

Comparison of different extraction methods of *Alternaria* allergens

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Spore and mycelial allergens of two species of Alternaria (A. brassicicola and A. alternata) extracted under two different conditions were analyzed by radiorocket immunoelectrophoresis, crossed radioimmunoelectrophoresis, and Western blotting with a pool of sera from Alternaria-allergic patients. More allergens were extracted after disruption of the cells if protease inhibitors and a phenol-binding component were included in the homogenization buffer. However, the 31 kDa major allergen was extracted in about the same amount, either by incubation or by cell breakage of the cells. This 31 kDa allergen was present in higher concentration in the mycelium than in the spore. The difference between the two species studied is less than between spore and mycelium from the same species. (J ALLERGY CLIN IMMUNOL 1990; 85:941-8.)

Previous studies on *Alternaria* allergens have demonstrated the high variability among the different preparations.¹⁻⁴ The reasons for such heterogeneity are not well understood. However, it is known that, among the numerous causes of variability, the type of species of strain, type of propagule (spore or mycelium), and extraction procedure are important in the extraction of fungal allergens.⁵ In the case of *Alternaria*, the choice of the propagule and the technique of extraction appear important.

Some authors^{6,7} found that spores contain specific allergens that are more reactive than mycelium, whereas other studies^{8,9} concluded that mycelium extracts produced the best allergenic preparations.

Previous studies^{3,10,11} tend to demonstrate that extraction is a critical step in obtaining good allergen extracts. Some allergens can be released by passive incubation in Coca's solution,¹² whereas other allergens can be extracted only after cell disruption.⁹ Moreover, two factors may cause problems during extraction of allergens of *Alternaria*: melanin and proteases. *Alternaria* spores are highly pigmented by melanin, which is a polyphenolic compound; moreover, phenols are known to precipitate with proteins. In the

Abbreviations used

PVP:	Polyvinylpyrrolidone
PMSF:	Phenylmethylsulfonyl fluoride
EDTA:	Ethylenediaminetetraacetic acid
RIE:	Rocket immunoelectrophoresis
RRIE:	Radiorocket immunoelectrophoresis
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
SDS:	Sodium dodecyl sulfate
PAGE:	Polyacrylamide gel electrophoresis
kDa:	Kilodalton

course of our work, one extract, which precipitated heavily during concentration, was demonstrated to contain few allergens. Precipitation after extraction was frequent and presumably caused by extracted pigments. To avoid precipitation of proteins with phenols, insoluble PVP, a phenol-binding component,¹³ was added to the extraction buffer. Proteases are also known to have a deleterious effect on protein extraction. To prevent enzymatic degradation of proteins, PMSF and EDTA were tested. PMSF reacts with the seryl residues at the active site of proteases like chymotrypsin, and EDTA, a chelating agent, inhibits divalent cation-dependent enzymes.

In the present study, spore and mycelial allergens of two species of *Alternaria* (*A. alternata* and *A. brassicicola*) were extracted under different conditions and were analyzed by conventional procedures and Western blotting to understand the influence of the fungal material and extraction techniques on the recovery of allergens of *Alternaria*.

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TABLE I. Allergenic potency of extracts obtained by different extraction procedures

	Strain 20297				Strain 1563			
	Spore		Mycelium		Spore		Mycelium	
	Coca	carb	Coca	carb	Coca	carb	Coca	carb
RAST*	NT	7.1	NT	9.3	8.6	7.3	7.6	5.1
Precipitins†	NT	10	6	8	10	10	5	13
Allergens‡	NT	3	2	3	3	2	3	3

NT, Not tested; Coca, incubation in Coca's solution; carb, disruption in carbonate buffer.

*Percent of total counts added measured with pool A diluted 10^{-1} . As an indication, this pool produced on old Pharmacia disks, 5.05%; on new Pharmacia disks, 8.6%.

†Precipitins determined with RIE.

‡Allergens determined with RRIE.

MATERIAL AND METHODS

Strains of *Alternaria*

Two strains of *Alternaria* were selected because their sporulation capacity is high and stable and their allergen production is good: IP 1563 (*A. alternata* (Fries) Keissler, Institut Pasteur, Paris) and MUCL 20297 (*A. brassicicola* (Schweinitz) Wiltshire, Université catholique de Louvain, Belgium).

Culture

Mycelium was grown for 3 days in liquid 2% malt extract with constant agitation (80 to 100 rpm) to prevent sporulation. Spores (90% to 96% pure) were harvested after 4 weeks of growth on V8 agar medium.¹⁴

Extraction

Two types of extraction procedure were tested. (1) Coca's extracts: spores and mycelium were incubated 24 hours at $+4^{\circ}\text{C}$ in Coca's solution (NaHCO_3 , 3 gm; NaCl , 9 gm; phenol, 5 gm; and H_2O , 11 L) and (2) Carbonate extracts: spores and mycelium were broken in carbonate buffer (NaHCO_3 , 50 mmol/L, and NaCl , 0.15 mol/L, pH 8.3) with a glass-bead cell homogenizer (MSK, B. Braun, Melsungen, West Germany).

Both types of extracts were centrifuged 1 hour at 20,000 g. The supernatant was dialyzed 24 hours at $+4^{\circ}\text{C}$ against phosphate-buffered saline, pH 7.2, and concentrated against polyethylene glycol, 20,000 molecular weight (Carbowax, Union Carbide Corp., Indianapolis, Ind.). When noted, the following additives were used during the extraction procedure: 1 mmol/L of PMSF, 5 mmol/L of EDTA, and 25% (wt/wt) of insoluble PVP (Sigma Chemical Co., St. Louis, Mo.).

Patient sera

The IgE antibody pools were derived from patients tested in different allergy centers in France. They had documented positive clinical histories of mold sensitivity, positive *Alternaria* skin tests, and elevated serum IgE antibodies to *Alternaria* (Phadebas RAST class, 2 to 4). Pool A and pool B were from 17 and 20 patients, respectively. Pool C was

from three other patients. A pool of sera from normal subjects having negative skin test and RAST toward *Alternaria* was included in the study as a nonallergic control.

Rabbit antibody

Antisera to *Alternaria* extracts were raised in rabbits (New Zealand white) by multiple intracutaneous immunizations with the crude allergenic extracts, as previously described.⁹

Immunoelectrophoresis

RIE, RRIE, CIE, and CRIE were performed as described by Weeke et al.¹⁵ and Weeke,¹⁶ and modified by Le Mao et al.¹⁷

SDS-PAGE

SDS-PAGE was carried out on a 12.5% acrylamide separating gel with 0.1% SDS and a 3% acrylamide upper stacking gel with the discontinuous buffer system of Laemmli.¹⁸ Samples (100 μg protein) heated at 100°C for 3 minutes in Tris HCl buffer, pH 6.8, containing 2% SDS, were electrophoresed at 30 mA for 1 hour, followed by 4 hours at 60 mA. After electrophoresis, the gel was stained with Coomassie brilliant blue G 250.

Immunoblotting

Proteins bands from the SDS-PAGE gel were electrophoretically transferred to a nitrocellulose membrane (0.2 μm , Schleicher & Schuell, Kassel, West Germany) in a 192 mmol/L or Tris glycine buffer with 20% methanol, as described by Towbin et al.¹⁹ After saturation of the nitrocellulose print, 0.5 cm wide strips were incubated with a pool of sera from patients or negative sera diluted 10^{-1} and then with isotope-labeled, ^{125}I -anti-IgE antibodies. After strips were washed and dried, they were put on an x-ray film and exposed for 10 and 30 days.

RAST

The *Alternaria* extracts were coupled to CNBr-activated paper disks, as described by Ceska et al.²⁰ The RAST was performed according to the principle described by Wide

et al.³¹ The results were expressed as percentage of bound radioactivity.

Enzyme screening

The extracts were screened with the Api ZYM system (Api Laboratories, Montalieu-Vercieu, France). Extracts (200 µg protein) were placed in each well and incubated overnight at 37° C before addition of the chromogenic reagent. Reactions were graded from 0 to 5 according to the intensity of the color reaction as compared with the manufacturer's color chart.

RESULTS

The two types of extracts (incubation of Coca's solution and disruption in carbonate buffer) of spore and mycelium of *A. alternata* (strain 1563) and *A. brassicicola* (strain 20297) were tested for allergenicity by RAST and RRIE (Table I; Fig. 1). All extracts produced similar allergenic activity measured by RAST and RRIE. This finding is evident, even for Coca's extracts, which produce very low IgG responses in the rabbit (Fig. 1, lanes 5 and 6).

SDS-PAGE patterns of spore and mycelium extracts demonstrated differences in the intensity and number of bands (Fig. 2). Low-molecular-weight compounds present in the Coca's extracts of the spores provoked distortion in the pattern (Fig. 2, lanes 2 and 3); Coca's extracts of spores demonstrated fewer numbers of Coomassie brilliant blue-staining bands than the Coca's extracts of the mycelium (Fig. 2, lane 4). Carbonate extracts of spores and mycelium produced a much more complex protein pattern with numerous Coomassie brilliant blue-stained bands that ranged in apparent molecular weight from 150 to 10 kDa (Fig. 2, lanes 5 to 7).

Allergenic patterns of the different extracts were examined by immunoblotting. ¹²⁵I-labeled anti-IgE immunoblot demonstrated that there was no major difference between Coca's and carbonate extracts without additives (Fig. 3, lanes c, d, g, and h). However, when additives (PVP, PMSF, and EDTA) were added to the carbonate buffer before and during breakage, protein extraction was more efficient (yield of three times more protein; data not presented), and a higher number of allergens was detected (Fig. 3, lanes a, b, e, and f). CRIE confirmed that carbonate extracts plus additives contained a higher number of allergens than Coca's extracts (Fig. 4). Controls with normal human sera or with sera of patients allergic to pollen elicited no binding with the different extracts (data not presented).

The efficiency of the additives, PMSF and EDTA, in the carbonate extracts can be explained by the presence of proteolytic activity in these extracts (Table II). Chymotrypsin and, to a lesser extent, the valine and cystine aminopeptidases of the carbonate extracts

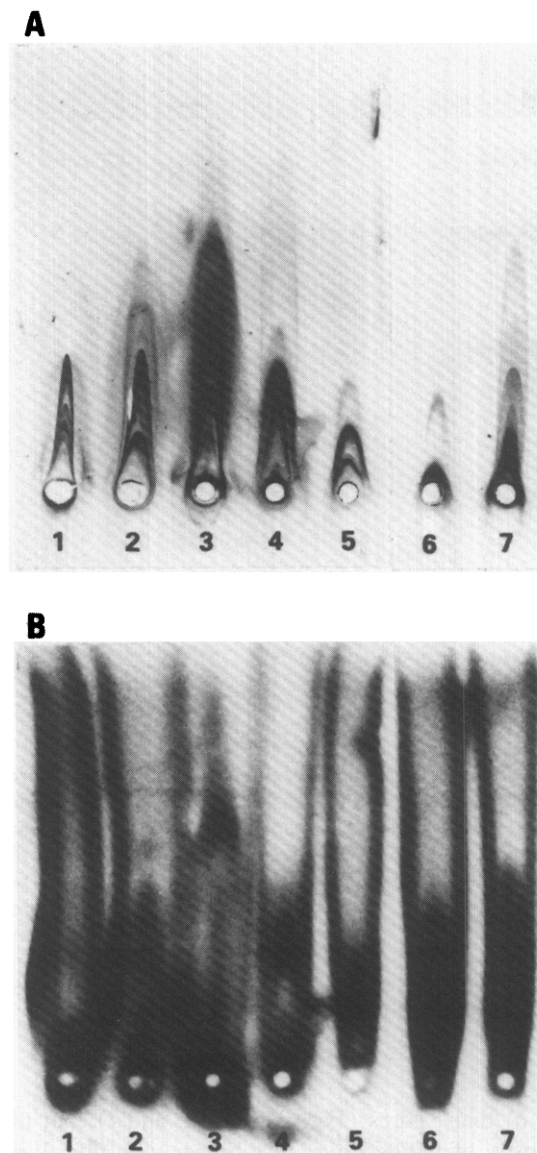


FIG. 1. A, RIE. B, RRIE. Different extracts of *Alternaria*. Carbonate extracts of spore of *A. alternata* strain 1563 (lane 1) of mycelium. *A. brassicicola* strain 20297 (lane 3) of mycelium strain 1563 (lane 4) and strain 20297 (lane 7). Coca's extracts of spore strain 1563 (lane 2) of mycelium strain 1563 (lane 5) and strain 20297 (lane 6). Pool A of patient sera.

of strain 1563 were inhibited by PMSF and/or EDTA. Leucine aminopeptidase activity was strong in both strains and was not inhibited by EDTA or PMSF. In contrast, Coca's extracts had no detectable proteolytic activity (not presented).

Differences between spore and mycelium could be observed in extracts obtained with the more efficient method (breakage in carbonate buffer plus additives). Mycelium contained fewer allergens (Fig. 3, lanes a

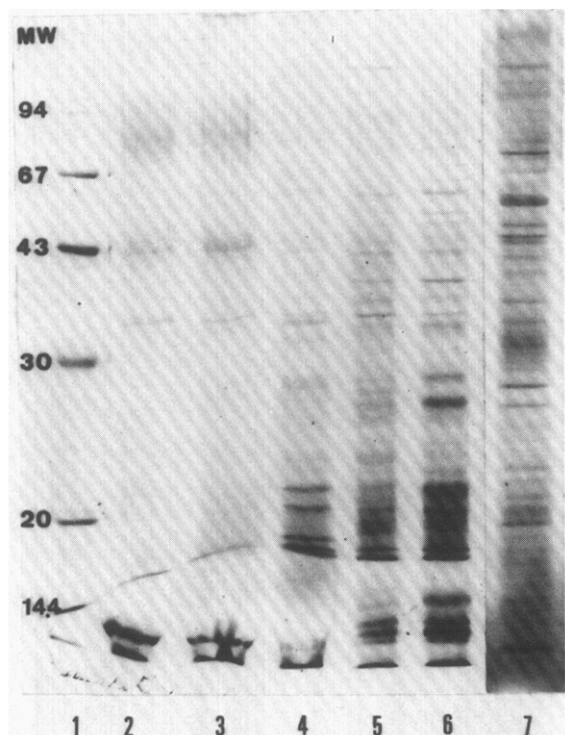


FIG. 2. SDS-PAGE patterns of *Alternaria* extracts after protein staining with Coomassie brilliant blue. Lane 1, molecular weight markers: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa). Coca's extracts of spores strain 1563 (lane 2) and strain 20297 (lane 3). Coca's extract of mycelium strain 1563 (lane 4). Carbonate extract of spores strain 1563 (lane 5) and strain 20297 (lane 6). Carbonate extract of mycelium strain 1563 (lane 7).

and b) than spores (Fig. 3, lanes e and f), but the intensity of radiostaining was higher with mycelium than with spore allergens. CRIE analysis demonstrated that a higher concentration of the major allergen⁹ (Fig. 4, arrows) was present in mycelium than in the spores, independent of the extraction method. This major allergen was not revealed in Coomassie brilliant blue staining of CIE (Fig. 4). Spore allergen patterns were different in the two species studied (Fig. 3, lanes e and f). In contrast, no major difference was found between mycelial extracts of the two species (Fig. 3, lanes a and b).

The 31 kDa allergen, which demonstrated the highest radiostaining, was present in the spore and mycelium of both species (Fig. 3). When mycelial extracts of strain 1563 and of strain 20297 were electrophoresed in the same well, only one band at 31 kDa was detected by immunoblotting (Fig. 5). This

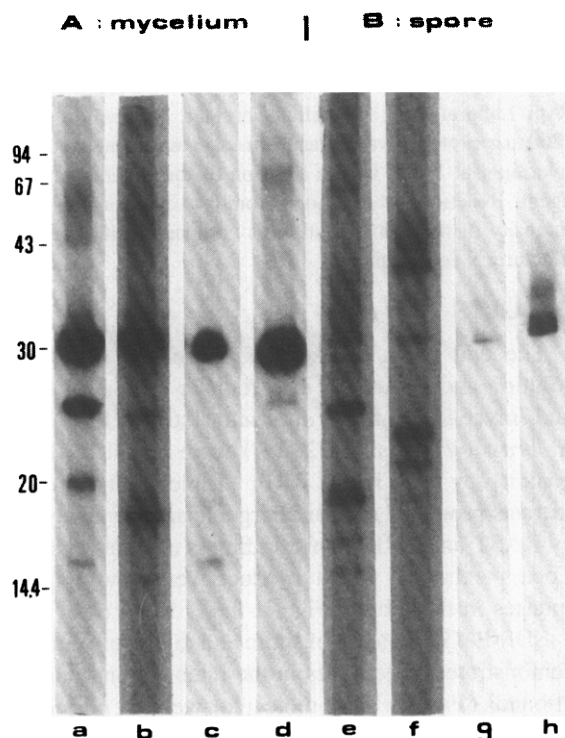


FIG. 3. IgE binding components. A, In mycelium. B, In spore. Extracts of *Alternaria* obtained by three extraction procedures: disruption in carbonate buffer with additives (lanes a, b, e, and f); disruption in carbonate buffer without additives (lanes c and g); incubation in Coca solution (lanes d and h). Strains used: *A. brassicicola* (20297), lanes a and e; *A. alternata* (1563), lanes b-d and f-h. Pool A of patient sera.

finding demonstrated that the molecular weight of this allergen was the same in both species. This result was confirmed by inhibition experiments demonstrating the disappearance of this 31 kDa band when a blot with the extract of one species was incubated with the serum pool adsorbed with the extract of the other species (Fig. 6, lanes 3 and 4).

DISCUSSION

Our results demonstrated that the best extraction method is the breakage of the cells in a carbonate buffer containing additives (PVP, PMSF, and EDTA) that prevent precipitation and enzymatic hydrolysis of proteins. These extracts contained a high number of allergens in CRIE similar to the allergens obtained by Steringer et al.⁴ The allergenicity of the extracts obtained without additives appears comparable to the extract proposed as the international reference stan-

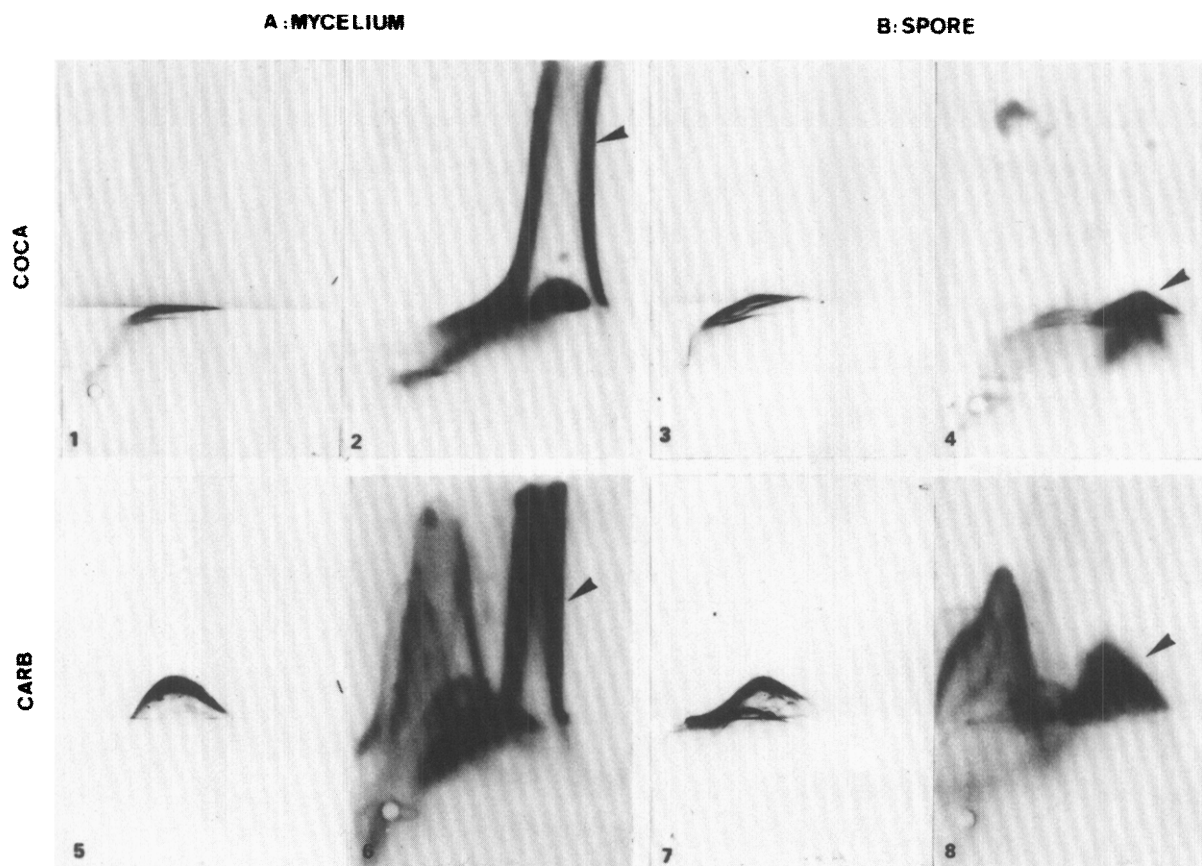


FIG. 4. *A. alternata* extracts analyzed by CIE and CRIE. Odd numbers: CIE plates of strain 1563 antigen (3 μ g of protein) against hyperimmune rabbit antisera, Coomassie brilliant blue stain. Even numbers: corresponding x-ray films (after 10 days of exposure) from the same CIE plates incubated with pool C of patient sera. Plates obtained with Coca's extracts (1 to 4) and plates obtained with carbonate extracts (*carb*) plus additives (5 to 8); mycelium (plates 1, 2, 5, and 6), spore (plates 3, 4, 7, and 8), and major allergen (*arrow head*).

TABLE II. Proteolytic enzymes detected by the Api ZYM system in *Alternaria* carbonate extracts

Proteolytic activity	Score							
	Spore				Mycelium			
	20297		1563		20297		1563	
	<i>carb</i> -		<i>carb</i> -	<i>carb</i> +	<i>carb</i> -	<i>carb</i> +	<i>carb</i> -	<i>carb</i> +
Exopeptidases								
Leucine arylamidase	5		5	5	5	5	5	5
Valine arylamidase	0-1		1	0-1	0-1	1	1-2	0
Cystine arylamidase	0-1		1	0-1	0-1	0-1	1	0
Endopeptidases								
Trypsine	0		0	0	0	0	0	0
α -Chymotrypsin	0		1-2	0	0-1	0	1-2	0

0, No activity; 0-1, residual activity; 1, low activity; 2, medium activity; 5, highest activity; *carb* -, disruption in carbonate buffer; *carb* +, disruption in carbonate buffer with additives (PSMF, EDTA, and PVP).

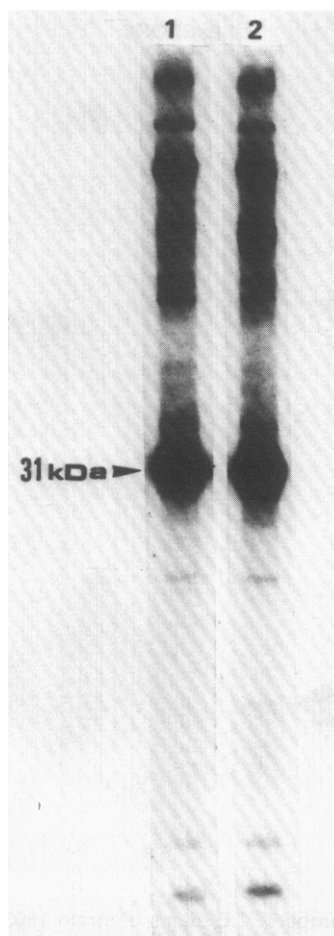


FIG. 5. Autoradiographs of immunoblots of mycelial extracts of *Alternaria* incubated with pool B of patient sera. *Lane 1:* mycelial extract of *A. alternata* (strain 1563); *lane 2:* comigration of mycelial extracts of *A. alternata* (strain 1563) and of *A. brassicicola* (strain 20297); 50 μ g protein of mycelial extract of strain 1563 and 50 μ g of mycelial extract of strain 20297 were mixed together and subjected to electrophoresis in the same well.

dard *Alternaria* extract³ (Fig. 1; Table I). RAST inhibition has demonstrated that our Coca's extracts contained about the same panel of allergens as the international standard (Yunginger JW. Personal communication). An important finding was that the major allergen was extracted in about the same amount either by incubation in Coca's extract or by breakage in carbonate extracts containing additives. This may suggest either an external position in the cell or a quick release from the cytoplasm.

The addition of proteolytic inhibitors and of a phenol-binding compound is efficient not only on the extraction but also on the stability of allergenic extracts of *Alternaria* as demonstrated by Vijay et al.²² who did not find any loss of activity of two commercial extracts. However, these investigators observed a del-

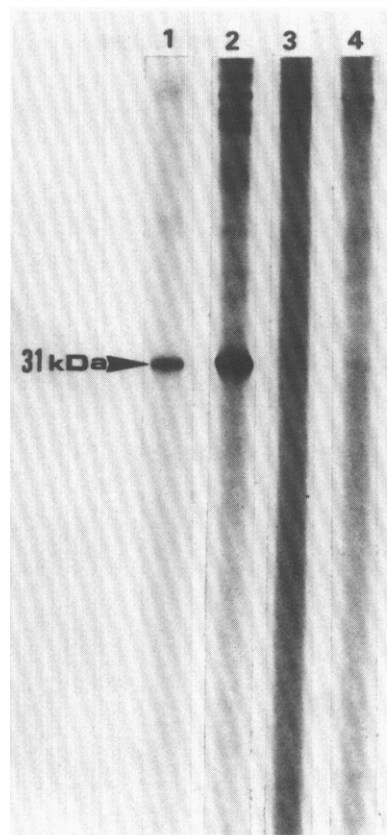


FIG. 6. Blotting-inhibition experiment. *Lanes 1 and 3:* mycelial extract of *A. alternata* (strain 1563). *Lanes 2 and 4:* mycelial extract of *A. brassicicola* (strain 20297). Strips were incubated with pool C containing essentially specific IgE against the 31 kDa protein. Serum was preincubated with mycelial extract of strain 20297 (*strip 3*) or strain 1563 (*strip 4*).

eterious effect of phenol (0.4% in saline) on stability of *Alternaria* extracts (loss of 50% activity measured by passive cutaneous anaphylaxis and RAST inhibition). Moreover Vijay et al.²³ found no endopeptidases in extracts obtained with Tris HCl, pH 7.8. Our Coca's extracts (0.5% phenol) tested by prick test appeared stable (Guinnepain MT, Fadel R. Personal communication), probably because these extracts have no proteolytic activity. We also observed a better stability of strain 20297 carbonate extracts compared to carbonate extracts of strain 1563. This finding may be explained by a lower proteolytic activity of this strain.

Our SDS-PAGE patterns are quite different from patterns obtained with commercial *Alternaria* extracts^{24, 25} in which the number of bands was always very low. Autoradiographs with these extracts demonstrate only very few allergens (six for Tee et al.²⁵; four for Bengtsson et al.²⁴). The extract partially pu-

rified by Kroutil and Bush²⁶ on diethylaminoethyl cellulose produced also a quite different pattern. In all cases, allergens do not produce bands positive in Coomassie brilliant blue staining.

CRIE and immunoblotting experiments demonstrated that spores and mycelium contained both specific and common allergens. Among the common allergens, the 31 kDa major allergen, was very reactive in immunoblotting and represents the major IgE binding peak in CRIE (Fig. 4, arrows).⁹ As observed by Aukrust et al.,⁸ this major allergen is present in higher amount in mycelium than in spore (Fig. 4, arrows). The presence of spore-specific allergen is consistent with the previous observations of Hoffman et al.⁷ and Solomon et al.⁶

Although our study was done with only one strain for each species, our results are similar to the results of Vijay et al.²⁷ in which *A. alternata* (*A. tenuis*) and *A. solani* appear to possess common and unique allergenic determinants. Helm et al.³ also found that the best commercial extract was obtained from eight strains belonging to four different species (*A. alternata*, *A. tenuissima*, *A. longipes*, and *A. paludii*). With this work there were essentially two pools used, and IgE binding components varied with the pool used. In mycelial extracts of both strains, the 31 kDa major allergen is detected as a strong IgE-binding component with pool A and B (Fig. 3, lanes a and b, and Fig. 5), but pool A detected also a few bands of lower molecular weight (Fig. 3, lanes a and b), whereas pool B detected numerous bands of higher molecular weight than 31 kDa (Fig. 5). Thus, this large disparity observed between pool A (Fig. 3) and pool B (Fig. 5), which is derived from different patients, may have minimized the variability between strains. The comigration and immunoblotting experiments demonstrated that the 31 kDa major allergen is present and appears identical in mycelium and spore of both species. Our results appear to favor the importance of the culture procedure. However, Bush and Yunginger²⁸ found that the structure of the major allergen might be quite variable, depending on the strain, the culture, and/or extraction procedure.

Results presented in this study confirmed that extraction is a critical step in obtaining good allergen extracts and that spores appear to contain more allergens than mycelium. However, whatever conditions are used, the major allergen is always present.

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Response to a *Haemophilus influenzae* type b diphtheria CRM₁₉₇ conjugate vaccine in children with a defect of antibody production to *Haemophilus influenzae* type b polysaccharide

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A defect in antibody response to immunization with Haemophilus influenzae type b (Hib) capsular polysaccharide vaccine has been reported in children with recurrent infections and normal immunoglobulin levels. We identified 15 children, aged 2 to 6 years, with this defect, and we evaluated their response to immunization with an Hib capsular oligosaccharide diphtheria CRM₁₉₇ protein-conjugate vaccine (HbOC). The children received a series of three vaccines: HbOC at 0 and 8 weeks, and the Hib polysaccharide vaccine at 16 weeks. Levels of antibody to the Hib capsular polysaccharide (polyribosyl ribitol phosphate, PRP) and to diphtheria toxoid were obtained before and 4 weeks after each vaccination. The geometric mean serum anti-PRP concentration was 0.17 µg/ml before immunization and 29.3 µg/ml after the second HbOC immunization (week 12). All 15 children had postvaccination anti-PRP antibody levels >1.0 µg/ml after receiving the second HbOC (week 12). In addition, booster responses were observed after the second Hib conjugate in 13 of the patients and after the Hib polysaccharide in four of the patients. All patients with low preimmunization diphtheria titers had a response to the diphtheria toxoid. These results suggest that conjugation of Hib polysaccharide with diphtheria CRM₁₉₇ overcomes the defective antibody response to Hib oligosaccharide in children who are initially observed with recurrent infections and inability to respond to the Hib polysaccharide vaccine. (J ALLERGY CLIN IMMUNOL 1990;85:948-53.)

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