

Immunologic characterization of natural and recombinant Mal f 1 yeast allergen

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Background: Individuals with atopic dermatitis (AD) often have IgE antibodies against protein components of *Malassezia furfur*. The cDNA encoding one of these proteins (Mal f 1) has recently been cloned and sequenced.

Objective: We sought to express recombinant Mal f 1 (rMal f 1) allergen in large quantities by using different expression systems. The primary aim was to characterize the IgE-binding properties of rMal f 1 in comparison with its natural counterpart in *M furfur* extract.

Methods: We have expressed and purified Mal f 1 from prokaryotic (*Escherichia coli*) and eukaryotic cells (baculovirus-infected insect cells). The rMal f 1 produced in both systems has been tested for the ability to be recognized by IgE from patients with specific serum IgE to *M furfur* by using immunoblotting and the Pharmacia CAP System RAST FEIA. **Results:** Sixty-one percent of sera from 95 patients showed positive RAST responses to the rMal f 1 produced in the baculovirus expression system and 43% to the *E coli*-produced rMal f 1. Both the *E coli*- and baculovirus-produced proteins can specifically inhibit IgE binding to a 36-kd protein band (Mal f 1) in immunoblotting, indicating that the recombinant proteins contain the majority, if not all, the IgE-binding epitopes of Mal f 1. Recombinant Mal f 1 is able to release histamine from basophils of an atopic individual.

Conclusion: We have expressed and purified rMal f 1, which can bind IgE in a way resembling natural Mal f 1. The ability to produce recombinant allergens with similar properties to their native counterparts has many potential uses, such as accurately diagnosing causes of IgE-mediated allergy. (*J Allergy Clin Immunol* 1999;103:877-84.)

Key words: *Malassezia furfur*, *Pityrosporum orbiculare*, fungi, recombinant allergen, expression system, atopic dermatitis, IgE, CAP RAST, in vitro translation

The lipophilic yeast *Malassezia furfur*, previously also known as *Pityrosporum orbiculare* (ovale), is a member of the human residential skin microflora and primarily

Abbreviation used

AD: Atopic dermatitis

colonizes the sebaceous areas of the skin.^{1,2} It can, under predisposing conditions, act as a pathogen.^{3,4} This yeast appears to induce a specific IgE reactivity in patients with atopic dermatitis (AD),⁵⁻⁹ which is rarely seen in patients with other atopic conditions,^{5,7} and a positive correlation between skin prick test and RAST results to *Malassezia* spp has been found.¹⁰

Different IgE-binding components of the yeast *M furfur* have been detected by SDS-PAGE and immunoblotting, and some of them were found to be major allergens (ie, recognized by >50% of patient sera).¹¹⁻¹³ We have previously reported the complete cDNA sequence of a novel protein from the yeast that shows the criteria of a major allergen in AD.¹⁴ This allergen has a molecular mass of 36 kd (determined from amino acid sequence) and has been designated as Mal f 1 because it is the first allergen cloned from *M furfur*. In immunoblotting of the crude extract, Mal f 1 appears as a double band, indicating that it may be glycosylated.¹⁴

Crude extracts of the yeast may vary in their allergen content, depending on the culture conditions and extraction.^{15,16} Moreover, the allergens may be lost during extraction by the activity of proteases. Expression of allergens as recombinant proteins in heterologous expression systems overcomes the problems of obtaining sufficient quantities and simplifies purification. With the availability of pure recombinant allergen, it is possible to characterize the allergenic properties of individual allergens. It is, however, necessary to compare the recombinant allergens with the native allergen to determine whether they have similar properties. In this study Mal f 1 has been expressed in an in vitro translation system, in a prokaryotic system (*Escherichia coli*), and in a eukaryotic (baculovirus) expression system. The recombinant allergens have been immunologically tested and compared with crude *M furfur* extract to determine their potential as tools in diagnostic analysis and research in AD.

METHODS

Yeast extract

M furfur from American Type Culture Collection (no. 42132) was cultured on glycerol monostearate/olive oil medium for 4 days at 37°C, and the extract was prepared as described earlier.¹⁷

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Patient sera

Among sera from patients with AD sent to the Division of Clinical Immunology at the Karolinska Hospital (Stockholm, Sweden) for IgE antibody analysis from January 1997 to February 1998, all sera with a sufficient volume (≥ 1 mL) were selected on the basis of a specific IgE reactivity to *M furfur*. The 95 sera with positive values in the Pharmacia CAP System (referred to as RAST in this article; Pharmacia & Upjohn Diagnostic AB, Uppsala, Sweden) to *M furfur* with a class of at least 1 (≥ 0.35 kU/L) had a median of 1238 kU/L total serum IgE value (range, 41 to 25,200 kU/L), as measured with RAST (Pharmacia). For negative controls, 14 sera with a median of 1465 kU/L total serum IgE (range, 930 to 20,000 kU/L) but a negative RAST response to *M furfur* whole extract were chosen. A human myeloma IgE (SB 790514) diluted in normal serum was used as an additional control. The study was approved by the Ethics Committee at the Karolinska Hospital.

Antibodies

A mouse mAb designated 9G9 of IgG₁ class, recognizing a 36-kd (previously described as a 37-kd allergen) protein allergen of *M furfur*, was used.¹⁷ The mAb was purified from hybridoma cell culture supernatant by using protein G-Sepharose (Pharmacia) to a concentration of 1.4 mg/mL as calculated from the absorbance measured at 280 nm.

Polyclonal antibodies to rMal f 1 were raised in rabbits and purified with protein A to a concentration of 9 mg/mL.

In vitro transcription and translation in reticulocyte lysate

The full-length cDNA for Mal f 1 was subcloned into XbaI, EcoRI-digested pGEM1 plasmid (Promega, Madison, Wis) by using standard techniques. The Mal f 1 was thus placed under control of the SP6 promoter in pGEM1 TCTAGAGGATCCATGC-GATAC (the XbaI site and initiator codon are underlined).

Synthesis of RNA by SP6 RNA polymerase and translation in reticulocyte lysate in the presence (or absence) of dog pancreas microsomes was performed as described.¹⁸ Translocation of polypeptides to the luminal side of the microsomes was assayed by resistance to exogenously added proteinase K and by prevention of N-linked glycosylation through competitive inhibition by addition of a glycosylation acceptor peptide but not by a nonacceptor tripeptide.¹⁹ The in vitro translation reactions were analyzed by SDS-PAGE and fluorography. The gels were visualized and quantified with a Fuji Bas 1000 image reader with the MacBAS (2.1) software (Fujifilm, Tokyo, Japan).

Recombinant Mal f 1 produced in the *E coli* expression system

The coding region for mature Mal f 1 containing 6 histidine codons at the C-terminal end of the protein was cloned into the pET 17b (Novagen, Abingdon, UK) expression vector. Briefly, the cDNA encoding mature Mal f 1 was amplified with Pfu polymerase by using PCR with the following 2 primers: 5'-GAGCATATG-GCTCTCCCTGATCAGATTGAT-3', which contains an Nde I site (underlined) followed by the first 7 codons of mature Mal f 1, and 5'-GGAAGATCTTTA(ATG)₆AAAGTTCAGGATGCTGTTGT-CAAGCT-3', which contains a Bgl II site (underlined) with codons for 6 histidine residues followed by the last 8 codons of Mal f 1. The amplified PCR product was purified, digested with Nde I and Bgl II, and ligated into pET 17b (Novagen), which had been digested with Nde I and Bam HI. The ligation was transformed into the non-expressing *E coli* strain BL21 (Novagen). Recombinant plasmids were confirmed with restriction enzyme digest and sequencing and

then transformed into the expression strain BL21 DE3 pLysS (Novagen).

For expression of rMal f 1 in *E coli*, overnight cultures of BL21 DE3 pLysS containing the recombinant pET plasmid were diluted 50-fold in prewarmed LB media containing 100 μ g/mL ampicillin and 30 μ g/mL chloramphenicol and grown to an absorbance of 600 nm of 0.6 at 37°C. Expression of rMal f 1 was induced by the addition of isopropyl-B-D thiogalactoside to a concentration of 0.4 mmol/L, and incubation at 37°C was continued for a further 2 hours. Cells were harvested, and inclusion bodies were prepared and solubilized in a urea-containing solution by using standard methods.²⁰

Recombinant Mal f 1 produced in the baculovirus expression system

The coding region of the Mal f 1 cDNA (including the signal sequence) was amplified by using PCR from a plasmid containing full-length cDNA with the following primers: an upstream primer 5'-GAGGGATCCATGCGATACAGCACCGTCTTGGCA-3' containing a Bam HI restriction site (underlined) and a downstream primer 5'-GGAAGATCTTTA-(ATG)₆AAAGTTCAGGATGCTGTTGTCAAGCT-3' containing a Bgl II restriction enzyme site (underlined) and 6 histidine codons. The PCR product encoding the full-length Mal f 1 was digested with the appropriate restriction enzymes (New England Biolabs) and ligated into pBlueBacIII vector (Invitrogen BV, Leek, The Netherlands). After transformation into competent XL2 Blue bacteria cells (Stratagene), plasmid DNA was prepared, and the construct was confirmed with restriction enzyme digest and DNA sequencing.

For expression of rMal f 1 in insect cells, the recombinant plasmid was cotransfected with Bac-N-Blue DNA (Invitrogen) into *Spodoptera frugiperda* (Sf9) cells (Invitrogen) according to the manufacturer's protocol. Media from the transfected cells were harvested after 3 days, and recombinant clones were isolated by a plaque assay with Blueo-gal (Gibco/BRL, Life Technologies, Grand Island, NY) to detect β -galactosidase-producing clones. Each positive clone was verified to be recombinant by PCR with the specific baculovirus primers provided by Invitrogen. The recombinant viruses from selected positive clones were transfected into the *Trichoplusia ni* (Hi-Five) cells (Invitrogen) grown in serum-free medium Ex-Cell 400 (AMS Biotechnology, Täby, Sweden). Supernatants were sampled daily for 5 days, and expression of rMal f 1 was tested by SDS-PAGE and immunoblotting.

Purification of rMal f 1

Purification of the rMal f 1 produced in *E coli* from the urea-solubilized inclusion bodies was performed by using TALON metal affinity resin (Clontech Laboratories, Palo Alto, Calif), according to the instructions of the manufacturer, and eluted with PBS containing 100 mmol/L imidazole (Sigma, St Louis, Mo). The eluted sample was dialyzed against PBS, and the concentration was determined by measurement of absorbance at 280 nm.

Supernatants from recombinant baculovirus grown in Hi-5 cells were collected after 5 days and were concentrated by ultrafiltration with a YM10 membrane (Amicon Corp, Beverly, Mass). The concentrated material was then purified with TALON metal affinity chromatography as mentioned above.

To confirm the cleavage of the signal sequence in the baculovirus expression system, the 5 N-terminal amino acids were sequenced. The purity of both *E coli*-produced and baculovirus-produced rMal f 1 was checked with SDS-PAGE.

IgE reactivity

The specific IgE reactivity was measured with Pharmacia CAP System RAST FEIA under standardized conditions, as recommend-

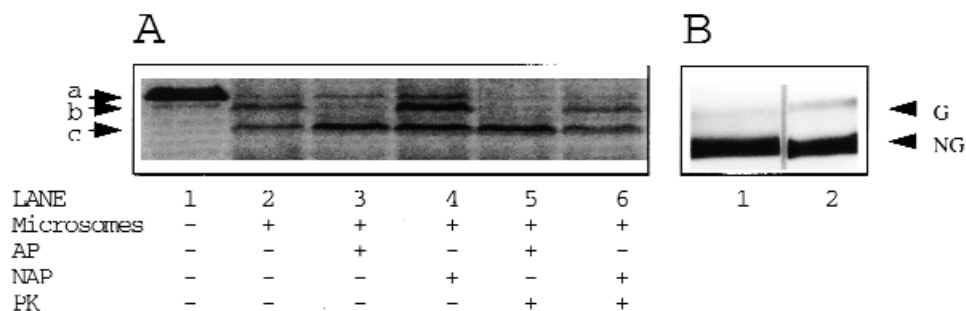


FIG 1. A, In vitro translation of in vitro transcribed mRNA was carried out in the presence (+) or absence (-) of microsomes, acceptor peptide (AP), or nonacceptor peptide (NAP). After translation, some of the reactions were treated with proteinase K (PK). The protein band at position *a* is the Mal f 1-containing signal sequence, at position *b* is the N-glycosylated mature Mal f 1, and at position *c* is the nonglycosylated mature Mal f 1. **B,** Immunoblotting of *M furfur* extract with monoclonal (lane 1) and polyclonal (lane 2) antibodies against Mal f 1. The Mal f 1 glycosylated (G) and nonglycosylated (NG) forms are indicated.

ed by the manufacturer (Pharmacia & Upjohn Diagnostics AB). The m70 ImmunoCAP (*M furfur* whole extract) was used. In addition, the *E coli*-produced and baculovirus-produced rMal f 1 were covalently coupled at the same concentration to the cellulose solid phase (ImmunoCAP). A positive RAST result was defined as a value of at least 0.35 kU/L.

SDS-PAGE and immunoblotting

Protein components of *M furfur* were separated according to molecular size by electrophoresis of SDS-treated and reduced samples either in gradient 7.5% to 20% or homogenous 12% polyacrylamide gels. *M furfur* extract was run at a protein concentration of 6 µg/mm gel, and rMal f 1 produced from different expression systems was run at a protein concentration of 0.6 µg/mm gel. Blotting and immunologic detection were performed as reported previously.¹⁷

For the immunoblot inhibition assay, 80 µL of pooled (5 sera) or individual sera from the patients with positive RAST results to *M furfur* detecting a 36-kd band in immunoblotting were used. The sera were incubated with 40 µg purified rMal f 1 obtained from different expression systems, with 0.25 mg of protein from the *M furfur* whole extract or with 3 mg of BSA (negative control) overnight at 4°C. The preadsorbed sera were then used in immunoblotting to determine whether IgE binding to *M furfur* whole extract or rMal f 1 allergens had been inhibited.

Histamine release assay

All experiments were performed as recommended by the manufacturer of the RIA for histamine (Immunotech, Marseille, France). Briefly, whole blood was challenged with different concentrations of rMal f 1 (produced by *E coli* or baculovirus) or *M furfur* extract (dose range, 0.1 to 10 µg/mL) for 30 minutes at 37°C. The released histamine in the supernatant was acetylated and assayed by RIA. Spontaneous histamine release was measured in an unchallenged sample, and total histamine in the blood samples was measured after cell lysis by freeze thawing twice. All measurements were performed in duplicate.

RESULTS

In vitro translation

In the absence of microsomes (Fig 1, A, lane 1), the Mal f 1 containing its signal sequence is translated. In the

presence of microsomes, the signal sequence is cleaved off, and 50% of Mal f 1 becomes N-glycosylated. The glycosylation can be inhibited by competition with an acceptor peptide for N-glycosylation (lane 3) but not with a nonacceptor peptide (lane 4). Both the glycosylated and nonglycosylated Mal f 1 are in the lumen of the microsomes as they are protected from digestion with proteinase K (lanes 5 and 6). These results indicate that in an in vitro translation/translocation system, Mal f 1 is efficiently translocated to the lumen of the endoplasmic reticulum but is inefficiently glycosylated. We believe this to be the case also in *M furfur* because a double band with the same sizes as found in the in vitro experiment can be seen when Western blots are probed with mAbs or polyclonal antibodies against Mal f 1 (Fig 1, B). It appears that glycosylation of the natural Mal f 1 is even less efficient than in the in vitro translation system.

Expression and purification of rMal f 1

Recombinant Mal f 1 was expressed and purified from *E coli* or the baculovirus expression system. The amounts of protein purified varied in individual experiments, but typical yields were 5 mg of pure protein per liter of bacterial culture and 20 mg of pure protein per liter in the baculovirus expression system. The purified rMal f 1 expressed in both systems was free from contaminating proteins as visualized on Coomassie blue-stained gel (Fig 2). The rMal f 1 produced in the baculovirus expression system appears to be partially glycosylated (Fig 2, lane 1), as can be seen in the in vitro translation system (Fig 1, A) and the *M furfur* extract (Fig 1, B).

IgE reactivity

Ninety-five patient sera selected on the basis of a RAST class of at least 1 (≥ 0.35 kU/L) to *M furfur* whole extract were tested in immunoblotting against *M furfur* and rMal f 1 (see Fig 3 and Table I for examples). Fifty-one of the patient sera showed IgE reactivity to a 36-kd protein in the extract (Table I). All 95 sera were tested in RAST to rMal f 1 produced in the *E coli* and baculovirus

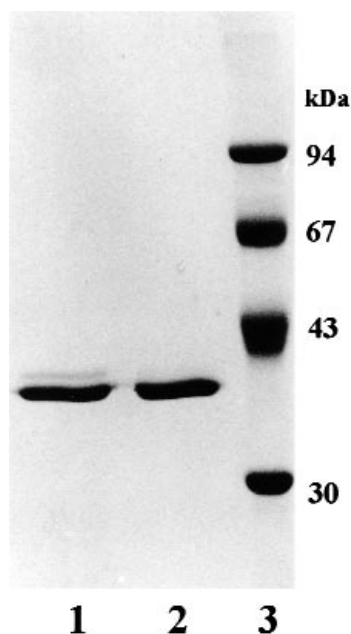


FIG 2. Coomassie brilliant blue-stained gel of purified rMal f 1 from the baculovirus (lane 1) and *E coli* (lane 2) expression systems. Low molecular weight standards are shown (lane 3).

expression systems (Table I). The *E coli* rMal f 1 and baculovirus rMal f 1 RAST results are positive for the majority (78% and 94%, respectively) of the 51 sera that recognize the 36-kd protein in whole extract. Of these 51 sera, 65% and 75% recognized the *E coli* and baculovirus rMal f 1 in immunoblotting, respectively.

The 44 sera that did not recognize a 36-kd protein in immunoblotting of *M furfur* whole extract did not recognize rMal f 1 in immunoblotting (for examples, see Fig 3, sera 5 and 9). However, 2% and 23% of these 44 sera showed positive RAST values to the *E coli* rMal f 1 and baculovirus rMal f 1, respectively.

Of the 14 RAST-negative sera to *M furfur* whole extract but with serum IgE levels from 930 to 20,000 kU/L, 13 sera were RAST negative to the *E coli* rMal f 1 (Table II). With the rMal f 1 produced in the baculovirus expression system, 5 sera were negative (see Table II and Discussion section). The myeloma IgE did not show a positive RAST value for either rMal f 1.

Inhibition

IgE antibodies in patient sera preincubated with either rMal f 1 produced in *E coli* or in the baculovirus system were selectively inhibited in recognition of a 36-kd band in the whole extract (Fig 4). However, the inhibition was not complete in all individual sera tested or pooled patient sera (Fig 4) (see Discussion section). Preincubation of sera with whole *M furfur* extract completely inhibited IgE recognition of rMal f 1 produced in either of the heterologous expression systems in all cases (Fig 4). The rMal f 1 produced in *E coli* could completely inhibit IgE binding to the baculovirus-produced rMal f 1 and vice versa.

Histamine release

The results of histamine release are shown for 4 individuals with AD and who have positive RAST results to *M furfur* extract (Fig 5). Histamine was released on challenge with *M furfur* whole extract in all cases. However, rMal f 1 only released histamine on challenge of 3 of the individuals. Challenge with polyclonal anti-human IgE was used as a positive control and was shown to release histamine (Fig 5). Two control subjects with negative RAST results to *M furfur* extract did not release histamine when challenged with either *M furfur* extract or rMal f 1 (data not shown).

DISCUSSION

The Mal f 1 allergen has been expressed in different expression systems in order to characterize its properties. In an in vitro translation system in the presence of microsomes, the signal sequence of Mal f 1 is cleaved off, and about 50% of the molecules become N-glycosylated. It appears that only a small proportion (even less than 50%) of Mal f 1 in its natural form is glycosylated. The reason for the molecule being only partially glycosylated is most likely to be caused by the suboptimal acceptor site (N-E-S) for N-glycosylation.²¹ Because the way in which glycosylation affects IgE binding to Mal f 1 was not known, it was decided to express Mal f 1 in both a prokaryotic (*E coli*) and a eukaryotic expression system. The Mal f 1 protein does not contain any cysteine residues, and therefore formation of correct disulfide bonds was not an issue when selecting the expression systems. As far as we know, there are no other posttranslational modifications on natural Mal f 1 apart from signal sequence cleavage and partial N-glycosylation that may affect IgE-binding epitopes.

The purified rMal f 1 expressed in both systems could bind serum IgE from individuals with specific IgE against *M furfur* whole extract. This was tested in RASTs and immunoblots. The number of patients with positive RAST responses to the recombinant protein was higher than the number with positive responses in immunoblotting (Table I). The discrepancy between the 2 methods can be explained by the fact that in immunoblotting the sera were diluted 6-fold and in RAST they were used undiluted. However, other factors, such as differences in the conformation of the rMal f 1 when coupled to RAST or when transferred onto polyvinylidene fluoride membrane after SDS-PAGE, cannot be excluded.

In RAST to rMal f 1 produced in the *E coli* and baculovirus expression systems, 43% and 61%, respectively, of the 95 patient sera showed positive values. More than 80% of patients with a RAST class of greater than 3 to the whole extract recognize both *E coli*- and baculovirus-expressed rMal f 1. This makes rMal f 1 a potentially useful tool in detecting patients with high RAST classes to *M furfur* extract. It is possible that some of the patients with positive RAST responses to *M furfur* extract have actually been sensitized to other yeasts, such

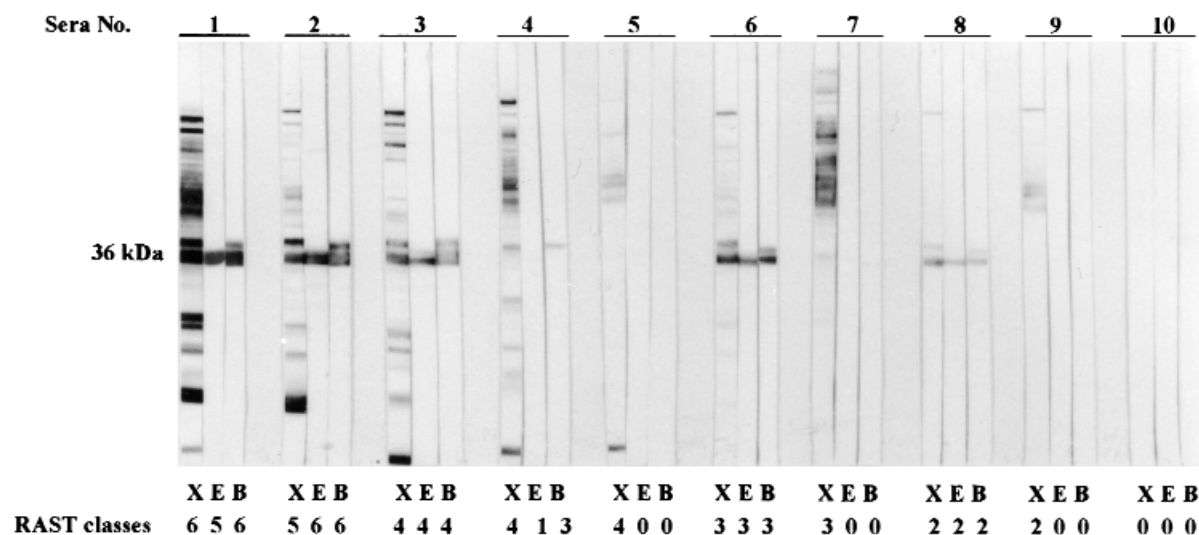


FIG 3. Immunoblot analysis of 10 sera from atopic patients with different RAST classes to *M furfur* whole extract. The specific IgE binding to *M furfur* whole extract (X), rMal f 1 produced in *E coli* (E), or rMal f 1 produced in the baculovirus expression system (B) was detected.

TABLE I. Results of RAST and immunoblotting to *M furfur* extract and rMal f 1 for 95 sera from patients with atopic disease

RAST class (kU/L) to <i>M furfur</i> whole extract	No. of patients	No. (%) of patients with positive RAST results to E rMal f 1	No. (%) of patients with positive RAST results to B rMal f 1	No. (%) of patients with positive immunoblotting results to:		
				36-kd protein in the whole extract	E rMal f 1	B rMal f 1
≥4 (≥17.5)	32	27 (84)	32 (100)	30 (94)	21 (66)	24 (75)
3 (≥3.5)	34	13 (38)	21 (62)	17 (50)	11 (32)	12 (35)
2 (≥0.7)	18	1 (5)	3 (17)	3 (17)	1 (5)	1 (5)
1 (≥0.35)	11	0 (0)	2 (18)	1 (9)	0 (0)	1 (9)
Totals ≥ 1 (≥0.35)	95	41 (43)	58 (61)	51 (54)	33 (35)	38 (40)

E, *E coli*; B, baculovirus.

as *Candida albicans*, which contain proteins that cross-react with *M furfur*.^{13,22} If this is the case, Mal f 1, for which there is no evidence for cross-reacting IgE-binding epitopes in *C albicans*¹⁷ and no known protein homologues in other organisms,¹⁴ could be used for detecting specific IgE against *M furfur*. Currently, crude extracts of *M furfur* are used, but these have the possible and likely disadvantage of giving false indication as to the organism that the IgE was raised against because of cross-reactivity.^{13,22} Another disadvantage is that crude extracts of *M furfur* vary in allergenic content depending on the method of preparation and are therefore difficult to standardize.^{12,15} Although rMal f 1 does not itself detect all patients with IgE raised against *M furfur*, we believe that in combination with other *M furfur*-specific recombinant allergens diagnosis of patients specifically sensitized to this yeast will be greatly improved and standardized. Organism-specific allergens are desirable diagnostic tools to enable determination of the cause of sensitization.

Some of the differences between the *E coli*- and bac-

TABLE II. Sera with negative RAST values to *M furfur* whole extract used as controls for rMal f 1 in RASTs

Serum no.	Total IgE content (kU/L)	RAST value to E rMal f 1, kU/L (class)	RAST value to B rMal f 1, kU/L (class)
1	930	<0.35	0.38 (1)
2	950	0.63 (1)	0.98 (2)
3	1003	<0.35	6.97 (3)
4	1200	<0.35	<0.35
5	1257	<0.35	1.5 (2)
6	1300	<0.35	<0.35
7	1460	<0.35	<0.35
8	1470	<0.35	9.44 (3)
9	1603	<0.35	<0.35
10	1604	<0.35	<0.35
11	1800	<0.35	0.4 (1)
12	5800	<0.35	0.76 (2)
13	8870	<0.35	2.61 (2)
14	20,000	<0.35	0.35 (1)
Myeloma IgE	1000	<0.35	<0.35

A positive RAST result is defined as a value of at least 0.35 kU/L.
E, *E coli*; B, baculovirus.

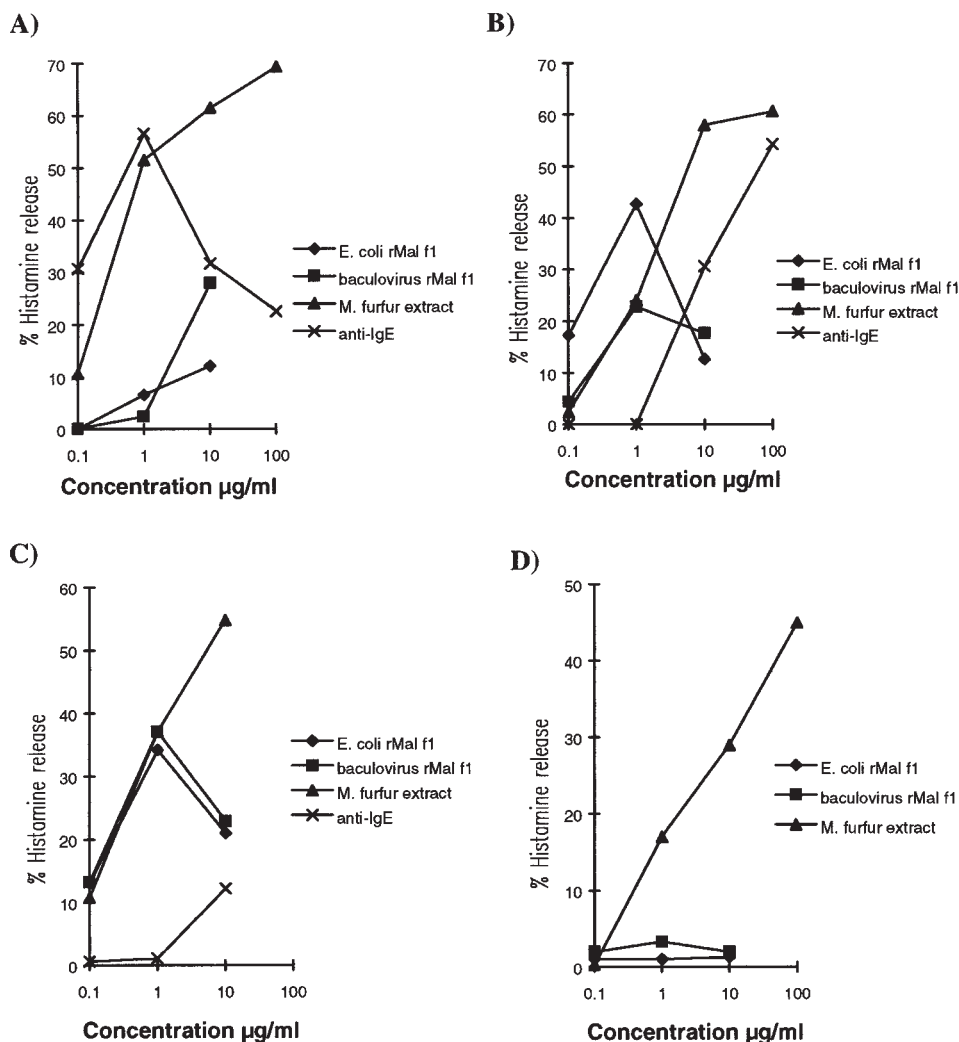


FIG 5. Histamine release on challenge with different concentrations of rMal f 1 and *M furfur* whole extract in 4 patients with AD (**A**, **B**, **C**, and **D**). Respective RAST values to whole extract, *E coli*-produced, and baculovirus-produced Mal f 1 were 20, 7.36, and 11.4 kU/L for **A**; 56.2, 51.2, and 91.3 kU/L for **B**; 83.3, 47.4, and 75.6 kU/L for **C**; and 2.4, 1.1, and 3.4 kU/L for **D**. In 3 patients a positive control with anti-human IgE polyclonal antibodies was included. The results are expressed as a percentage of total histamine.

furfur RAST-positive sera that cannot recognize nonglycosylated Mal f 1. The disadvantage is that the sugars added in insect cells may also create an additional epitope that is not related to *M furfur*. Taken together with economic considerations, these findings suggest that producing rMal f 1 in *E coli* may be the best option, especially if rMal f 1 is to be used in combination with other recombinant *M furfur* allergens.

The *M furfur* extract could completely inhibit IgE binding (in a pool of 4 sera) to rMal f 1, and the rMal f 1 could inhibit IgE binding to a 36-kD protein in the whole extract, but this inhibition was not complete in all of the sera tested. We believe that these sera also recognize another allergen with the same apparent molecular weight as Mal f 1 in the *M furfur* extract. The presence of another 36-kD allergen can also explain some of the differences between the whole extract and rMal f 1 in

immunoblotting. It cannot be ruled out that rMal f 1 does not have all the IgE-binding epitopes of the natural Mal f 1.

A further test to see whether rMal f 1 could behave as an allergen was the basophil degranulation (histamine release) assay, which closely resembles the cascade of the in vivo allergic effector reaction. The rMal f 1 produced in both expression systems was able to release histamine from 3 of the 4 patients with AD. A possible explanation for one patient not releasing histamine on challenge with the recombinant allergens is that this patient had the lowest RAST values (1.1 and 3.4 kU/L for *E coli*- and baculovirus-produced proteins, respectively) of the 4 tested. A correlation between RAST values and histamine release has been shown for birch pollen allergens.²³

In conclusion, we believe that purified rMal f 1 retains

sufficient allergenic properties to be useful as a diagnostic tool and in further research into AD.

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