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Identification of Der p 23, a Peritrophin-like Protein, as a New Major *Dermatophagoides pteronyssinus* Allergen Associated with the Peritrophic Matrix of Mite Fecal Pellets

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Abstract

The house dust mite (HDM) *Dermatophagoides pteronyssinus* is one of most important allergen sources and a major elicitor of allergic asthma. We screened a *D. pteronyssinus* expression cDNA

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The nucleotide sequence of clone 30 has been deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/nuccore/EU414751>) under accession number EU414751.

library with IgE Abs from HDM allergic patients. A cDNA coding for a new major allergen was isolated, which showed sequence homology to peritrophins, which contain chitin-binding domains and are part of the peritrophic matrix lining the gut of arthropods. The mature Der p 23 allergen was expressed in *Escherichia coli* as an 8-kDa protein without its hydrophobic leader sequence and purified to homogeneity. It reacted with IgE Abs from 74% of *D. pteronyssinus* allergic patients ($n = 347$) at levels comparable to the two major HDM allergens, Der p 1 and Der p 2. Thus, Der p 23 represents a new major *D. pteronyssinus* allergen. Furthermore, rDer p 23 exhibited high allergenic activity as demonstrated by upregulation of CD203c expression on basophils from *D. pteronyssinus* allergic patients. Immunogold electron microscopy localized the allergen in the peritrophic matrix lining the midgut of *D. pteronyssinus* as well as on the surface of the fecal pellets. Thus, we identified a new major *D. pteronyssinus* allergen as peritrophin-like protein. The high allergenic activity of Der p 23 and its frequent recognition as respiratory allergen may be explained by the fact that it becomes airborne and respirable through its association with mite feces. Der p 23 may be an essential component for diagnosis and specific immunotherapy of HDM allergy.

Immunoglobulin E-associated allergy is one of the most important immunological hypersensitivity diseases affecting >25% of the population (1). Today, house dust mites (HDMs) are well established as the most important source of indoor allergens in allergic patients and a major cause of perennial asthma worldwide (2). Already in 1922, Cooke (3) recognized that house dust represents a major allergen source associated with asthma. A few years later, Dekker (4) reported the occurrence of HDMs in the beds of asthmatic patients and that their elimination reduced asthmatic symptoms. Yet, it took decades until HDMs, in particular the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, could be identified as major producers of house dust allergens (5-7).

Using allergen-specific IgE Abs from HDM allergic patients for quantitative measurements, it was found that mite feces represent the major source of HDM allergens (8, 9). In fact, one reason why mite-derived fecal pellets are potent elicitors of asthma is that they are small enough to become deposited not only in the nasal pharyngeal but also in the bronchial mucosa. Hydration of the fecal pellets leads to the release of HDM proteins on the airway epithelial cells resulting in allergic sensitization (8). In this context, it has been demonstrated that certain HDM allergens exhibit protease activity and thus induce the release of proinflammatory cytokines from respiratory epithelial cells and disturb the epithelial barrier function (10, 11). Furthermore, there is evidence that major HDM allergens such as Der p 1 and Der p 2 may increase allergic immune responses through intrinsic properties such as cleaving IgE receptors and boosting the innate immune response, respectively (12, 13).

Although several important HDM allergens were purified and characterized by classical protein chemical techniques (14, 15) it has been demonstrated that important allergens are lacking even in allergen extracts used for allergy diagnosis (16) and that it is difficult to produce large amounts of pharmaceutical grade natural allergens for immunotherapy (17). A major breakthrough toward the characterization of HDM allergens has been the application of molecular biological and recombinant technologies for allergen characterization (18). Since the isolation of the first cDNA coding for the major HDM allergen Der p 1 in 1988

(19), the sequences and biological functions of several HDM allergens have been revealed. Furthermore, it became possible to establish new forms of diagnostic tests based on purified recombinant HDM allergens (20) and to develop new forms of immunotherapy based on native-like recombinant HDM allergens, epitopes thereof, and recombinant hypoallergenic mite allergens (21-23).

To identify new important HDM allergens as components for diagnosis and specific immunotherapy, we used a discovery approach based on the screening of an expression cDNA library prepared from *D. pteronyssinus* with serum IgE Abs from HDM allergic patients. Unexpectedly, we discovered a cDNA that coded for a novel major HDM allergen, designated Der p 23. In this study, we report the expression and purification of the recombinant allergen in *Escherichia coli*, its immunological and structural characterization as well as its localization in the feces of HDMs by immunogold electron microscopy.

Materials and Methods

Isolation of a cDNA clone coding for Der p 23, expression, and purification of the allergen

A λ gt11 expression cDNA library of *D. pteronyssinus* was immunoscreened with pooled serum IgE from mite-allergic patients (24), and the clone 30 that coded for an IgE-reactive protein was isolated as described previously (25). Both DNA strands were sequenced (MWG, Ebersberg, Germany), the amino acid sequence was deduced, and the DNA and protein sequences were compared with the sequences deposited in GenBank using the BLASTN and BLASTP program, respectively. The clone 30 cDNA coding for the predicted mature Der p 23 (nt 89–295 with an additional ATG at the N terminus) was PCR amplified using the forward primer 5'-

GGAATTCCATATGCCAATGATAATGATGATGATCCTAC-3' and the reverse primer 5'-*GAATTCTTAAGTGCATGTTTCTTCATCTTCATTCCATCG*-3'. The PCR product was cut with the restriction enzymes EcoRI (*italics*) and NdeI (*underlined*), subcloned into the expression vector pET-17b (Novagene, Madison, WI), and transformed into *E. coli* BL21 (DE3) cells (Stratagene, La Jolla, CA). The bacterial cells were grown overnight in Luria-Bertani medium containing 100 mg/l ampicillin at 28°C, and expression of the recombinant protein was induced by adding isopropyl- β -thiogalactopyranoside to a final concentration of 0.5 mM. After cultivation for additional 3 h at 37°C, *E. coli* were harvested by centrifugation (15 min, 3000 rpm, 4°C; Sorvall RC5C) and lysed as described previously (26). The lysed bacterial cells were centrifuged at 18,000 rpm, 20 min, 4°C, and proteins of the soluble fraction containing Der p 23 were treated with 60% ammonium sulfate for 1.5 h at 4°C. Precipitated proteins were separated by centrifugation (18,000 rpm, 20 min, 4°C), and the soluble fraction containing Der p 23 was dialyzed against 2 M ammonium sulfate, 50 mM sodium phosphate (pH 7), and 10 mg/l PMSF and applied to a HiTrap Phenyl FF (high sub) column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Der p 23 was eluted by a 500- to 0-mM ammonium sulfate gradient, and fractions containing Der p 23 were pooled. After dialysis against 20 mM Tris-Cl (pH 8) and 10 mg/l PMSF, the sample was applied to a HiTrap DEAE Sepharose FF column (GE Healthcare Bio-Sciences). Der p 23 was eluted by a 0- to 500-mM NaCl gradient, and fractions containing >90% pure Der p 23 were pooled and dialyzed against 20 mM Tris-Cl (pH 8). A protein sample was analyzed for purity by

14% SDS-PAGE and Coomassie brilliant blue protein staining (27). The protein concentration was measured with the Micro Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL).

DNA and protein sequence analysis and MALDI mass spectrometry

The nucleotide and the deduced amino acid sequence were compared with the sequences deposited in the National Center for Biotechnology Information databases including GenBank, SwissProt, PIR, PRF, and Brookhaven Protein Data Bank. The deduced amino acid sequence was also compared with domains deposited at the conserved domain database at National Center for Biotechnology Information and the Pfam database at the Sanger Institute. The protein sequence of Der p 23 was analyzed with tools of the ExPASy proteomics server, and the protein secondary structure prediction was performed on the PSIPRED protein structure prediction server (28).

Purified Der p 23 was analyzed by MALDI mass spectrometry (piChem, Research and Development, Graz, Austria) as described previously (29).

Allergic patients' sera and IgE binding frequency to rDer p 23

Residual serum samples from allergic patients who had undergone routine allergy diagnosis in Austria ($n = 67$), France ($n = 55$), and Italy ($n = 67$) were used for serological analyses. The diagnosis of allergy to *D. pteronyssinus* was based on case history, typical indoor symptoms (rhinitis, conjunctivitis, and/or asthma), and the presence of *D. pteronyssinus*-specific IgE Abs > 0.7 kU_A/l in serum as determined with the ImmunoCAP System (ThermoFisher/Phadia, Uppsala, Sweden). Sera from nonallergic individuals were used for control purposes (30). The IgE binding frequency of Der p 23 was determined in a nondenaturing dot-blot assay. The allergen (0.25 µg/µl) was dotted in 2-µl aliquots onto nitrocellulose strips (0.2 µm BA83 Protran; Whatman pIc, Maidstone, U.K.), blocked with 50 mM sodium phosphate (pH 7.4), 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, and 0.05% (w/v) sodium azide and incubated overnight at 4°C with 1:10 diluted sera from HDM allergic patients (26). Bound IgE Abs were detected with 1:15 diluted ¹²⁵I-labeled anti-human IgE Abs (Specific IgE RIA [RAST]; Demeditec Diagnostics, Kiel, Germany) and visualized by autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany).

In addition, rDer p 23 was microarrayed onto glass slides and assessed by ImmunoCAP immuno solid phase allergen chip (ISAC) technology (ThermoFisher/Phadia, Uppsala, Sweden) for IgE reactivity with sera from 158 additional HDM allergic patients.

Basophil activation assay

Heparinized blood samples from HDM allergic patients were obtained with approval from the ethics committee of the Medical University of Vienna. For the measurements of CD203c expression on basophils, peripheral blood was collected from allergic patients with IgE Abs both to Der p 23 and Der p 1 in heparinized tubes after informed consent was obtained. Natural Der p 1 was isolated by affinity chromatography using the mAb 4C1 (31). Aliquots (100 µl) of the blood were incubated with serial dilutions (10^{-4} to 10 µg/ml) of nDer p 1, rDer p 23, anti-IgE (1 µg/ml), or PBS for 15 min at 37°C. After incubation, cells were

washed in PBS containing 20 mM EDTA and incubated with 10 μ l PE-conjugated mAb 97A6 (CD203c; Immunotech, Beckman Coulter, Fullerton, CA) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with FACS Lysing Solution (BD Biosciences, San Jose, CA). Cells were then washed, resuspended in PBS, and analyzed by flow cytometry on a FACSCalibur (BD Biosciences), using a Paint-a-Gate Software (32). Allergen-induced upregulation of CD203c was calculated from mean fluorescence intensities (MFI) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells and expressed as stimulation index (SI = MFI_{stim}:MFI_{control}) (32).

IgE ELISA competition assay

A rabbit antiserum was raised against rDer p 23 using Freund's adjuvant (Charles River Laboratories, Kisslegg, Germany), and the ability of the Der p 23-specific rabbit Abs to inhibit binding of *D. pteronyssinus* allergic patients' IgE to the allergen was examined by ELISA competition assays (29). ELISA plate-bound Der p 23 (5 μ g/ml) was preincubated with 1:100 in PBS, 0.05% (v/v) Tween 20 (PBST), and 0.5% (w/v) BSA diluted rabbit anti-Der p 23 Abs, or rabbit preimmune serum and incubated at 4°C overnight. Subsequently, the plate was exposed to 1:5 in PBST and 0.5% (w/v) BSA diluted sera from *D. pteronyssinus* allergic patients overnight at 4°C. Bound IgE Abs were detected with HRP-coupled goat anti-human IgE Abs (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:2500 in PBST and 0.5% BSA. The color reaction was performed and quantified as described previously (29). The degree of inhibition was calculated as follow: percent inhibition of IgE binding = $100 - \text{OD}_{\text{anti Der p 23 serum}} \times 100 / \text{OD}_{\text{preimmune serum}}$.

Immunogold transmission electron microscopy

Rabbit IgG Abs specific for Der p 23 were isolated by affinity chromatography (ImmunoPure IgG Purification Kit, Pierce) from the serum of an immunized rabbit. The preimmune Igs were used as control. Mites were grown in 150-cm² vented cell culture flasks (Corning Incorporated, Corning, NY) under controlled humidity (75% relative humidity) and temperature (25°C) conditions. Adult *D. pteronyssinus* mites were pierced with a fine needle for penetration of fluids and fixed for 6 d at 4°C with 6% (v/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde in 0.2 M PIPES buffer (pH 7.3). After dehydration in an ascending series of ethanol, the mites were embedded in Lowicryl K4M resin polymerized at -35°C using UV irradiation. Ultrathin sections (silver color) were placed on nickel grids and were exposed successively to the following solutions (room temperature, moist chamber, PBS buffer, 0.1% (w/v) BSA, pH 7.3, Tris buffer, 0.1% (w/v) BSA (pH 8.2) (1): 5% (w/v) BSA in PBS buffer, 15 min (2); rabbit anti-Der p 23 Abs, diluted 1:100 in PBS buffer, 2 h (3); PBS buffer, 2 \times 5 min, Tris buffer, 1 \times 5 min (4); goat anti-rabbit IgG Abs coupled to 10nm colloidal gold particles (BritishBioCell, Plano, Wetzlar, Germany), 1 h (5); Tris buffer, 1 \times 5 min, distilled water, 2 \times 5 min. Controls were run (1) by replacing the specific anti-Der p 23 Abs by the preimmune Abs and (2) by omitting the anti-Der p 23 Abs from the labeling protocol. After immunolabeling, sections were stained with uranyl acetate and lead citrate and analyzed in a Hitachi H 500 transmission electron microscopy (TEM) (Nissei Sangyo, Tokyo, Japan) and Philips EM 410 (FEI Company, Eindhoven, The Netherlands) at 75 and 80 kV, respectively.

Extracts from mite feces and immunoblots

Aliquots of 0.12 g feces from the mite *D. pteronyssinus* were shaken in 2 ml distilled water containing 10 µg/ml PMSF over night at 4°C and insoluble material in the protein extract was removed by centrifugation for 10 min and 4°C at 14,000 rpm. Alternatively, mite feces, which were resuspended in water as above, were additionally treated with an Ultra-Turrax T25 Basic disperser (IKA, Staufen, Germany) before the incubation period. The quality of the extracts was analyzed by SDS-PAGE and Coomassie brilliant blue (Bio-Rad) staining (26). Comparable amounts of the extracts were separated by 14% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell). Membranes were blocked with 50 mM sodium phosphate (pH 7.4), 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, and 0.05% (w/v) sodium azide and incubated with 1:1000 diluted rabbit anti-sera specific for Der p 2, Der p 23, or with the preimmune serum of the Der p 23-immunized rabbit overnight at 4°C. Bound Abs were detected with 1:1000 diluted ¹²⁵I-labeled donkey anti-rabbit Abs (GE Healthcare Bio-Sciences AB) and visualized by autoradiography (Kodak XOMAT film; Kodak).

House dust collection and ELISA—Dust samples were collected from the mattresses of 18 mite-allergic individuals. The collection was performed by vacuuming the surface of the mattress for 2 min using paper filters adjusted to a modified portable vacuum cleaner as described previously (33). The dust (100 mg) was shaken in 2 ml PBS overnight at 4°C, and insoluble material was removed by centrifugation (5 min, 4°C, 14,000 rpm).

ELISA plates (Nunc, Roskilde, Denmark) were then coated with the aqueous dust extracts (1:10 in PBS) and incubated with a 1:10,000 diluted rabbit anti-Der p 2 antiserum, a rabbit anti-Der p 23 antiserum or the preimmune serum from the Der p 23-immunized rabbit. Bound Ig Abs were detected with 1:2000 diluted HRP-conjugated donkey anti-rabbit Ig Abs (GE Healthcare Bio-Sciences) as described previously (29). The OD values obtained with the preimmune serum were subtracted from the values obtained with the immune sera. Displayed are mean OD levels of duplicate determinations corresponding to bound IgG Abs with a variation of <5%.

Results

Identification of a new HDM allergen, Der p 23, as a peritrophin-like protein

Several IgE-reactive cDNA clones were isolated from a *D. pteronyssinus* λgt11 cDNA library by IgE immunoscreening. Sequence analysis of the clone 30 cDNA showed that it coded for a protein comprising 90 aa with a calculated molecular mass of 10.34 kDa (Fig. 1A). Analysis of the deduced amino acid sequence revealed that the N-terminal sequence has characteristic features of a hydrophobic signal peptide required for membrane translocation. The putative cleavage site of the signal peptide was predicted to be located between aa 21 and 22 (34). The molecular mass of the mature protein calculated from the amino acid sequence is 7.98 kDa and the calculated isoelectric point 4.32. The clone 30-encoded allergen was submitted to the International Union of Immunological Societies Allergen Database and, according to the allergen nomenclature, was designated Der p 23. The clone 30-derived protein has been given the official name Der p 23 by the International Union of Immunological Societies allergen nomenclature subcommittee. The comparison of

the Der p 23 amino acid sequence with the sequences deposited in the National Institutes of Health protein database using the BLASTP program revealed similarity of the C terminus of Der p 23 with two conserved domains (Fig. 1B). Residues 45–89 of Der p 23 showed 35% identity to chitin-binding domain type 2 (ChtBD2; smart00494) and residues 47–90 were 36% identical with the chitin-binding peritrophin-A domain (CBM_14; pfam01607). CBM_14 is found in chitin-binding proteins particularly in peritrophic matrix (PM) proteins of insects and in animal chitinases (35–37). Within the homologous regions of the two domains, five conserved cysteines can be found, which are involved in chitin-binding, of which four were also present in Der p 23 (Fig. 1B) (38, 39). Between residues 29 and 41, the sequence of Der p 23 is rich in threonine (T) and proline (P) and thus strongly resembles proline, glutamate, serine and threonine (PEST)-like regions typical for insect chitinases (40) (Fig. 1A, underlined). PEST-like regions are often extensively glycosylated (41). In fact six threonines in the PEST-like region and one at the C terminus of Der p 23 may represent *O*-glycosylation sites according to sequence analysis and prediction (Fig. 1A).

Purification of recombinant Der p 23

The mature Der p 23 was expressed as recombinant nonfusion protein in the soluble fraction of *E. coli* and purified to homogeneity using ammonium sulfate precipitation and two different column chromatography steps. The purified allergen migrated at ~14 kDa in SDS-PAGE (Fig. 2A), but the analysis of the molecular mass by MALDI mass spectrometry showed that the protein had a molecular mass of 8 kDa, which corresponded to the molecular mass calculated from its amino acid sequence (Fig. 2B).

The secondary structure of Der p 23 was determined by far-UV circular dichroism analysis and indicated a predominantly random coil structure (data not shown). The circular dichroism results corresponded to the PSIPRED results based on the amino acid sequence, which predicted that Der p 23 contained five β -strands (23%) and predominantly random coil elements.

Der p 23 represents a new, major *D. pteronyssinus* allergen

The IgE binding frequency of Der p 23 was determined in three populations of European HDM allergic patients (Austria: $n = 67$; Italy: $n = 67$; France: $n = 55$). Fig. 3A shows the IgE-reactivity of dot-blotted rDer p 23 with 37 representative sera from the Austrian population. Seventy percent of the Austrian sera showed specific IgE-reactivity to Der p 23 (i.e., 47 of 67 sera), 80% of the French sera (i.e., 44 of 55), and 87% of the Italian sera (i.e., 58 of 67). In total, 149 of the 189 tested sera (i.e., 79%) showed specific IgE reactivity to Der p 23 demonstrating that Der p 23 represents a major *D. pteronyssinus* allergen. Serum from a non-allergic individual (N) as well as buffer without serum (BC) showed no IgE reactivity to Der p 23 (Fig. 3A, lanes N, BC). None of the individuals showed IgE reactivity to BSA (Fig. 3A).

Microarrayed rDer p 23 was also evaluated for chip-based IgE serology in the ISAC system (ThermoFisher/Phadia, Uppsala, Sweden) in a population of 158 HDM allergic patients. Fifty-eight percent of the 53 patients suffering only from allergic rhinoconjunctivitis and 72% of the 105 patients who also had asthma showed specific IgE reactivity to rDer p 23

(i.e., >0.3 ISAC standardized units [ISU]). The Der p 23-specific IgE levels measured for the 158 HDM allergic patients were of similar magnitude as those specific for the major HDM allergens, Der p 1 and Der p 2 (Fig. 3B–D). There was an association of specific IgE levels between Der p 23 and Der p 1 and Der p 2, respectively, but several patients reacted only with Der p 23, Der p 2, or Der p 1 (Fig. 3C, 3D). In fact, 6 of the 158 HDM allergic patients showed exclusive IgE reactivity to Der p 23 but not to any of the other tested HDM allergens (i.e., Der p 1, Der p 2, Der p 5, Der p 7, Der p 10, and Der p 21).

Der p 23 has high allergenic activity

To determine the allergenic activity of Der p 23, basophils from *D. pteronyssinus* allergic patients were incubated with increasing concentrations of nDer p 1 and rDer p 23 and the allergen-induced upregulation of the basophil activation marker CD203c was measured. Fig. 4 shows results from three representative patients. In these patients, Der p 23 was even more allergenic than Der p 1. Der p 23 induced dose-dependent upregulation of CD203c expression at concentrations between 0.001 and 0.1 µg/ml, whereas Der p 1 induced upregulation at concentrations between 0.001 and 1 µg/ml. No upregulation was obtained with buffer alone (unstimulated), whereas anti-IgE (positive control) induced CD203c upregulation in all patients (Fig. 4).

The allergenic activity of rDer p 23 has been demonstrated in basophil activation experiments for additional 10 patients (data not shown).

IgG Abs induced with Der p 23 block mite-allergic patients' IgE binding to the allergen

To evaluate whether Der p 23 can induce upon immunization protective Abs that block the binding of allergic patients' IgE to Der p 23, an ELISA competition assay was performed (Table I). ELISA plate-coupled Der p 23 was preincubated with rabbit antiserum raised against Der p 23 or with the preimmune serum and thereafter incubated with sera from 14 *D. pteronyssinus* allergic patients (Table I). For all patients with the exception of patient number 11, a strong inhibition of IgE binding of >70% was observed after preincubation with the rabbit Abs. In half of the patients, an inhibition of IgE binding of 90% or more was obtained (Table I).

Der p 23 is localized in the PM of *D. pteronyssinus*

Fig. 5 shows a schematic drawing of the digestive tract of the mite *D. pteronyssinus*. The digestive tract is divided into esophagus (ES), anterior and posterior midgut (AMg and PMg), colon (CO), rectum (RE), and anus (AN). Colon and rectum are equivalent to anterior and posterior hindgut as defined by other authors (42, 43). Because the major *D. pteronyssinus* allergens Der p 1 and Der p 2 are derived from the digestive tract of the mite and concentrated in the feces (9, 44), ultrathin *D. pteronyssinus* sections of the midgut, hindgut, and the fecal pellets were incubated with IgG Abs specific for Der p 23. Bound Abs were detected with Abs coupled to colloidal gold particles by TEM. Der p 23 is synthesized in the midgut epithelial cells where the allergen was localized in vesicles (Fig. 6A, 6B). In this part of the midgut the long microvilli of epithelial cells are partially adhering to the forming PM. The digested food stuff is placed loose and more centrally in the stomach (Fig. 6A). In the hindgut, the food stuff is enveloped by the PM and subsequently packed in the

colon to form a fecal pellet (Fig. 7A, 7B). In fact, Der p 23 was found in the PM surrounding the fecal pellet (Fig. 7C). Lower concentrations of Der p 23 were also detected in the microscopic lighter parts of the digested material of the fecal pellet (Fig. 7C). Fig. 7D shows the hindgut after incubation with control Abs and no unspecific binding of the Abs was detectable. Defecated fecal pellets (Fig. 8A) were incubated with IgG Abs specific for Der p 23 and Der p 23 was again detected in high concentrations on the surface of the fecal pellet in the PM (Fig. 8B).

Der p 23 is present in feces of *D. pteronyssinus* and in house dust samples

To compare the occurrence of Der p 23 with that of the major mite allergen, Der p 2, extracts from the feces of *D. pteronyssinus* were prepared. Whereas large amounts of Der p 2 were eluted from the feces upon hydration, only tiny amounts of Der p 23 were eluted, which were increased when the aqueous extract was treated mechanically with an Ultraturrax (Fig. 9A). The comparison of rDer p 23 (Fig. 2) and natural Der p 23 (Fig. 9A) showed that both proteins exhibited comparable apparent molecular weights indicating that the natural protein does not contain extensive post-translational modifications.

Harsh treatment of the feces by boiling in SDS-sample buffer and by mechanical treatment, which also eluted water insoluble proteins from the feces, further increased the amount of extracted Der p 23 (data not shown). However, the overall amount of Der p 23 in feces from *D. pteronyssinus* appeared to be considerably lower than that of Der p 2. The preimmune serum of the Der p 23-immunized rabbit did not show reactivity with any of the feces extracts (Fig. 9A). Allergen extracts which were obtained from the bodies of *D. pteronyssinus* contained large amounts of Der p 2, but no Der p 23 could be detected (data not shown).

We also investigated the presence of Der p 23 in house dust samples collected from mattresses of 18 HDM allergic patients (Fig. 9B). Interestingly, Der p 23 was detected in each of the dust samples, albeit at considerably lower levels than Der p 2 in 16 of the 18 samples (Fig. 9B).

Discussion

In search for new and important HDM allergens, we chose a discovery approach that was based on the screening of an expression cDNA library constructed from the HDM *D. pteronyssinus* with IgE from *D. pteronyssinus* allergic patients. One of the isolated IgE-reactive cDNA clones coded for a novel, so far unknown HDM allergen with a molecular mass of 8 kDa, which was submitted to the International Union of Immunological Societies allergen nomenclature subcommittee and designated Der p 23. Interestingly, Der p 23 showed homology at the C terminus to the chitin-binding domain type 2 and the chitin-binding peritrophin-A domain. Chitin-binding domains were also identified in two other mite allergens, Der p 15 and Der p 18 (45). Der p 15 contains each of the three domains typical for insect chitinases (family 18 chitinases), an N-terminal glycosyl hydrolase catalytic region, a PEST-like domain, which is rich in amino acids proline (P), glutamate (E), serine (S), and threonine (T) and a C-terminal chitin-binding domain (46). Der p 18 has a truncated N-terminal glycosyl hydrolase catalytic region and the C-terminal chitin-binding

domain but lacks the PEST-like region (45). However, Der p 23 is different from the two allergens because it lacks a catalytic region but possesses a PEST-like region and a chitin binding peritrophin-A domain. In fact, we also did not find any evidence for cross-reactivity between Der p 23, Der p 15, and Der p 18 when tested with patients' IgE or rabbit antisera raised against these allergens, confirming that Der p 23 represents a new allergen (Y. Resch and S. Vrtala, unpublished observations).

The in situ localization of Der p 23 by immunogold electron microscopy on ultrathin mite sections with allergen-specific Ab probes showed that Der p 23 is a protein of the peritrophic matrix that lines the midgut and surrounds the digested food stuff and the fecal pellets. In mites, as in most *Arthropods*, fecal pellets are surrounded by the peritrophic matrix. The peritrophic matrix is synthesized along the whole midgut of the HDMs and functions as a permeability barrier between the ingested food and midgut epithelium. It enhances digestive processes and protects the midgut epithelium from mechanical disruption as well as from attacks by toxins and pathogens (35, 47). The peritrophic matrix is thought to be secreted by the microvilli of gut epithelial cells and is composed of a meshwork of chitin fibrils embedded in a matrix of proteins, proteoglycans, and glycoproteins (40). In fact, the in situ localization suggests that Der p 23 is indeed synthesized in the midgut epithelial cells where the allergen is localized in vesicles.

Peritrophins are the most abundant proteins present in the peritrophic matrix (i.e., 35–55%) (48). They are thought to link chitin microfibrils via their chitin-binding domains and mediate binding to other glycoproteins (40).

Taken together, the results of the sequence analysis and of the in situ localization data, Der p 23 shows typical features of peritrophins (i.e., Der p 23 is localized in the peritrophic matrix), it contains a peritrophin-A domain and a PEST-like region in its sequence, it resembles an acidic isoelectric point and exhibits an extended structure caused by the amino acids proline and threonine and therefore migrates at higher molecular mass in SDS-PAGE (35), and it shows a strong interaction with the peritrophic matrix (48). In this context, we found that Der p 23 was not easily eluted from mite feces with physiological buffers (Fig. 9A). We therefore suggest that Der p 23 is a peritrophin-like protein or a peritrophin.

The close association of Der p 23 with fecal pellets may be one reason why it represents a major allergen and exhibits high allergenic activity. In fact, we determined the IgE binding frequency of Der p 23 using 347 sera from well characterized HDM allergic patients from different countries using two different technologies (i.e., classical RAST-based testing and ImmunoCAP ISACmicroarray technology) and consistently found that >70% of the patients showed specific IgE reactivity to rDer p 23. The frequency of IgE recognition in patients with HDM-induced rhinoconjunctivitis and atopic dermatitis was similar whereas it was higher in patients suffering from HDM-induced asthma (data not shown).

The Der p 23-specific IgE levels of the tested patients were comparable to the IgE levels to Der p 1 and Der p 2, which are known as major HDM allergens with high clinical relevance. Furthermore, basophil activation assays performed with basophils from HDM allergic patients showed that Der p 23 has high allergenic activity. In certain patients, Der p 23–

induced basophil activation even at >10-fold lower concentrations than the major allergen, Der p 1. Thus, Der p 23 represents one of the most important HDM allergens besides Der p 1 and Der p 2.

In fact, mite feces are a major source of HDM allergens, and it is supposed that sensitization to HDMs occurs primarily via fecal pellets (8). Fecal pellets are 10–40 µm in diameter and can therefore be deposited not only on the mucosa of the upper respiratory tract but also enter the lung (8, 49). Interestingly, the mechanism of release of Der p 1 and Der p 2 from fecal pellets seems to be different from that of Der p 23. Der p 1 and Der p 2 elute rapidly from the fecal pellets in aqueous solutions (8), whereas Der p 23 elutes slowly and in smaller amounts. However, it is possible that Der p 23 may directly activate the patient's immune system such as Der p 1 and Der p 2 (11-13), because it has been reported recently that peritrophins can directly activate the innate immune system (50). Although only small amounts of Der p 23 are present in fecal pellets and in house dust, the low exposure is sufficient to sensitize HDM allergic patients and to induce specific IgE levels which are comparable to the major allergens. Furthermore, several patients were sensitized only to Der p 23 but not to Der p 1 or Der p 2. These findings indicate that Der p 23 is a very potent allergen.

Because of its high frequency of IgE recognition and allergenic activity, Der p 23 must be considered as an essential component for the diagnosis and specific immunotherapy of HDM allergy.

Regarding immunotherapy, several new molecular strategies relying on defined recombinant allergens, hypoallergenic derivatives and peptides, which allow a selective targeting of the patient's immune system have been shown to be clinically effective (51, 52). In fact, it is known that a mix of Der p 1 and Der p 2 allows diagnosing the majority of HDM allergic patients (30). However, a molecular or epitope mixture for specific immunotherapy certainly needs to include Der p 23 because of its high allergenic activity, its high frequency of recognition and due to the high levels of IgE-antibodies directed to this allergen.

Der p 23 therefore must be considered as an important allergen for the diagnosis and specific immunotherapy of HDM allergy.

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Abbreviations used in this article

CBM_14	chitin-binding peritrophin-A domain
ChtBD2	chitin-binding domain type 2
HDM	house dust mite
ISAC	immuno solid phase allergen chip

ISU	immuno solid phase allergen chip standardized unit
MFI	mean fluorescence intensity
PBST	PBS, 0.05% (v/v) Tween 20
PEST	proline, glutamate, serine and threonine
PM	peritrophic matrix
TEM	transmission electron microscopy

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A

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3 atatcttctgcttgctttttgaaa ATG AAA TTC AAC ATA ATC ATC ca
      M K F N I I I 7
47 GTT TTT ATT TCG TTG GCC ATT TTG GTC CAT TCA TCA TAT
      V F I S L A I L V H S S Y 20
86 GCC GCC AAT GAT AAT GAT GAT GAT CCT ACC ACA ACC GTT
      A A N D N D D D P T* T* T* V 33
125 CAT CCA ACA ACA ACC GAA CAA CCA GAT GAT AAA TTT GAA
      H P T* T* T* E Q P D D K F E 46
164 TGT CCA AGT AGA TTT GGT TAT TTT GCC GAT CCA AAA GAT
      C P S R F G Y F A D P K D 59
203 CCA CAT AAA TTT TAT ATC TGT TCA AAT TGG GAA GCT GTA
      P H K F Y I C S N W E A V 72
242 CAT AAA GAT TGT CCA GGT AAT ACA CGA TGG AAT GAA GAT
      H K D C P G N T R W N E D 85
281 GAA GAA ACA TGC ACT TAAtaatgcaataaaattatgatttattatg
      E E T C T*
327 gtaattcataaatcaacgtttcaacaaaaaatcataaatttttattccaata
379 aattcatttttatggttgattacatgcttgtcaattttattacaaaataata
431 aaattatttatttacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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B

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D.p 23 1 MKFNIIIVFI SLAILVHSSY AANDNDDDPT TTVHPTTTEQ PDDKFEECPSR 49
ChtBD2 1 -----
D.p 23 50 -FGYFADPKD PHKFYICSNW EAVHKDCPGN TRWNEDEETC T 90
ChtBD2 7 GDGLYPHPTD CSKYYQCSNG RPIVMSCPAG LVFDPATQTC - 46

D.p 23 1 MKFNIIIVFI SLAILVHSSY AANDNDDDPT TTVHPTTTEQ PDDKFECPSR 49
CBM_14 1 -----
D.p 23 50 -FGYFADPKD PHKYICSNW EAVHKDCPGN TRWNEDEETC T 90
CBM_14 5 PDGLYADPGD CSKYYVCSNG QAVVFTCPAG LYFDPELGIC D 45

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FIGURE 1.

Nucleotide and deduced amino acid sequence of Der p 23 and comparison with chitin-binding domains. (A) 5' and 3' noncoding sequences are printed in lowercase letters, sequences representing the start and stop codons are in bold, and the predicted cleavage site of the signal peptide (between aa 21 and 22) is in italic. The sequence with similarity to PEST-like sequences is underlined and putative O-glycosylation sites are indicated by asterisks. The numbers on the left side indicate the numbers of the nucleotides and those on the right side the amino acid numbering. (B) The Der p 23 amino acid sequence was aligned with the chitin-binding domain type 2 (ChtBD2) and the chitin-binding peritrophin-A

domain (CBM_14). Identical amino acids between Der p 23 and the two domains were displayed in rectangular boxes. Dashes within the sequences represent gaps.

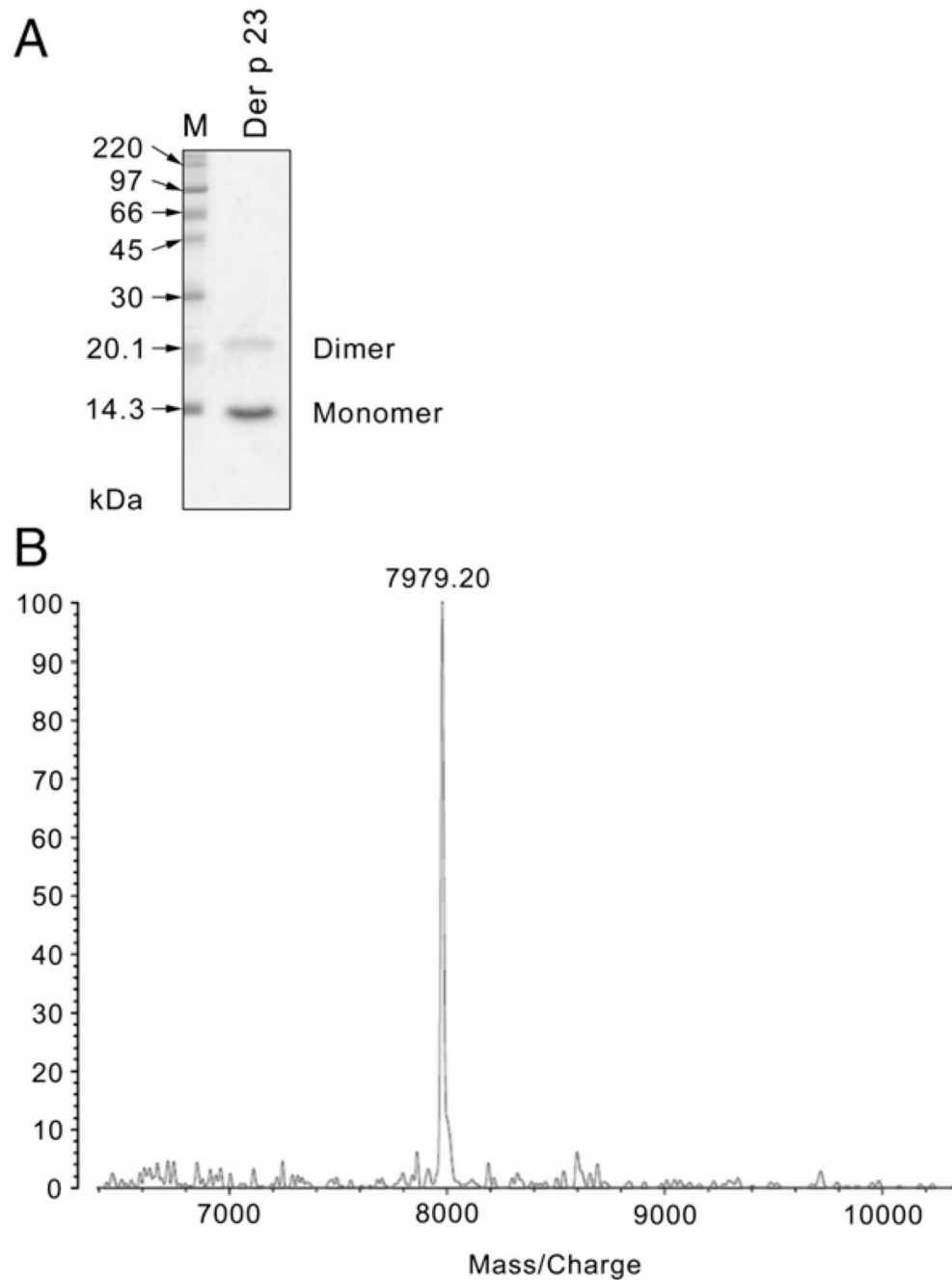
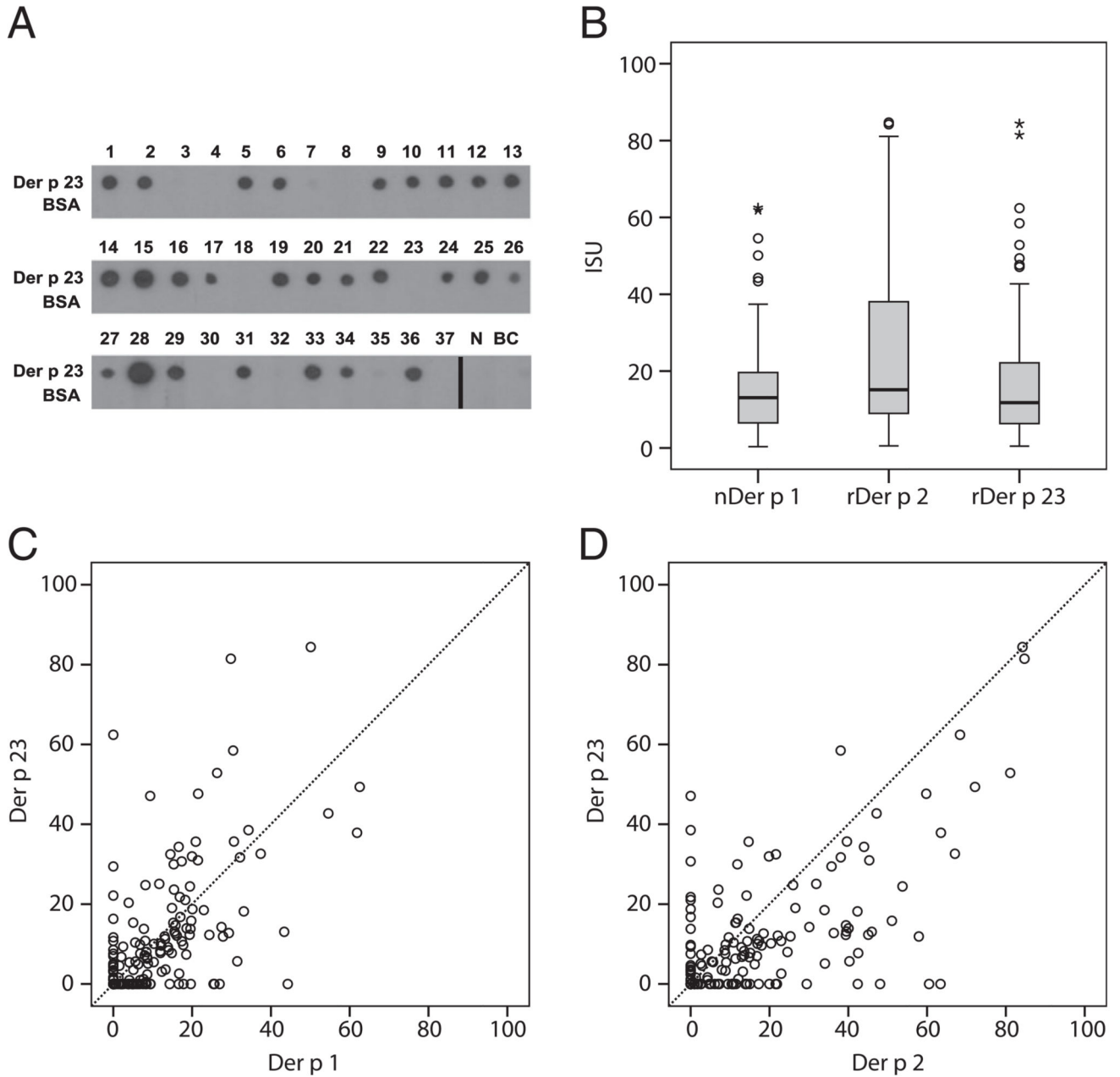


FIGURE 2.

Analysis of purified recombinant Der p 23. **(A)** A molecular mass marker (M) and 3 μ g of the purified protein (Der p 23) were separated by 14% SDS-PAGE and stained with Coomassie brilliant blue. **(B)** The mass spectrometry analysis of the purified allergen shows the mass/charge ratio on the x-axis and the signal intensity is displayed on the y-axis as the percentage of the most intense signal obtained in the investigated mass range.

**FIGURE 3.**

IgE reactivity of Der p 23. **(A)** Der p 23 and BSA as control were dotted onto nitrocellulose strips and incubated with sera from *D. pteronyssinus* allergic patients (1-37), from one nonallergic individual (N) and buffer alone (BC). Bound IgE Abs were detected with ^{125}I -labeled anti-human IgE Abs and visualized by autoradiography. **(B)** Levels of specific IgE Abs to nDer p 1, rDer p 2, and rDer p 23 (ISU per milliliter) from 158 HDM-allergic patients are displayed as box plot diagram where 50% of the values are within the boxes and nonoutliers between the bars. Lines within the boxes indicate the median values. Open circles and asterisks indicate outliers and extreme values. **(C and D)** Association of Der p

23-specific IgE levels (y-axes) (ISU/ml) from 158 HDM allergic patients with Der p 1 (C) or Der p 2 (D)-specific IgE levels (x-axes).

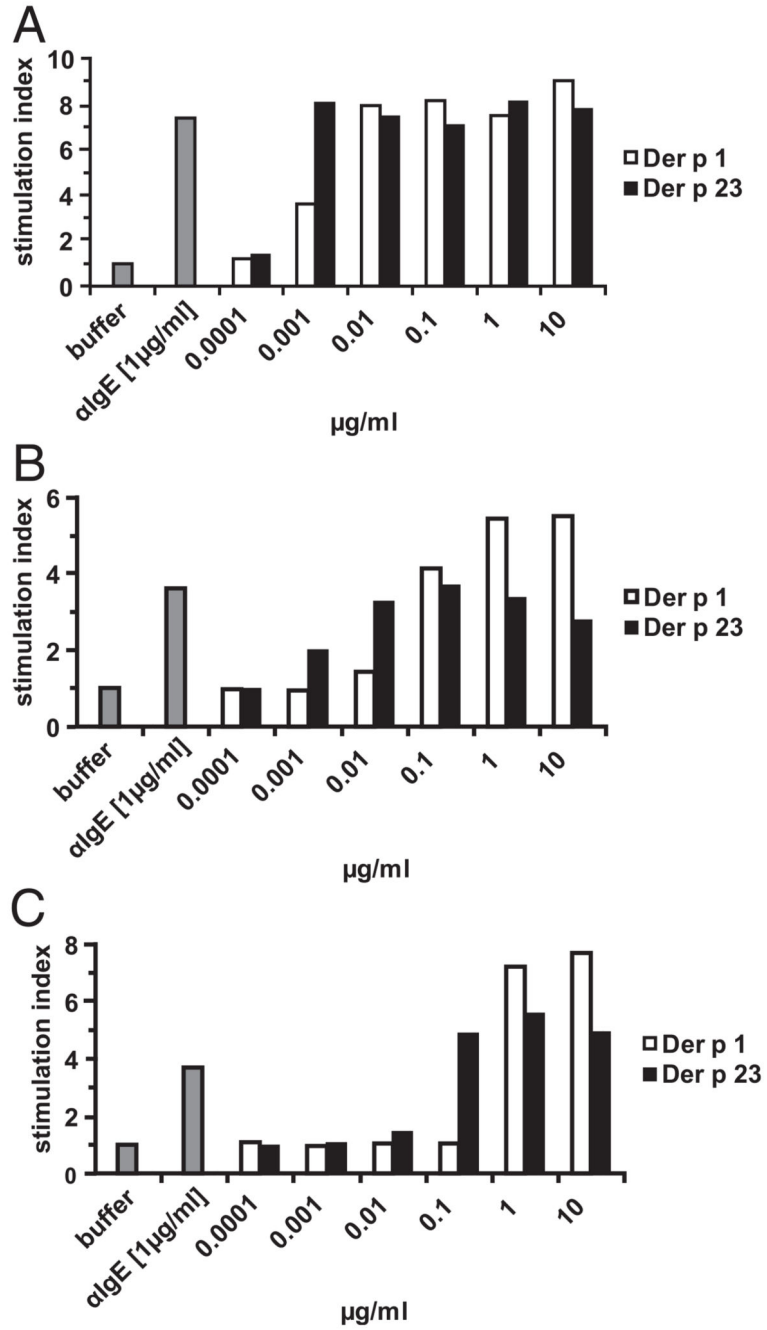


FIGURE 4. Allergenic activity of Der p 23 determined by basophil activation assays. Blood samples from three mite-allergic patients (A–C) were incubated with 0.0001–10 µg/ml nDer p 1 and rDer p 23, 1 µg/ml anti-IgE, or buffer (x-axes). Allergen- or anti-IgE-induced upregulation of expression of CD203c on basophils is displayed as stimulation index (y-axes).

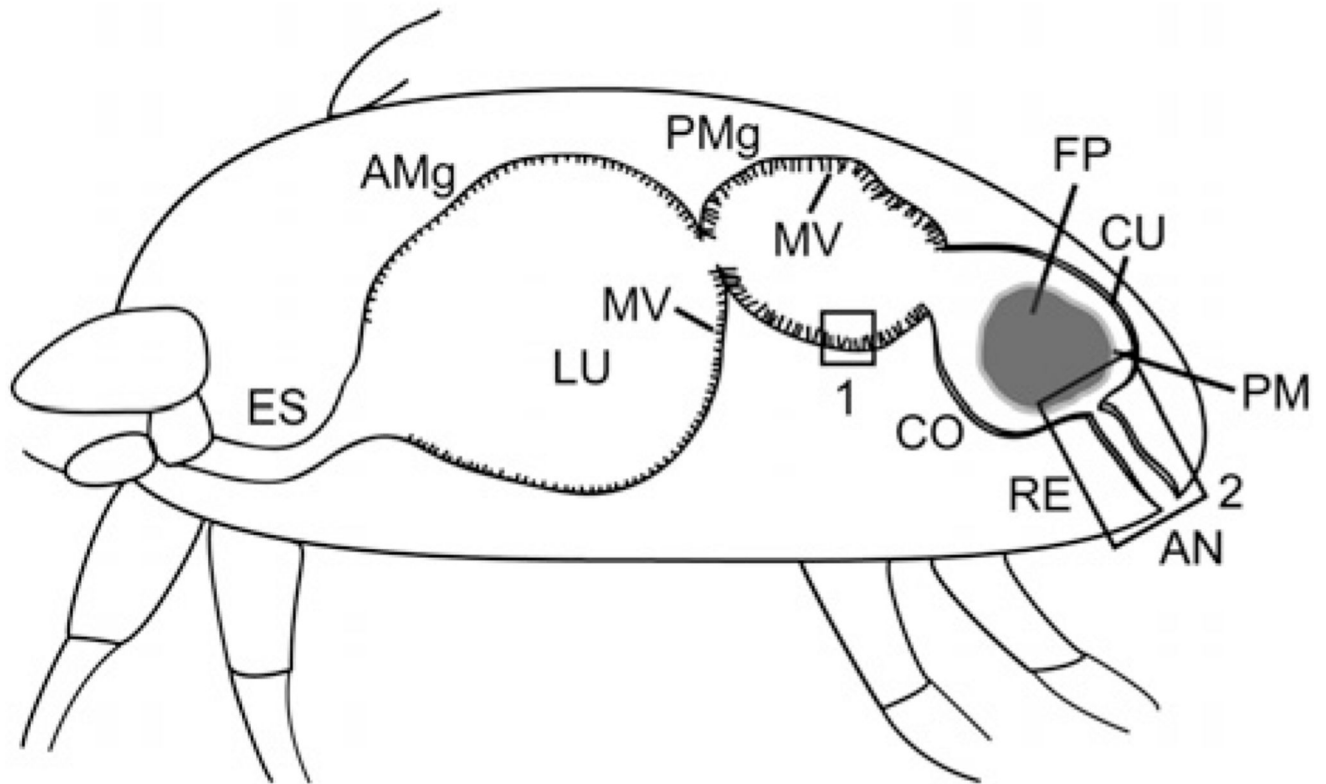
**FIGURE 5.**

Illustration of the digestive tract of the HDM *D. pteronyssinus*. Sections of the midgut and the hindgut with a fecal pellet are represented. The areas delineated in the box (1) and the rectangle (2) are shown in Figs. 6A and 7A, respectively, at higher magnifications. AMg, Anterior midgut; AN, anus; CU, cuticle; CO, colon; ES, esophagus; FP, fecal pellet; LU, lumen; MV, microvilli; PM, peritrophic matrix; PMg, posterior midgut; RE, rectum.

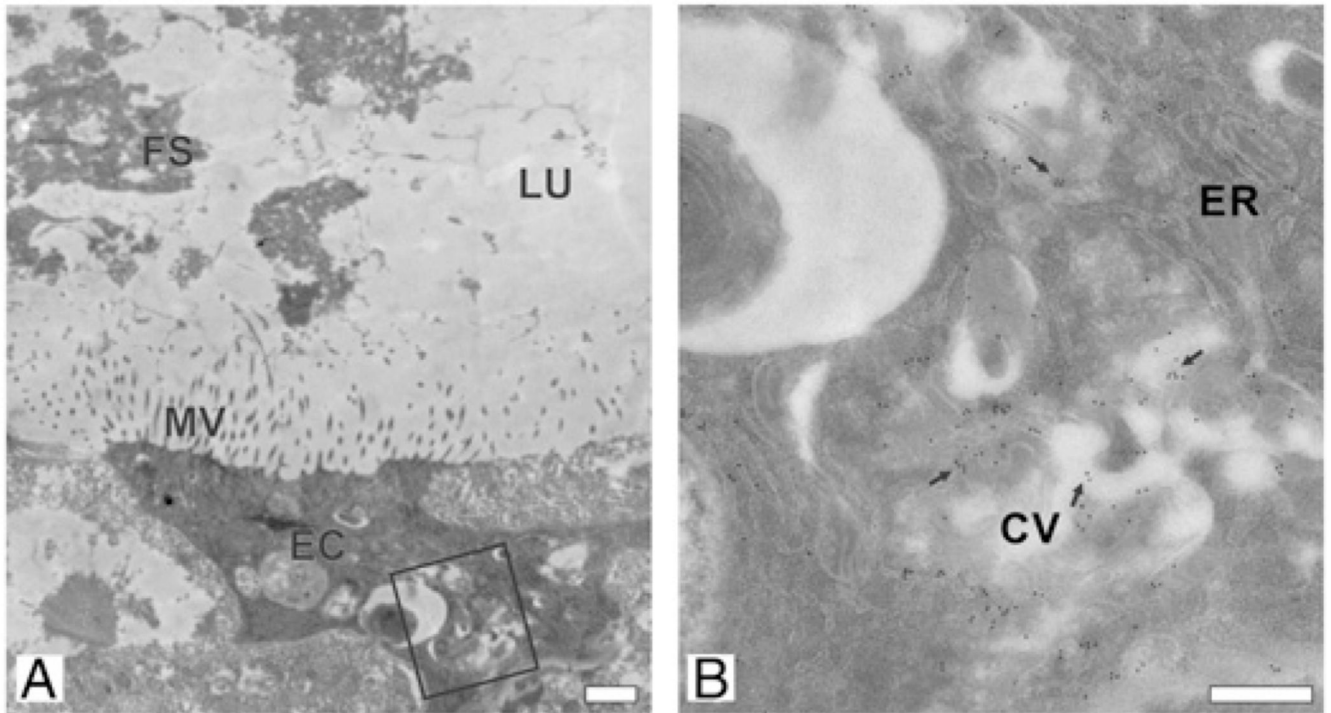


FIGURE 6.

TEM micrograph from the midgut of *D. pteronyssinus*. (A) A midgut epithelial cell (EC) with long microvilli (MV) and digested food stuff (FS) in the gut lumen (LU) is shown. The area delineated in the rectangle is shown in (B) at higher magnification. (B) In the epithelial cell, Der p 23 is localized (gold particles: arrows) in the endoplasmatic reticulum (ER) and in high concentrations in complex vacuoles (CV). Scale bars, 1 μm (A); 0.2 μm (B).

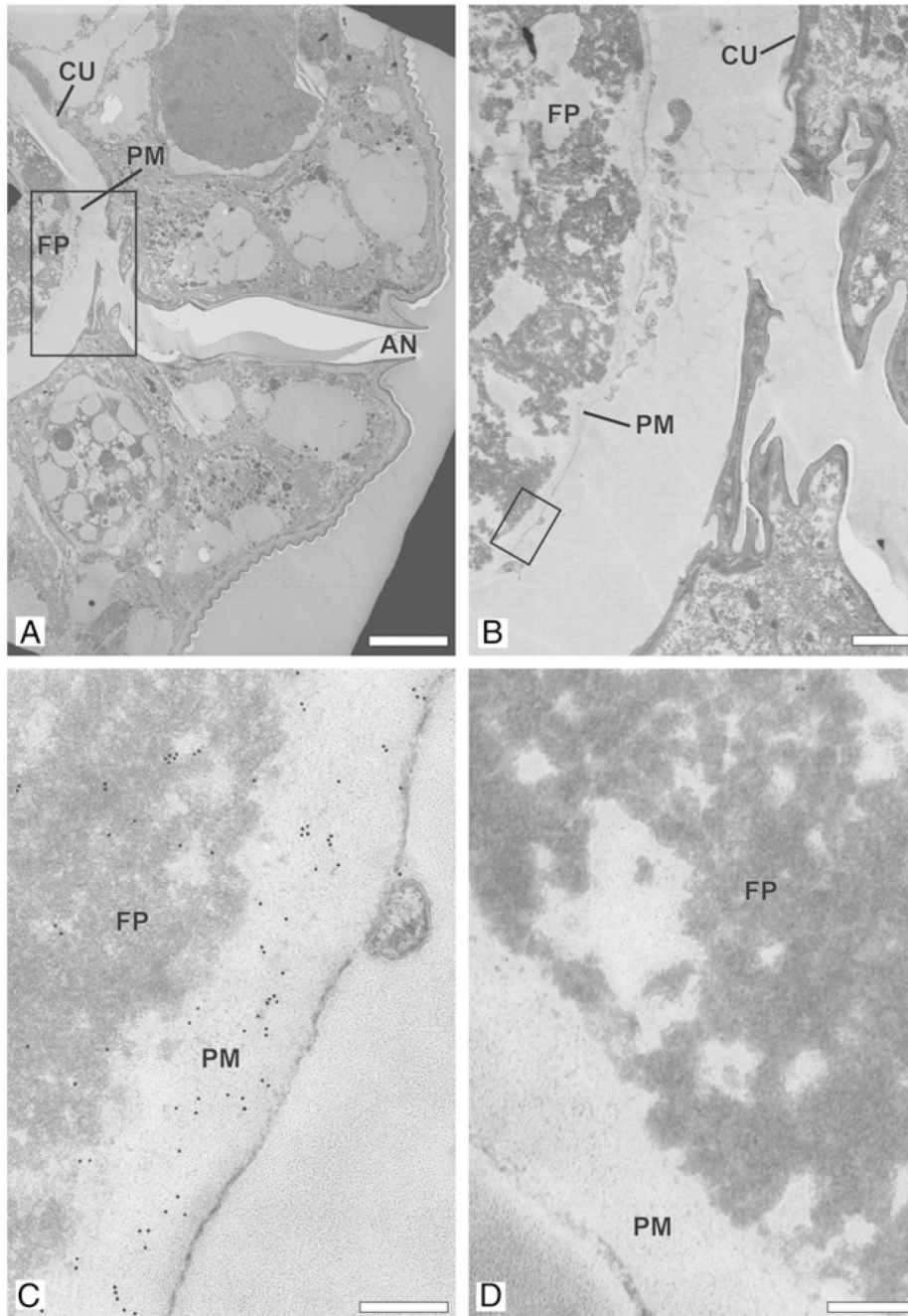
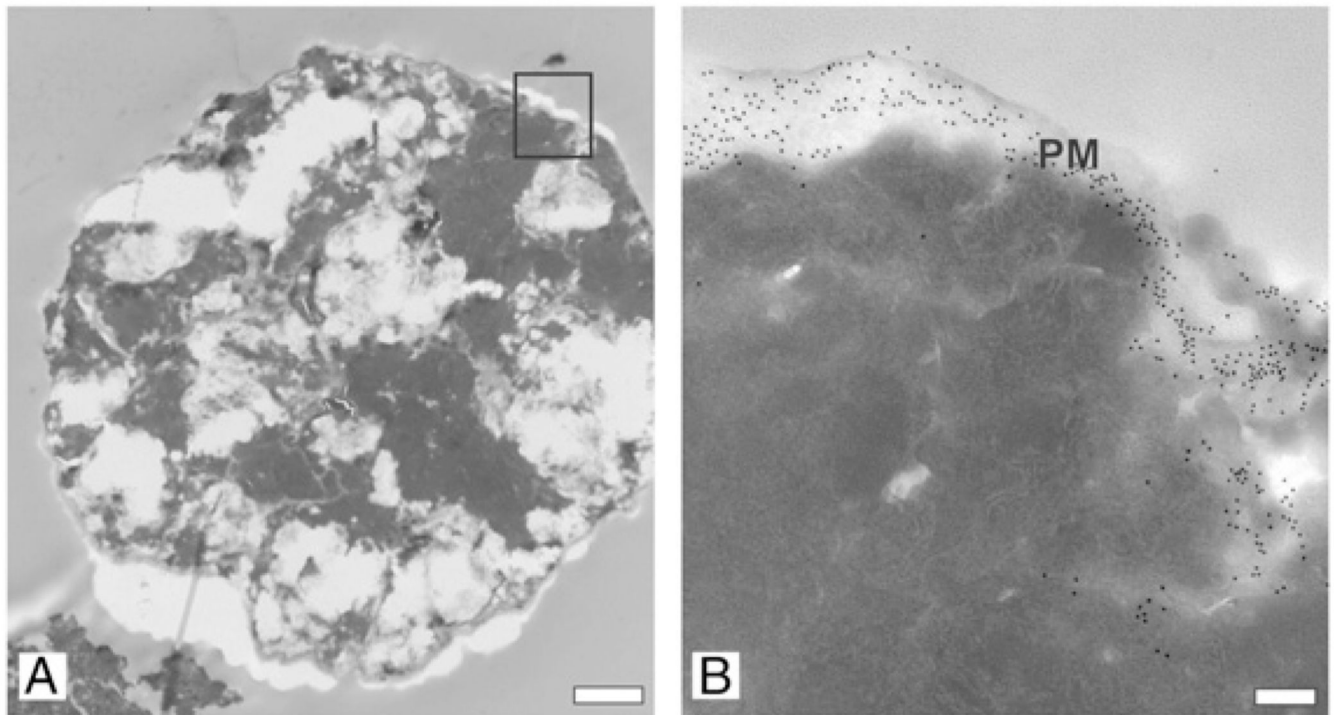


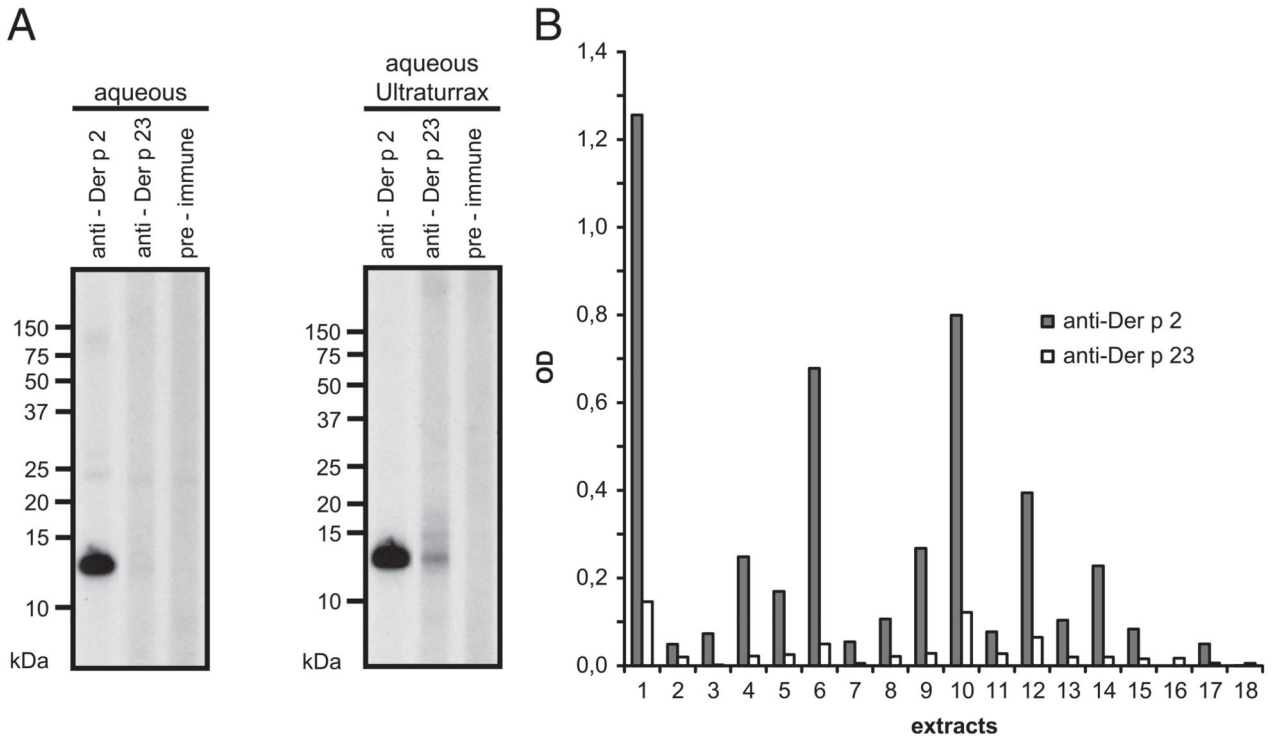
FIGURE 7.

Electron micrograph from the hindgut of *D. pteronyssinus* with colon, rectum, and anus. (A) Section of the hindgut is filled with a fecal pellet (FP) that is surrounded by the peritrophic matrix (PM). The cuticle (CU) is lining the hindgut with the anus (AN). (B) Magnification of the area demarcated by the rectangle in (A) is shown. The FP is enveloped by the PM and the hindgut is lined by the CU. (C) Magnification of the area demarcated by the rectangle in (B) is shown. Localization of Der p 23 by anti-Der p 23 Abs and colloidal gold-coupled

secondary Abs in the PM and the FP is shown. **(D)** Control Abs show no binding to the hindgut. Scale bars, 10 μm (A); 1 μm (B); 0.25 μm (C); 0.25 μm (D).

**FIGURE 8.**

Electron micrograph from a fecal pellet from *D. pteronyssinus*. (A) The fecal pellet is composed of dense and light material that is surrounded by the peritrophic matrix. The area delineated in the rectangle is shown in (B) at higher magnification. (B) Gold particles indicating the localization of Der p 23 in the fecal pellet are predominantly found in the PM and in association with light parts of the pellet material. Scale bars, 2 μm (A); 0.2 μm (B).

**FIGURE 9.**

Detection of Der p 23 in house dust samples and in feces from *D. pteronyssinus*. **(A)** Nitrocellulose-blotted extracts obtained from feces by aqueous elution or mechanical treatment were incubated with rabbit anti-sera raised against Der p 2 and Der p 23 and with the preimmune serum of the Der p 23-immunized rabbit (preimmune). Bound IgG Abs were detected with ^{125}I -labeled anti-rabbit Abs and visualized by autoradiography. Molecular weights are indicated in kilodaltons at the left margins. **(B)** Dust samples (1–18) were incubated with rabbit anti-sera raised against Der p 2 or Der p 23 and with the preimmune serum from the Der p 23-immunized rabbit. The ODs corresponding to the levels of bound Abs are displayed on the y-axis after deduction of the OD values obtained for the preimmune serum.

Table I
Rabbit anti-Der p 23 IgG Abs inhibit IgE binding from mite-allergic patients' sera to Der p 23

Preincubation with	Patient No.														Mean
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Preimmune serum (OD values)	0.569	1.14	0.451	0.982	2.52	0.628	0.79	0.347	0.484	0.269	0.553	1.333	1.638	1.038	0.91
Anti-Der p 23 serum (OD values)	0.06	0.083	0.07	0.1155	0.22	0.039	0.071	0.101	0.029	0.078	0.415	0.047	0.362	0.137	0.133
Percent inhibition of IgE binding	90	93	85	84	91	94	91	71	94	71	25	97	78	87	82