

Characterization of the allergens of the house dust mite *Euroglyphus maynei*

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Background: The house dust mite *Euroglyphus maynei* inhabits homes in many parts of the world and is the source of many allergens.

Objective: The purpose of this study was to biochemically and immunologically characterize the major allergens of *E. maynei*.

Methods: Proteins in an extract of *E. maynei* were separated into 20 fractions by using preparative isoelectric focusing. Most proteins and allergens were contained in fractions 7 to 17 with pH of 4.8 to 8.0. The fractions were further characterized by nonreducing sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting with the serum of 16 individuals sensitive to *E. maynei*.

Results: Molecular weights and isoelectric points were assigned to 47 IgE-binding proteins in an *E. maynei* extract, and the frequency of IgE binding to each allergen was determined. Twenty-two of the allergens were recognized by more than 50% of the 16 sera, and all but one of the subjects had IgE that bound to more than 10 allergens (range, 0 to 32).

Conclusion: *E. maynei* is the source of at least 47 individual allergens that have been characterized by molecular weight, isoelectric point, and IgE-binding properties. (J Allergy Clin Immunol 1997;100:222-8.)

Key words: *Euroglyphus maynei*, house dust mite, allergens, characterization

The house dust mite *Euroglyphus maynei* is common in homes in England, the southern United States, and other parts of the world.¹⁻⁸ *E. maynei* mites are usually found in homes that are also inhabited by the mites *Dermatophagoides farinae*, *D. pteronyssinus*, and *Blomia tropicalis*.¹ *E. maynei* is the source of multiple potent species-specific allergens and allergens that are cross-reactive with *D. farinae*, *D. pteronyssinus*, and *B. tropicalis*.⁹⁻¹³ Therefore the allergenicity of *E. maynei* and the cross-reactivity between *E. maynei* and other mite species are important considerations for diagnosis of mite-induced allergy and use of immunotherapy.

Using crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE), we pre-

Abbreviations used

CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmuno-electrophoresis
IEF:	Isoelectric focusing
MW:	Molecular weight
pI:	Isoelectric point
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

viously determined that *E. maynei* is the source of at least 33 antigens, 15 of which are allergens.⁹ Ninety-one percent of the reference patients showed serum IgE directed at three or more of the 15 allergens. Of the patients tested, 84%, 78%, 56%, and 50% had IgE binding to allergens 33, 4x, 2x, and 6x, respectively. The major allergens have not been biochemically and immunologically purified and characterized. Characterization and purification of the major *E. maynei* allergens are required for the development of specific allergenic assays that are needed for: (1) clinical evaluation of patients, (2) quantitation of *E. maynei* in house dust, and (3) determination of the relationship between the allergens of *E. maynei* and the allergens of other mite species.

The purpose of this study was to biochemically and immunologically characterize the major allergens of *E. maynei*. The molecular weight (MW), isoelectric point (pI), and IgE binding properties of 47 allergens of *E. maynei* were determined.

METHODS

Mite extracts

Pure *D. farinae*, *D. pteronyssinus*, and *E. maynei* mites were harvested from laboratory cultures, killed by freezing, and then dried. The dried mite bodies were ground, defatted, extracted 1:10 (wt/vol) in glass distilled water, and centrifuged; the supernatant (extract) was filter-sterilized and stored at 4°C until used as previously described.^{9, 14, 15} Protein concentrations were determined by the Bradford method.¹⁶

A second *E. maynei* extract was prepared in the presence of various protease inhibitors.¹⁷ Dried, ground, and defatted mites were extracted 1:10 (wt/vol) as described above except that the extracting solution contained 1% (vol/vol) aprotinin, 1 µg/ml pepstatin A, 0.1 mmol/L leupeptin, 100 µg/ml bacitracin, and 1 mmol/L phenylmethylsulfonyl fluoride in Dulbecco's phosphate-buffered saline (Sigma Chemical Co., St. Louis, Mo.).

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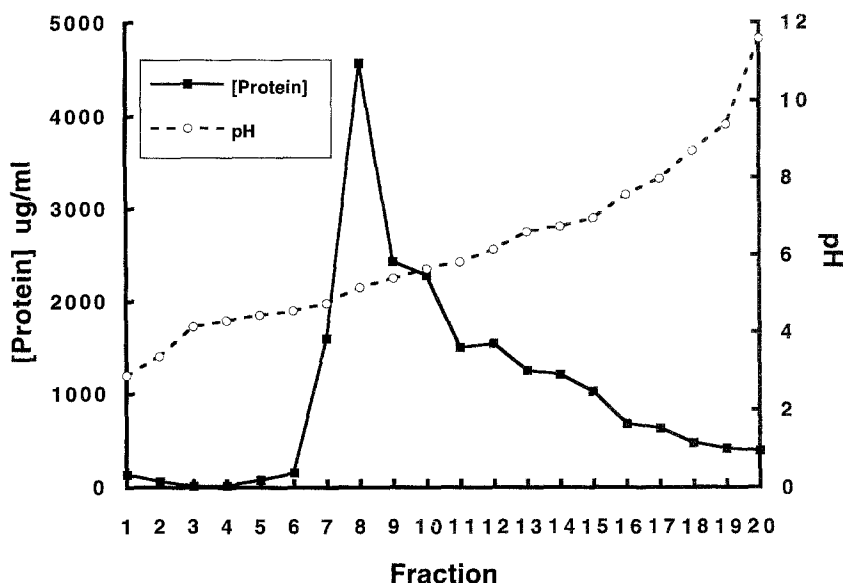


FIG. 1. Protein concentration and pH of 20 fractions obtained from preparative IEF of an extract of *E. maynei* whole bodies.

Antisera

Antisera to *D. farinae*, *D. pteronyssinus*, and *E. maynei* were produced previously by immunizing New Zealand White rabbits.^{9, 14, 15}

Human sera

Human sera were obtained from 12 patients in Tampa, Florida, an area where *E. maynei* is prevalent.^{1, 6} All sera were previously determined to have circulating IgE directed at *E. maynei*.^{9, 10} Four sera were obtained from residents of Barbados whose exposure to *E. maynei* was confirmed by the identification of this mite in dust samples collected from their homes. None of the subjects in this study had received immunotherapy with any species of house dust mite before the collection of serum. A reference serum pool was also prepared by using serum from 17 individuals with previously confirmed *E. maynei* sensitivity.⁹ The pooled serum had total IgE greater than 1000 U/ml and was RAST class V to both *D. farinae* and *D. pteronyssinus*.

Preparative isoelectric focusing (Rotofor fractionation)

Whole *E. maynei* extract was subjected to preparative isoelectric focusing (IEF) in a Bio-Rad Rotofor apparatus (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions. The cell was loaded with 48 mg of *E. maynei* protein in a solution that contained BioLyte 3/10 (2% wt/vol final) and 5% (vol/vol) glycerol. Focusing was performed at 5°C for 5 hours at 12 W constant power. Proteins were separated into 20 fractions, and the pH and protein content of each fraction were determined.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed to determine the MWs of proteins and IgE-binding allergens contained in the whole *E. maynei* extract and in the different fractions obtained by Rotofor fractionation. Electrophoresis was carried

out on 12% acrylamide gels under nonreducing (no reducing agent added to sample buffer) conditions by using the method of Laemmli.¹⁸ To ensure uniformity, gels were prepared in large batches by using a Bio-Rad multi-gel casting chamber. Proteins on representative gels were visualized by silver staining.^{19, 20} SDS-PAGE-resolved proteins were also subsequently electrophoretically transferred to Pro-Blott polyvinylidene difluoride membrane (Applied Biosystems, Foster City, Calif.) in 10 mmol/L 3-(cyclohexylamino)-l-propanesulfonic acid, 10% methanol, pH 11 according to the membrane manufacturer's recommendations. Blots were blocked overnight with a solution composed of 8 mol/L urea, 0.1 mol/L 2-mercaptoethanol, and 5 mg/ml bovine serum albumin.^{19, 20} After blocking, blots were washed three times for more than 5 minutes each with 100 ml of phosphate-buffered saline with 0.5% Tween-20. This washing procedure was repeated after each incubation step. Each blot was incubated overnight at room temperature in 2 ml of serum diluted to 50 ml with 0.1 mol/L phosphate buffer, pH 7.5, with 2 mg/ml bovine serum albumin and 1% Tween-20 (RAST buffer). IgE-binding allergens were visualized as previously described^{19, 20} by incubating blots with 1.3 million cpm iodine 125-labeled anti-human-IgE (Kallestad Allergoat RAST isotope reagent; Sanofi Diagnostics Pasteur, Chaska, Minn.) diluted to 50 ml with buffer. Blots were washed, dried, and placed in cassettes with tungsten-enhancement screens and Kodak X-OMAT LS film at -80°C. Films were exposed for 1, 3, 7, or 10 days. To standardize and to make comparisons, all blots were processed identically in one batch with one lot of reagents.

After autoradiography, IgG antibody binding to the blots was detected by using peroxidase-labeled goat anti-human-IgG (Fisher Scientific, Pittsburgh, Pa.) at 1:2000 in RAST buffer. All blots were developed for 5 minutes by using the 4-chloronaphthol/3,3'-diaminobenzidine/H₂O₂ chromogenic substrate of Young.²¹

A representative blot was also probed with the mouse monoclonal antibodies 1D8, 7A1, and α DpX (obtained from Dr. Martin D. Chapman, University of Virginia, Charlottesville) to identify group 2 allergens.²² Murine antibody was diluted 1:400 in RAST buffer and applied to the blot for 3 hours

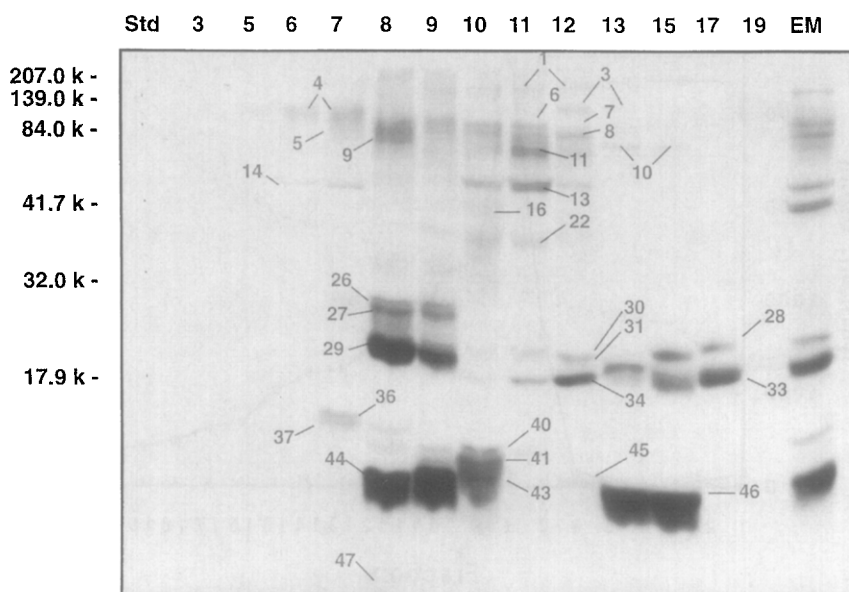


FIG. 2. Autoradiogram of SDS-PAGE-separated IEF fractions. Proteins were subjected to 12% nonreducing SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was probed with the reference serum pool. IgE-binding allergens were detected by using ^{125}I -anti-human IgE.

at room temperature. Antibody binding was detected by using alkaline phosphatase-labeled goat anti-mouse-IgG (Fisher Scientific) at 1:2000, followed by development for 30 minutes with Biomed 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium chromogen (Fisher Scientific). This yielded a purple precipitate that was clearly distinguishable from the red-brown bands produced in the human IgG development reaction.

CIE

CIE was performed as previously described.^{9,10,14,15} Fifteen microliters of mite extract was electrophoresed in the first dimension 1% agarose gel for 30 minutes at 200 V. Antigen-containing lanes were excised and placed transversely on a sheet of GelBond (FMC BioProducts, Rockland, Maine). An upper gel (anodic) containing 300 μl of rabbit antiserum in 3 ml of 1% agarose and a lower gel (cathodic) with 50 μl of serum in 1 ml of agarose were poured and electrophoresed overnight at 50 V. Homologous (e.g., *E. maynei* extract reacted with *E. maynei* antiserum) reactions were performed for *E. maynei*, *D. farinae*, and *D. pteronyssinus*. Gel plates were washed, pressed, and dried; and representative gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.).

Immunoabsorption

Unstained CIE gels were used to remove species-specific IgE and IgG from aliquots of pooled mite-allergic serum by immunoabsorption.¹⁰ Two 0.5 ml aliquots of serum were diluted to 20 ml with RAST buffer. One sample was incubated sequentially with 14 *E. maynei* homologous gels for 8 to 24 hours each to remove *E. maynei*-reactive IgE and IgG. The second sample was depleted of *Dermatophagoides*-reactive IgE and IgG by similar incubation with *D. farinae* and *D. pteronyssinus* homologous gels. ^{125}I -anti-human IgE binding to the last gel in each series was negligible. An *E. maynei* homologous gel was then incubated overnight in the *Dermatophagoides*-depleted serum, and *D. farinae* and *D. pteronyssinus* gels were placed in the *E. maynei*-depleted serum. IgE binding to all gels was visualized by using ^{125}I -anti-human IgE and

autoradiography as outlined above for immunoblots, and all gels were exposed to film at the same time.

In parallel to the two serum samples depleted of *Dermatophagoides*- or *E. maynei*-specific antibodies and used to probe CIE gels as outlined above, a second pair of serum samples was depleted of mite-specific antibody. These samples, along with fresh serum, were then used to probe blots containing lanes with *D. farinae*, *D. pteronyssinus*, and *E. maynei*. Blots were processed and IgE binding was visualized as described earlier.

RESULTS

Extract fractionation and allergen identification

Preparative IEF of an extract of *E. maynei* with an ampholyte mixture (pH 3 to 10) yielded 20 fractions of pH 2.9 to 11.6. Most proteins were distributed in fractions 7 to 17 with pH 4.8 to 8.0 (Fig. 1). CIE and CRIE of each fraction confirmed that most antigens and allergens were also present in fractions 7 to 17 (data not shown). When samples of the fractions were subjected to nonreducing SDS-PAGE, followed by electrophoretic transfer and immunoblotting with a reference serum pool (Fig. 2), as well as with the individual sera of 16 subjects sensitive to *E. maynei*, at least 47 individual *E. maynei* allergens were detected. The MWs and pIs assigned to each allergen are listed in Table I. Twenty-two and nine of the allergens were recognized by 50% or more and 75% or more of the sera, respectively (Table I). The reference serum pool contained IgE that bound to 31 bands, and all but one of the individual subjects had IgE that bound to 10 or more allergens (range, 0 to 32) (data not shown). Twelve of the 16 sera (75%) had IgE directed at more than 20 allergens. The mouse monoclonal antibody 1D8 (that recognizes Der p 2 but

not Der f 2²²) bound to a protein with an MW of 14.0 kd and a pI of 6.6 to 7.0. This protein (no. 46) was identified as the allergen Eur m 2 on the basis of this binding. Eighty-one percent of the individual subjects tested had circulating IgE directed at this allergen (Table I). No binding to *E. maynei* allergens was observed when two other anti-group 2 monoclonal antibodies (7A1 or α DpX) were used.²²

Effect of protease inhibitors

Because so many IgE-binding bands were seen on the SDS-PAGE immunoblots of IEF-fractionated *E. maynei* extract, an *E. maynei* extract was prepared in the presence of a variety of protease inhibitors. When SDS-PAGE immunoblots of the extracts prepared in the absence and presence of these inhibitors were probed with the reference serum pool, the resulting autoradiograms were identical (data not shown).

Immunoabsorption experiments

Immunoabsorption experiments were also performed to investigate the presence of species-specific and cross-reactive epitopes on *E. maynei* allergens. In one experiment homologous CIE gels of *D. farinae* and *D. pteronyssinus* were sequentially incubated in the mite-sensitive serum pool to deplete the serum of IgE and IgG that recognized *D. farinae*- and *D. pteronyssinus*-reactive epitopes. After 14 cycles, little IgE binding was observed on the autoradiograms. When an *E. maynei* homologous CIE gel was then incubated in the *Dermatophagoides*-adsorbed serum, most of the allergens visualized with fresh (unadsorbed) serum were also seen on the gels incubated in the serum from which *Dermatophagoides*-reactive antibody had been depleted (Fig. 3). Only a few of the IgE-binding *E. maynei* proteins visualized in fresh serum were not visible when the adsorbed serum was used. The reciprocal experiment was also performed. CIE gels containing *E. maynei* proteins were sequentially reacted with the reference serum pool until IgE antibody directed at epitopes on *E. maynei* allergens was removed from the serum. When CIE gels containing *Dermatophagoides* allergens were then incubated in the *E. maynei*-depleted serum, most IgE-binding allergens of *D. farinae* and *D. pteronyssinus* were still visualized (Fig. 3). Few allergens that were visualized with fresh serum had disappeared after removal of IgE that recognized epitopes of *D. farinae* and *D. pteronyssinus*. A similar experiment was performed with *Dermatophagoides*- and *E. maynei*-depleted serum to probe SDS-PAGE immunoblots of the three mite species. On these blots very few allergens were visualized on either of the blots probed with mite-adsorbed serum (Fig. 4). Although a few bands were seen in the *D. farinae* and *D. pteronyssinus* lanes on the blot probed with *E. maynei*-depleted serum, almost no IgE binding was observed on the blot probed with the *Dermatophagoides*-adsorbed serum.

DISCUSSION

We have determined the MWs, pIs, and IgE-binding frequencies of proteins present in an extract of *E.*

TABLE I. Isoelectric points, molecular weights, and frequency of IgE binding to the 47 *E. maynei* allergens

Allergen No.	Fractions	pI	MW (kd)	IgE binding frequency (%)
1	9-12	5.4-6.1	180.0	75
2	13-17	6.6-8.0	110.0	38
3	12-13	6.1-6.6	105.0	56
4	5-7	4.4-4.8	103.0	81
5	6-7	4.6-4.8	97.0	56
6	9-11	5.4-5.8	89.0	69
7	10-12	5.6-6.1	79.0	75
8	11	5.8	76.0	69
9	8	5.2	74.0	81
10	13-15	6.6-7.0	73.0	44
11	10-12	5.6-6.1	67.0	69
12	6-8	4.6-5.2	66.0	13
13	10-12	5.6-6.1	50.0	38
14	6-7	4.6-4.8	49.5	63
15	5-7	4.4-4.8	45.5	13
16	10	5.6	43.0	25
17	7	4.8	40.5	13
18	3-5	4.2-4.4	38.2	25
19	9-10	5.4-5.6	37.5	13
20	19	9.4	37.0	31
21	3-5	4.2-4.4	36.5	25
22	9-11	5.4-5.8	36.5	88
23	7-9	4.8-5.4	35.2	69
24	15-17	7.0-8.0	34.0	19
25	7-9	4.8-5.4	33.8	63
26	8-9	5.2-5.4	28.8	88
27	8-9	5.2-5.4	27.7	81
28	15-17	7.0-8.0	24.6	31
29	8-9	5.2-5.4	24.0	69
30	11-12	5.8-6.1	24.0	13
31	12-13	6.1-6.6	23.0	13
32	8	5.2	22.5	63
33	15-17	7.0-8.0	22.0	31
34	11-12	5.8-6.1	21.7	31
35	3	4.2	21.0	6
36	7	4.8	18.9	63
37	7-8	4.8-5.2	18.1	44
38	17-19	8.0-9.4	18.0	31
39	17-19	8.0-9.4	16.7	25
40	8-11	5.2-5.8	16.7	56
41	9-10	5.4-5.6	15.6	38
42	19	9.4	15.6	13
43	9-10	5.4-5.6	15.1	50
44	8-9	5.2-5.4	14.9	81
45	12	6.1	14.8	38
46	13-15	6.6-7.0	14.0	81
47	7-8	4.8-5.2	10.4	6

maynei. Twenty-two of these proteins were major allergens because they were recognized by IgE present in 50% or more of the reference sera tested. An IgE-binding protein of 14.0 kd and pI 6.6 to 7.0 was also identified as Eur m 2 on the basis of monoclonal antibody binding.

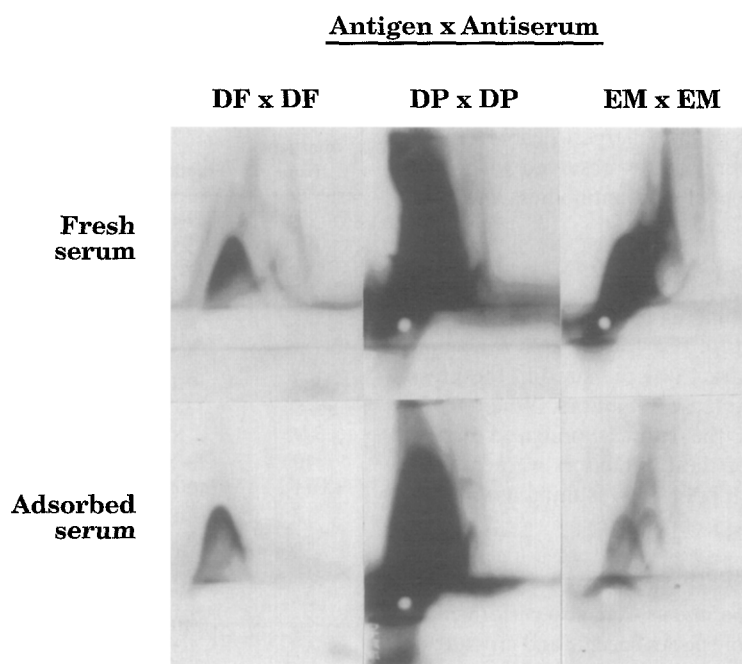


FIG. 3. Homologous CIE gels (*DF* $\times$ *DF*, *D. farinae* extract reacted with anti-*D. farinae*; *DP* $\times$ *DP*, *D. pteronyssinus* extract reacted with anti-*D. pteronyssinus*; *EM* $\times$ *EM*, *E. maynei* extract reacted with anti-*E. maynei*) incubated in fresh serum (*top row*) or in serum adsorbed to remove cross-reactive IgE (*bottom row*) as described in Methods.

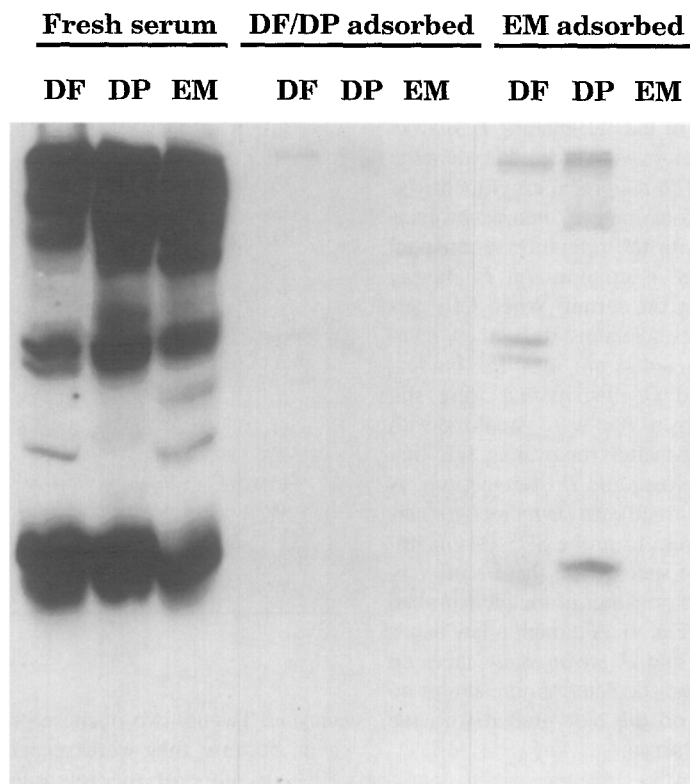


FIG. 4. Autoradiograms of immunoblots with lanes containing extracts of *D. farinae* (*DF*), *D. pteronyssinus* (*DP*), and *E. maynei* (*EM*) probed with fresh serum or mite-adsorbed serum.

Previous studies with CIE and CRIE demonstrated 15 allergens in an extract of *E. maynei*.⁹ Likewise, SDS-PAGE separation of proteins in *E. maynei* extracts and immunoblotting demonstrated the presence of 25 to 30 IgE-binding allergens.^{11, 23-25} This study demonstrated more allergens (47) than previously reported because the *E. maynei* extract was subjected to IEF before SDS-PAGE fractionation. This allowed proteins of similar MWs but different pIs to be resolved and characterized. For example, there are at least four allergens of 14 to 15 kd, which are not resolvable by one-dimensional SDS-PAGE alone. It is possible that some of the allergens were resolvable because of the presence of multiple isoforms of a more limited number of core proteins. However, proteins of different pIs must differ chemically (e.g., amino acid substitutions, variations in glycosylation) and hence are not identical proteins. Isoforms may be highly homologous, yet one may lack an IgE-binding epitope present on another. The identification of isoforms and the relationships among them would require primary sequence analysis of many of the allergens, which was beyond the scope of this study.

Using the monoclonal antibody 1D8 (that recognizes Der p 2 but not Der f 2²²), we demonstrated the presence of a group 2 allergen, Eur m 2, in the *E. maynei* extract. The MW (14 kd) and pI range (6.6 to 7.0) of this protein were consistent with those of the *Dermatophagoides* group 2 allergens.^{13, 26} This protein was also recognized as an allergen by 81% of the sera from subjects tested, a binding frequency similar to the 90% reported for the *Dermatophagoides* group 2 allergens.¹³ Two other anti-group 2 monoclonal antibodies, 7A1 and α DpX, did not bind to this *E. maynei* protein. It is interesting that of the three monoclonal antibodies tested, only 1D8 bound to the Eur m 2, indicating that Eur m 2 does not share two other allergenic epitopes present on the *Dermatophagoides* group 2 allergens. This observation also explains the earlier reports that *E. maynei* contained no group 2 allergen²⁷ because the two-site ELISAs used the antibodies 7A1 or α DpX,²² neither of which were observed in this study to recognize Eur m 2.

Our immunoadsorption experiments also indicated that *E. maynei* shares some cross-reactive epitopes with the *Dermatophagoides* mites but that most of its allergens also contain species-specific epitopes. The SDS-PAGE immunoblots probed with adsorbed serum showed very little antibody binding to proteins from either the mite species against which the serum had been adsorbed or the other mite species. Because sodium dodecylsulfate treatment denatures proteins so that only primary sequence epitopes remain intact on a blot, the lack of binding to the blots by the adsorbed serum indicated that there are many cross-reactive primary sequence epitopes present on most mite allergens. In the CIE experiment, however, the level of IgE binding to gels incubated in adsorbed serum was similar to that seen when fresh serum was used. However, CIE is a nondenaturing technique. The CIE data in combination

with the immunoblots therefore suggest that the species-specific epitopes on mite allergens are predominantly found in higher orders (secondary, tertiary, and quaternary) of protein structure. Whether the three-dimensional structure of allergens is altered during extraction on mucous membranes is an interesting question that has not been investigated.

In summary, this study indicates that the mite *E. maynei* is the source of a large number of potent allergens. Because most *E. maynei* proteins appear to contain allergenic epitopes unique to this mite, it is essential that individuals allergic to mites be tested with and treated for all mite species present in the local environment.

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