other Metazoa 2016_10 (10510 sequences) database. Peptide mass tolerances of 200 ppm and 0.3 Da were used for precursor and fragment ions, respectively. The other search parameters were 2 allowed missing cleavages, oxidation of methionine as a variable modification, and carbamide methylation as a fixed modification.

Determination of IgE specific to Der p 20

His-tag recombinant full length Der p 20 was obtained as described (8). An experimental Der p 20 ImmunoCAP test was developed by biotinylation of Der p 20 using EZ-link sulfo-NHSLC-biotin (Thermo Scientific) according to the manufacturer's instructions. Results were presented as kilounits of allergen per liter (kAU/l) (cut-off for positivity 0.10 kAU/L).

Statistical analysis

Patients' main characteristics were described as median (interquartile range, IQR) for continuous variables or proportions for categorical variables. Continuous variables were compared using the Wilcoxon rank sum test. Differences between proportions were tested by the *chi*-squared or Fisher exact test. Multivariate logistic regression models were performed to identify factors associated with the risk of symptoms. The multivariate model was performed in a stepwise manner with entry and removal criteria at 0.05 level. All analyses were conducted using SAS statistical software version 9.4 (Statistical Analyses System Inc, Cary, NC, USA).

RESULTS

Symptoms following exposure to mite dust and sensitization to inhalant allergens in HDM-sensitized symptomatic and asymptomatic patients

In a group of 62 reports of consecutive patients with positive skin prick test for HDM, 18 (29%) did not describe any symptoms of rhinitis when environmentally exposed to house dust, based on clinical records. The correlation between allergen exposure and triggering of symptoms could not be re-assessed in all patients, following a dedicated telephone call, due to no answer and/or unwillingness

Table I. *Results of skin prick test and diagnosis of study subjects*

			Skin Prick Test				
Symptomatic (12)	**sex**	**age**	***HDM***	***grass***	***trees***	***ragw***	**diagnosis**
S0301	*m*	*15*	*pos*	*pos*	*neg*	*neg*	*AR+AA*
S0315	*m*	*54*	*pos*	*neg*	*neg*	*neg*	*AR*
S0308	*f*	*45*	*pos*	*neg*	*neg*	*neg*	*AR+AA*
S0304	*f*	*9*	*pos*	*pos*	*pos*	*neg*	*AR*
S0309	*f*	*22*	*pos*	*neg*	*neg*	*neg*	*AR*
S0311	*f*	*19*	*pos*	*pos*	*neg*	*neg*	*AR*
S0307	*m*	*22*	*pos*	*neg*	*neg*	*neg*	*AR+AA*
S0302	*f*	*45*	*pos*	*pos*	*neg*	*neg*	*AR*
S0303	*f*	*35*	*pos*	*pos*	*pos*	*neg*	*AR+AA*
S0304	*m*	*19*	*pos*	*pos*	*neg*	*neg*	*AR*
S0305	*f*	*21*	*pos*	*neg*	*neg*	*pos*	*AR+AA*
S0306	*f*	*19*	*pos*	*pos*	*neg*	*neg*	*AR*
Asymptomatic (11)							
A331	m	8	pos	neg	neg	neg	AR+AA
A332	f	16	pos	pos	pos	neg	AR
A333	m	19	pos	neg	neg	neg	no respiratory allergy
A334	f	19	pos	neg	neg	neg	no respiratory allergy
A335	m	24	pos	pos	pos	neg	AR
A336	f	45	pos	pos	neg	pos	AR
A337	f	35	pos	pos	neg	neg	AR
A338	m	38	pos	pos	neg	neg	AR+AA
A310	m	53	pos	neg	neg	neg	no respiratory allergy
A339	m	51	pos	pos	neg	neg	AR
A333	f	18	pos	neg	neg	neg	no respiratory allergy

AA: Allergic Asthma; AR: Allergic Rhinitis; ragw: ragweed; Grass, trees and ragweed: result of positive prick test to Phleum p., Betula v., Ambrosia a.

to participate in the study. Twenty-three patients, including 12 subjects who were symptomatic following exposure to house dust and 11 who were not, provided informed consent and could be further studied. Results of skin prick test and diagnosis of patients included in the present study are reported in Table I.

Diagnosis in the symptomatic group was allergic rhinoconjunctivitis (AR) (N=12, 100%), associated with allergic asthma (AA) in 5 patients (41%). In the house dust mite prick test positive, asymptomatic group diagnosis was AR in 7 patients (64%), associated with allergic asthma in 2 of them (18%). Four subjects (36%) who did not report any symptoms at all correlated to AR with or without AA were screened as family members of subjects with respiratory allergies. At categorical analysis, the higher frequency of AR (with or without AA) in the symptomatic group was non-significant (p=0.061). IgE to commercially available Der p 1, 2, 10 and 23 allergen components were measured in sera. One subject of the asymptomatic group (patient 0310) did not display measurable IgE reactivity to any of these allergens. He had a positive prick test to house dust mite, which was confirmed by specific IgE in serum (0.89 kAU/l), and by a positive patch test to house dust mite (Rapid Patch Test, ALK Abellò). Bronchial provocation test with methacholine scored negative.

Identification of Der p 20 as the only IgE reactive allergen from the house dust mite proteome in one patient

In order to identify the allergen recognized by IgE from patient 0310, a previously described approach based on the evaluation of reactivity to the relevant allergen proteome was used. One protein spot (#1) of ≅ 35.5 kDa of molecular weight specifically reacted with patient 0310 serum, but not with the anti-human secondary antibody used as a probe or with serum from HDM-allergic patient 0301 serving as control (Fig. 2A). The protein spot corresponding to IgE reactivity was excised from a preparative gel and analysed by MS for protein identification. At LC-ESI-MS/MS analysis, the spot with isoelectric point of 7.98 and relative mass of 35.5 (kDa) yielded unique identification consistent to protein Der p 20 allergen (sequence coverage 11% on 4 fragmented peptides and Mascot score 40) and belonging to the arginine kinase-like protein 2 family (accession B2ZSY4). Small differences between theoretical and experimental isoelectric point (8.42

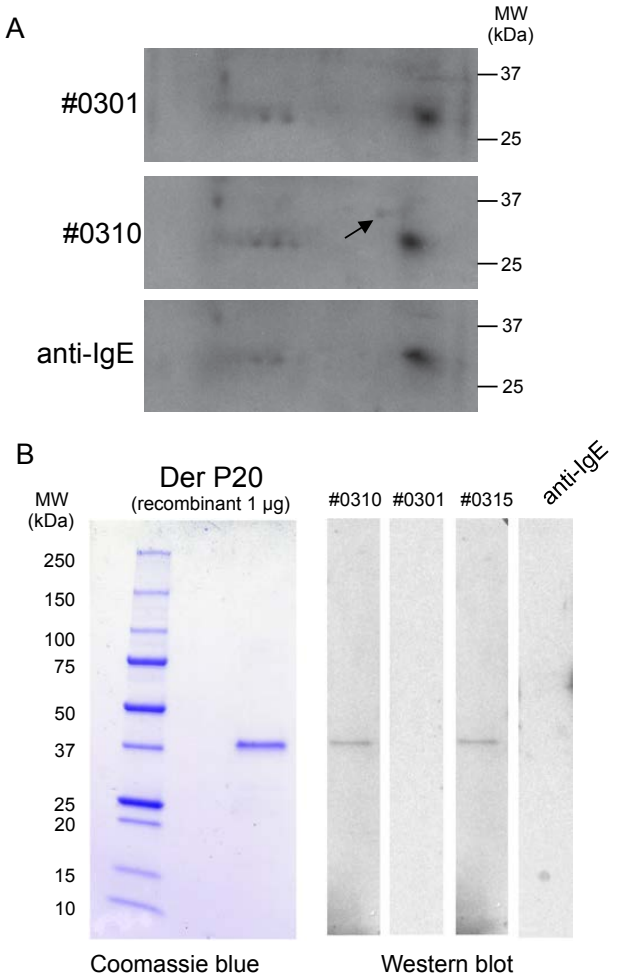

Fig. 2. ***A)*** *Reactivity of patient 0310 serum to the* Dermatophagoides pteronyssus *proteome.* D. pteronyssus *extracts were resolved by 2D-gel electrophoresis and then probed by WB with sera. IgE reactivity of patient 0310 serum was compared with the reactivity of another HDM-allergic patient (0301), and with the reactivity of the anti-human IgE secondary antibody as control. The arrow indicates the spot recognized by patient 0310 sera, that underwent to mass spectrometry analysis for protein identification.* ***B)*** *Reactivity of patient 0310 serum against recombinant Der p 20 allergen. The IgE reactivity of patient 0310 serum and of other two HDM allergic patient (0301, 0315) was tested against recombinant Der p 20 protein allergen resolved by SDS-PAGE. The anti-human IgE secondary antibody was used as control. Panel on the left shows the purity of the recombinant Der p 20 allergen resolved by SDS-PAGE and stained with blue Coomassie.*

vs 7.98) and relative mass (40.5 *vs* 35.5 kDa) could be due to protein fragmentation. However, the full-length recombinant Der p 20 allergen migrate in SDS-PAGE with an apparent relative mass below the 37 kDa reference standard (Fig. 2B on the left), suggesting that the 2DE reacting spot might represent the intact molecule.

Validation of IgE reactivity to Der p 20

His-tag recombinant full length Der p 20 expressed in *E. coli (8)* (1 μg) was used to test the reactivity with 0310 serum and with sera from 2 HDM allergic patients (0301 and 0315) serving as controls by mean of WB. IgE reactivity against the Der p 20 recombinant protein was observed with sera from patient 0310 and 0315 (Fig. 2B). In order to check allergen integrity, recombinant Der p 20 was resolved by SDS-PAGE and stained with Coomassie blue. There was no evidence of protein fragmentation (Fig. 2B). Titres of IgE levels to allergen components are individually reported in Table II and as median and interquartile range (IQR) in Table III.

IgE levels were unchanged in all the comparison, except for IgE to Der p 20, which was significantly higher in asymptomatic subjects (p=0.042). At categorical analysis (subjects considered positive when IgE > 010 kAU/l) no significant difference was observed with any allergen (not shown).

Table II. *Total and specific IgE levels to allergen components (individual values)*

Symptomatic	Der p 1	Der p 2	Der p 10	Der p 20	Der p 23	IgE to Dt. pt	total IgE
S0301	*0.45*	*2.12*	*0.35*	2.24	*1.12*	*2.35*	*56.00*
S0315	*3.20*	*7.22*	*0.00*	9.56	*0.00*	*5.12*	*46.00*
S0308	*1.23*	*1.20*	*0.00*	0.31	*2.45*	*3.45*	*34.00*
S0304	*0.00*	*42.03*	*0.00*	0.56	*0.00*	*22.45*	*220.00*
S0309	*0.00*	*5.56*	*0.00*	0.41	*0.56*	*1.52*	*157.00*
S0311	*2.23*	*2.12*	*0.00*	0.19	*1.23*	*6.45*	*159.00*
S0307	*0.00*	*2.22*	*0.00*	4.12	*0.00*	*9.53*	*42.00*
S0302	*5.78*	*3.96*	*0.00*	1.24	*0.00*	*12.45*	*45.00*
S0303	*2.45*	*5.12*	*0.00*	0.00	*1.11*	*2.23*	*424.00*
S0304	*1.05*	*1.23*	*0.00*	0.89	*0.85*	*7.89*	*350.00*
S0305	*4.02*	*19.45*	*0.00*	2.15	*2.12*	*12.56*	*442.50*
S0306	*5.56*	*7.45*	*0.00*	*0.00*	*1.22*	*4.98*	*33.00*
Asymptomatic							
A331	0.00	0.00	0.00	12.45	1.12	0.33	63.00
A332	0.00	1.45	0.00	4.56	0.56	0.98	78.00
A333	2.45	5.45	1.23	0.98	0.00	15.47	51.00
A334	0.00	3.45	0.00	1.23	0.59	3.32	68.00
A335	1.56	14.23	0.00	11.98	0.00	11.45	54.00
A336	1.23	7.45	0.00	6.23	0.00	1.68	78.00
A337	0.89	1.12	0.00	11.45	1.45	5.44	78.00
A338	0.89	1.25	0.00	0.78	0.00	0.98	89.00
A310	0.00	0.00	0.00	11.45	0.00	0.89	124.00
A339	4.45	6.41	0.00	0.98	0.00	7.55	94.00
A333	1.14	11.54	0.65	0.19	1.24	1.12	154.00

Results are expressed as AU/ml for allergens and IU/ml for total IgE

Table III. *Median and interquartile range of specific and total IgE values*

Vaariable	Category	Symptomatic	Aymptomatic	P-value
IgE to Der p 1	AU/ml	1.73 (0.23 - 3.61)	0.89 (0 - 1.56)	0.274
IgE to Der p 2	AU/ml	4.54 (2.12 - 7.34)	3.45 (1.12 - 7.45)	0.460
IgE to Der p 10	AU/ml	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.462
IgE to Der p 20	AU/ml	0.73 (0.25 - 2.20)	4.56 (0.98 - 11.45)	**0.042**
IgE to Der p 23	AU/ml	0.98 (0.00 - 1.23)	0.00 (0.00 - 1.12)	0.289
IgE D. pt. extract	AU/ml	5.79 (2.90 - 10.99)	1.68 (0.98 - 7.55)	0.079
total IgE	IU/ml	106.5 (43.5 - 285)	78 (63 - 94)	0.926

DISCUSSION

Up to 45% of subjects with a positive skin prick test (SPT) and/or positive level of serum sIgE for *D. pteronyssinus* do not suffer any allergic rhinitis (AR) symptoms (9). Here, we found that among patients with a positive skin prick test for HDM, 29% did not report any symptoms of rhinitis with or without conjunctivitis when environmentally exposed to house dust. Evaluation of reactivity to single allergen components in these subjects did not allow to highlight significant differences as compared to HDM-allergic subjects (1). One asymptomatic patient did not have any measurable IgE reactivity to the allergen components we analysed (Der p 1, Der p 2, Der p 10 and Der p 23). With a proteome-based approach, IgE to Der p 20 (arginine kinase) were identified in his serum. Arginine kinase allergens from different allergen sources show high sequence conservation and potential for cross reactivity. Notably, IgE to arginine kinase were previously correlated with mite sensitization not associated with allergy. Indeed, there is 75% and 80% identity to sequences of insects and crustaceans that have major inhalant (10) and food (11) arginine kinase allergens, respectively, but the HDM arginine kinases did not appear to be important HDM allergens (8). Der p 20 is a highly abundant protein in mites and, being the invertebrate equivalent of creatine kinase in muscle, is a candidate to induce high immune responses to a burrowing parasite. High levels of IgG and IgE to scabies were reported in patients with active infestation to this parasite (12). Along this line, in remote Australian Aboriginals IgE antibodies to Der p 20 reflected sensitisation to scabies mites and not to house dust mites (12). Our results suggest that non-species-specific, cross-reactive allergens, such as Der p 20, may act as markers of asymptomatic sensitization to house dust mite.

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