

Detection of *Alternaria* allergens by Western blotting

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The technique of Western blotting (immunoblotting) is being increasingly recognized as a means of identifying and characterizing allergens in complex mixtures. Because the allergens are immobilized on nitrocellulose paper, they can be probed for binding of IgE from patients' sera. In addition, the blotting technique provides useful biochemical information, such as the molecular weight of the allergen being studied. In this study we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate antigens in a crude Alternaria preparation, electrophoretically transferred these antigens to nitrocellulose, and incubated the nitrocellulose strips with individual serum and pooled sera from 13 Alternaria-sensitive subjects, sera from three nonallergic individuals, and sera from three individuals allergic to ragweed but not Alternaria. The strips were then washed, incubated with ¹²⁵I-labeled antihuman IgE, washed again, and exposed to autoradiography film. We observed IgE binding to 20 allergens. Five or more sera from Alternaria-sensitive patients recognized allergens in four molecular weight regions: (1) 55,000 to 60,000, (2) 40,000 to 45,000, (3) 35,000 to 40,000, and (4) 15,000 to 20,000. With pooled sera, the most intense IgE binding was directed at allergens with molecular weights above 40,000. No IgE binding was observed with sera from individuals who were not sensitive to Alternaria. We conclude that Western blotting is an effective method for detecting allergens in complex fungal mixtures. (J ALLERGY CLIN IMMUNOL 1987;80:170-6.)

Allergenic extracts of fungal origin have been difficult to characterize and standardize, caused in part by the complexity and high degree of variability of the organisms.¹ CRIE has been used to identify individual allergens in *Alternaria*,^{2,3} but the procedure requires the availability of a rabbit antiserum capable of recognizing all of the relevant allergens in a given preparation.

The transfer of antigens from polyacrylamide gels to nitrocellulose, or "Western blotting" (immunoblotting),⁴ allows antigens resolved in the gel to be probed with various antisera. IgE binding to allergens in cereal grains,⁵ house dust,⁶ mites, and silk proteins⁷ has been detected after electrophoretic transfer to nitrocellulose. Since the allergens are immobilized on nitrocellulose, they can be probed with sera from allergic individuals without the need for a rabbit antiserum. Also, use of the blotting technique can provide information about biochemical characteristics of the

Abbreviations used

CRIE:	Crossed radioimmuno-electrophoresis
DEAE:	Diethylaminoethyl
BSA:	Bovine serum albumin
PAS:	Periodic acid-Schiff stain
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
TBS:	Tris-buffered saline

allergens, such as molecular weight or isoelectric point. We used SDS-PAGE to separate antigens in a crude preparation from *Alternaria* and then used the Western blotting technique to detect *Alternaria* allergens and estimate their molecular weights.

MATERIAL AND METHODS

Alternaria strain

A. alternata, strain 46582, held by the American Type Culture Collection, Rockville, Md., was a local isolate originally obtained from the Wisconsin State Laboratory of Hygiene in Madison, Wis.

Preparation of *Alternaria* extract

Alternaria was cultured for 3 weeks at room temperature in stationary Roux bottles. One liter of medium contained the following ingredients: NaNO₃, 2 gm; KH₂PO₄, 1 gm; MgSO₄·7H₂O, 0.5 gm; KCl, 0.5 gm; FeSO₄, 0.01 gm; glucose, 15 gm; maltose, 15 gm; and Bacto-Casitone (Difco

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TABLE I. DEAE-cellulose fractionation of *Alternaria* extracts

Pool	Eluting buffer (pH 8.0) (mol/L)	% RAST inhibition, 100 µg antigen*
I	phosphate, 0.005	25.0
II	phosphate, 0.008, NaCl, 0.03	36.7
III	phosphate, 0.025, NaCl, 0.21	86.4
IV	phosphate, 0.04, NaCl, 0.4	40.2

*Sorbent: 70% (NH₄)₂SO₄ precipitate of crude *Alternaria* extract (2 mg of protein per milliliter) coupled to CNBr-activated Sepharose 4B. RAST inhibition was performed as described previously.⁸ Lyophilized pools from batch DEAE are dissolved in RAST buffer; % inhibition is given in the presence of 100 µg of antigen: % Inhibition = $100 - \frac{\text{cpm in presence of inhibitor} - \text{cpm bound nonspecifically}}{\text{cpm in absence of inhibitor} - \text{cpm bound nonspecifically}} \times 100$.

Laboratories, Detroit, Mich.), 10 gm. *Alternaria* mats predominantly comprising mycelia were harvested by filtration, defatted with acetone, and air-dried. Defatted *Alternaria* powder was extracted overnight at 4° C in 0.05 mol/L of Tris HCl, pH 8.0 (1:20 w/v) (Sigma Chemical Co., St. Louis, Mo.). The crude extract was dialyzed overnight against 0.1 mol/L NH₄HCO₃, lyophilized, resuspended in 0.005 mol/L of sodium phosphate, pH 8.0 (14 mg/ml), absorbed to DEAE cellulose (DE52, Whatman, Inc., Clifton, N. J.), and eluted stepwise with buffers of increasing ionic strength (Table I). The eluates were pooled, as presented in Table I, dialyzed against 0.1 mol/L NH₄HCO₃, and lyophilized. Pool III was found by RAST inhibition to be the most potent inhibitor (Table I).

Human serum—direct RAST

For serum incubations we used pooled sera from 13 *Alternaria*-sensitive subjects demonstrating IgE binding 3.5 to 29.8 times that of a normal control serum in the RAST. We also used the individual serum from *Alternaria*-allergic subjects that were included in the pool. Sera from three nonallergic individuals and from three individuals allergic to ragweed but not *Alternaria* were included as controls. The direct RAST procedure used a 70% (NH₄)₂SO₄ precipitate of crude *Alternaria* extract (2 mg of protein per milliliter) coupled to CNBr-activated Sepharose 4B as the solid-phase sorbent and was performed as previously described.⁸

SDS-PAGE analysis of *Alternaria* extract

The discontinuous buffer system of Laemmli⁹ was used for SDS-PAGE with a 3.9% polyacrylamide stacking gel and a 12.5% resolving gel. DEAE pool III was treated at 100° C for 2 minutes in the presence of SDS and 2-mercaptoethanol. The protein content of the 50 µl samples was 2 mg/ml, as determined according to the method of Bradford¹⁰ with a commercial assay kit (Bio-Rad Labora-

TABLE II. Direct RAST to *Alternaria*

Source of serum	cpm X/cpm normal*
<i>Alternaria</i> -sensitive patients	
A1	3.50
A2	22.88
A3	10.47
A4	4.80
A5	8.60
A6	7.79
A7	5.26
A8	17.74
A9	6.36
A10	29.81
A11	17.42
A12	10.95
A13	18.65
AP†	12.97
Ragweed-sensitive control subjects	
RW1	0.68
RW2	0.97
RW3	0.88
Normal control subjects	
N1	1.00
N2	1.08
N3	0.89

Sorbent: 70% (NH₄)₂SO₄ precipitate of crude *Alternaria* extract (2 mg of protein per milliliter) coupled to CNBr-activated Sepharose 4B. Direct RAST performed as previously described.⁸

* $\frac{\text{cpm X}}{\text{cpm normal}} = \frac{\text{counts per minute bound by test serum}}{\text{counts per minute bound by normal serum}}$

†Pooled *Alternaria*, sensitive sera, A1 to A13.

tories, Richmond, Calif.) with bovine gamma globulin as standard. Uninoculated culture medium (50 µl of sample) was used as a control. The inoculated culture medium was not tested. Electrophoresis was performed at a constant current of 30 mA per gel until the bromphenol blue tracking dye was about 1 cm from the bottom of the gel. A portion of the gel was sliced and stained with Coomassie blue R250. The remainder of the gel was used for the Western blot.

Western blot procedure

Antigens were transferred from the SDS gel to nitrocellulose (BA83, 0.20 micropore size, Schleicher & Schuell, Inc., Keene, N. H.) with a slight modification according to the method of Burnette.⁴ Before transfer, the unstained portion of the gel was washed for 30 minutes in 20 mmol/L of Tris base, 150 mmol/L of glycine (pH 8.3), 20% methanol, and 0.5% Triton X-100 (Sigma Chemical Co.).¹¹ Transfer to the nitrocellulose was performed in the 20 mmol/L of Tris, 150 mmol/L of glycine, and 20% methanol transfer buffer at 70 V for 17 to 20 hours with cooling, with a Hoefer Transphor unit (Hoefer Scientific Products, San Francisco, Calif.). After transfer, a portion of the nitrocellulose was stained (1) for protein with 0.1% amido black in 45% methanol and 10% acetic acid and rapidly destained (5 minutes maximum) in 90% methanol and 2%

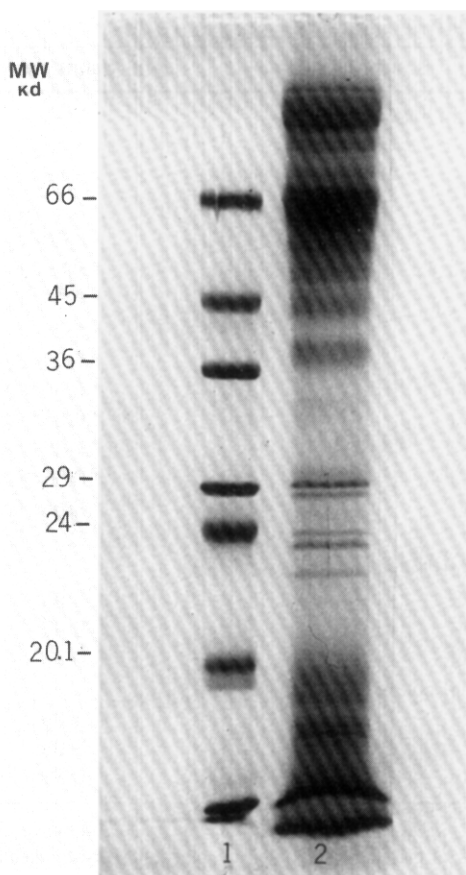


FIG. 1. SDS-polyacrylamide gel, 12.5%, stained with Coomassie blue R250. *Lane 1*, protein molecular weight markers from top: BSA (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000 sub-unit), carbonic anhydrase (29,000), trypsinogen (24,000), and soybean trypsin inhibitor (20,100). *Lane 2*, *Alternaria* DEAE pool III, approximately 100 μ g of protein.

acetic acid,¹² or (2) for carbohydrate with PAS reagent. The gel was stained with Coomassie blue to detect any proteins that had not been transferred.

Detection of IgE binding to *Alternaria* allergens

IgE binding to *Alternaria* allergens transferred to the nitrocellulose was detected as follows: Unoccupied sites on the nitrocellulose were blocked with 3% BSA in 10 mmol/L of Tris HCl, 0.9% NaCl (pH 7.4) (TBS), plus 0.02% NaN₃ for 1 hour at 40° C with gentle shaking. The nitrocellulose was cut into strips corresponding to the sample lanes and transferred to a multiwell incubation tray for serum incubations. Strips were incubated overnight with sera diluted in 1:5 in TBS plus BSA plus NaN₃ (total volume, 5 ml). A control strip of nitrocellulose without antigens was blocked with BSA and incubated with TBS plus BSA plus NaN₃ instead of serum. The strips were washed once with TBS for 10 minutes twice with TBS plus 0.05% Nonidet P-40 (Sigma Chemical Co.) for 10 minutes each, and once more with TBS. Strips were then incubated overnight

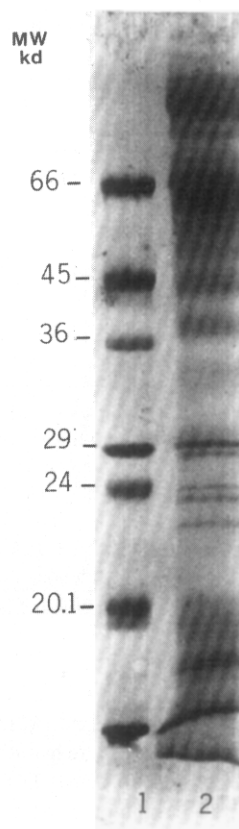


FIG. 2. Protein (amido black) staining pattern on nitrocellulose after transfer. *Lane 1*, protein molecular weight markers. *Lane 2*, *Alternaria* DEAE pool III.

with 3.5×10^5 to 4.0×10^5 cpm per strip of ¹²⁵I-labeled anti-IgE (Phadebas RAST isotope unit, Pharmacia Diagnostics, Piscataway, N. J.). After a final set of washes, the strips were wrapped in plastic film and placed on XAR-5 x-ray film (Eastman Kodak Co., Rochester, N. Y.) for autoradiography at -20° or -70° C.

CRIE

The ability to detect IgE-binding components in the *Alternaria* extract by Western blotting was compared to CRIE results by methods previously described² with sera of two patients. Ten microliters of crude *Alternaria* extract (50 mg/ml) diluted 1:1 in 0.05 mol/L of Tris HCl, pH 8.0, was applied to a 3 mm well in a gel (1% agarose in 0.073 mol/L of Tris base, 0.024 mol/L of barbital, 0.006 mol/L of calcium lactate, pH 8.6) on a 10 by 10 cm glass plate. The first dimension electrophoresis was carried out for 25 minutes at 10 V/cm. Gel strips (1 by 5 cm) were cut from the plate and transferred to individual 5 by 7 cm plates. Two milliliters of agarose solution containing 400 μ L of pooled anti-*Alternaria* rabbit serum was poured onto the plate to act as a second dimension reference gel; 100 μ L of the rabbit antiserum in 1 ml of agarose was pipetted onto the space below the first dimension gel to detect potentially migrating antigens. Second dimension electrophoresis was

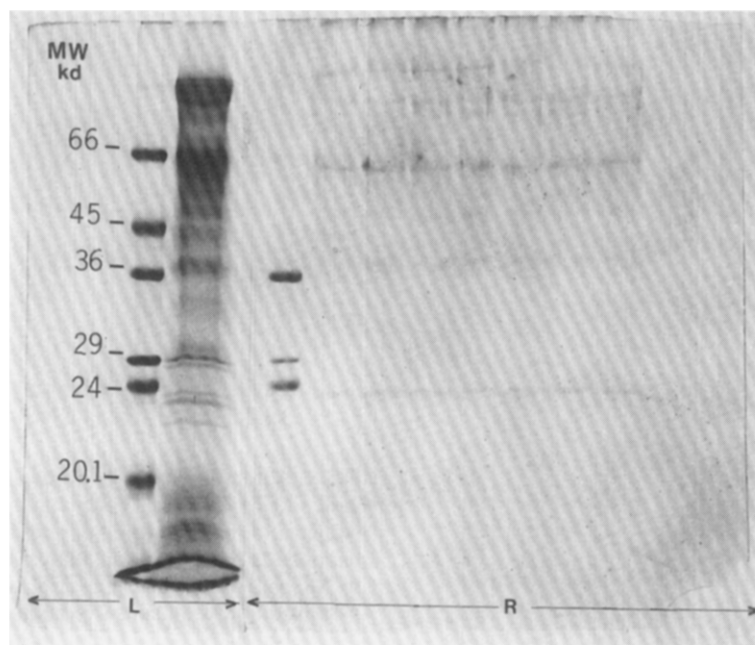


FIG. 3. Coomassie blue stain of SDS-polyacrylamide gel after transfer to nitrocellulose. *Left*, protein staining pattern of portion of gel not subjected to transfer. *Right*, protein staining pattern of remainder of gel after transfer.

conducted at 2 V/cm for 18 hours. After washing and pressing, the gels were incubated with 400 μ L of patient serum in 6.6 ml of 0.07 mol/L of sodium phosphate buffer, 0.15 mmol/L of NaN_3 , pH 7.0, at 25° C for 24 hours. The gels were washed and then incubated with ^{125}I -labeled antihuman IgE (Pharmacia) (2×10^5 counts per plate) in 0.05 mol/L of sodium phosphate buffer, pH 7.5, 0.3% BSA, 15 mmol/L NaN_3 , and 2.7 mmol/L Na-EDTA for 24 hours. The gels were then washed, dried, and exposed to Kodak XRP-1 film for 1, 3, 7, 14, 30, and 60 days at -20° C.

RESULTS

Human serum—direct RAST

RAST results for the sera used in the immunoblotting procedures are presented in Table II.

SDS-PAGE analysis of *Alternaria* extract

Fig. 1 illustrates a typical protein separation pattern of DEAE pool III in a 12.5% gel. At least 30 different protein bands can be identified, the most prominent bands being a pair of bands with apparent molecular weights around 66,000. There was no observable Coomassie blue staining with the uninoculated culture medium.

Western blot detection of IgE-binding *Alternaria* allergens

The staining pattern after antigen transfer from the gel to nitrocellulose is illustrated in Fig. 2. It can be observed that a faithful replica of the protein sepa-

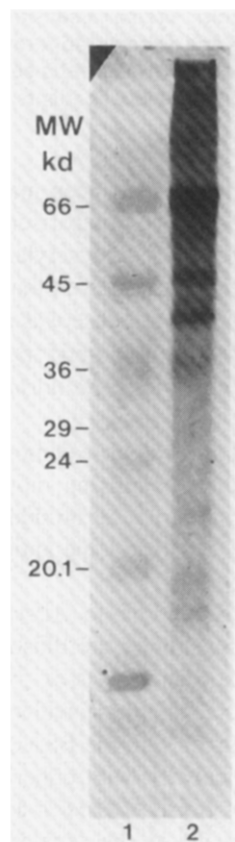


FIG. 4. PAS staining pattern on nitrocellulose after transfer. *Lane 1*, molecular weight markers. *Lane 2*, *Alternaria* DEAE pool III.

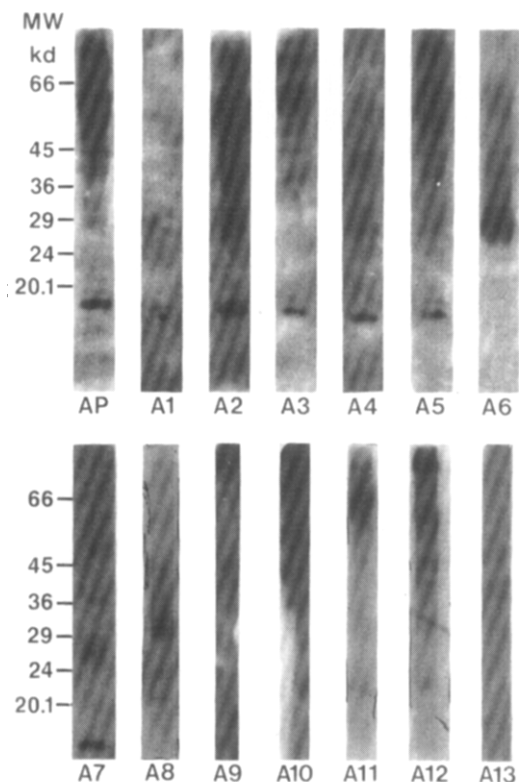


FIG. 5. Autoradiographs of nitrocellulose strips incubated with sera from *Alternaria*-sensitive subjects and ^{125}I -labeled anti-IgE and exposed to x-ray film for 1 to 3 days. AP, pooled sera from *Alternaria*-sensitive subjects; A1 to A13, sera from individual patients.

ration pattern of the gel has been made on nitrocellulose. Fig. 3 illustrates a comparison of the protein staining pattern of an SDS-polyacrylamide gel before (*left*) and after (*right*) transfer. Although transfer of the proteins to the nitrocellulose was not complete in 17 to 20 hours, the electrophoretic transfer procedure was effective in removing *Alternaria* proteins from the gel.

PAS staining of nitrocellulose demonstrated dye uptake by carbohydrate-containing moieties with molecular weights primarily at 66,000, 45,000, and 40,000. This suggests that some antigens are glycoproteins (Fig. 4).

Patterns of IgE binding to *Alternaria* allergens transferred to nitrocellulose are illustrated in Figs. 5 and 6. Only background levels of ^{125}I binding were detected with serum from the nonallergic or ragweed-allergic individuals or with the BSA control strip. In contrast, strips incubated with either pooled sera or individual serum from *Alternaria*-allergic subjects demonstrated that 20 of the separated components bound ^{125}I . In the serum pool of 13 *Alternaria*-

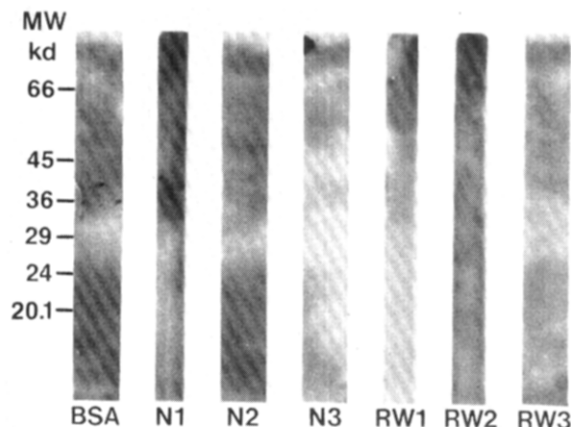


FIG. 6. Autoradiographs of nitrocellulose strips incubated with sera from normal control subjects, ragweed-allergic subjects, or no serum (BSA) and ^{125}I -labeled anti-IgE and exposed to x-ray film for 3 days. BSA, control strip; N1 to N3, sera from normal control subjects; RW1 to RW3, sera from ragweed-sensitive subjects.

sensitive individuals, IgE is directed mainly against allergens with molecular weights $>40,000$. Strips incubated with individual serum included in the pool demonstrate more clearly the individual components in *Alternaria* that bind IgE and the real differences in IgE-binding patterns between patients. Autoradiographs for individual serum and pooled sera from *Alternaria*-allergic subjects and control subjects are illustrated in Fig. 5. An allergogram demonstrating the molecular weights of the IgE-binding allergens is presented in Table III.

CRIE

Results of our CRIE studies in *Alternaria*-sensitive patients have been previously reported.² Autoradiographs with serum from subjects A3 and A11 are illustrated in Fig. 7. Both subjects had IgE binding to a similar component, and serum from subject A11 also recognized a second component.

DISCUSSION

The transfer of antigens from a polyacrylamide gel to nitrocellulose has become widely used in immunology and is perhaps the ideal method for identifying and characterizing allergens in complex mixtures. We have used this technique to detect IgE binding to allergens from extracts of *Alternaria* mycelia that were separated by SDS-PAGE. Four distinct molecular weight regions of IgE binding were observed: (1) 55,000 to 60,000, (2) 40,000 to 45,000, (3) 35,000 to 40,000, and (4) 15,000 to 20,000. IgE binding was detected only when the nitrocellulose strips were incubated with sera from individuals who had specific

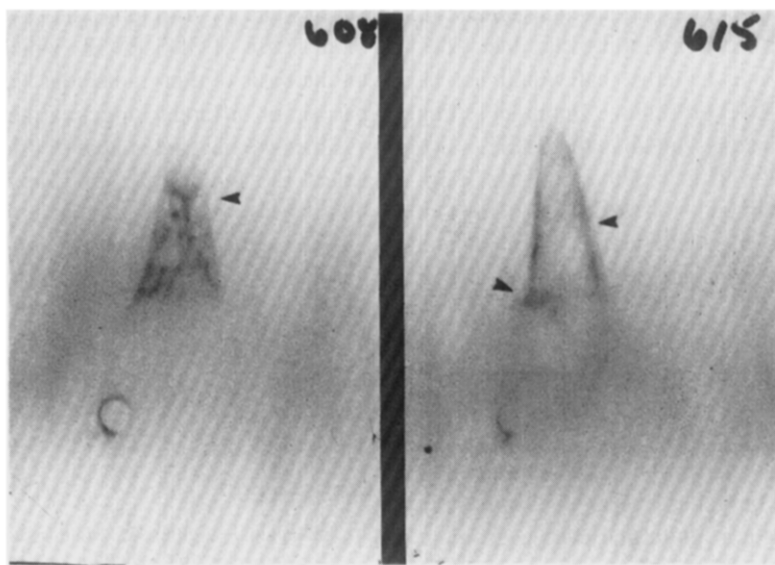


FIG. 7. CRIE autoradiograph with serum from patient A3 (left) and patient A11 (right), demonstrating IgE binding to crude *Alternaria* extract antigens (arrows).

TABLE III. Allergogram of IgE-binding antigens in *Alternaria* extracts

Patient	Molecular mass (kilodaltons)													
	>15- 20	>20- 25	>25- 30	>30- 35	>35- 40	>40- 45	>45- 50	>50- 55	>55- 60	>60- 65	>65- 70	>70- 75	>75- 80	>80- 85
1	X													
2	X		XX		X			X	X			X		
3	X					X	X	X	X		X		X	
4	X		X		X									
5	X				X			X	X			X		
6			XX			X		X						
7	X		XX		X									
8		X			X	X					X			
9					X				X					
10						X			X			X		
11						X			X		X			X
12					X	X	X					X		
13					X		X							
Total responders	6	1	4*	0	8	6	3	4	6	0	3	4	1	1

*Three subjects responded to two antigens having molecular masses between 25 and 30 kd.

IgE to *Alternaria*. Nitrocellulose strips incubated with sera from patients with IgE directed against ragweed but not *Alternaria* did not exhibit any nonspecific IgE binding to *Alternaria* antigens transferred to the nitrocellulose.

As can be observed from Fig. 7, sera from both subjects (A3 and A11) demonstrated IgE binding to a similar allergen (as demonstrated by electrophoretic

mobility pattern). Serum from subject A11 also had IgE binding to a second allergen. In contrast, Western blotting demonstrated that serum from subject A3 recognized seven IgE-binding components separated by SDS-PAGE, and serum from subject A11 recognized four binding components (Table III). Therefore, Western blotting may permit better discrimination of IgE-binding components in complex mixtures.

We also demonstrated that among individuals allergic to *Alternaria*, Western blotting can detect differences in IgE-binding patterns that would not be possible with the RAST procedure. Immunoblotting elicits more discriminating data than the RAST, since it allows identification of the reactive antigens. Repetition of these experiments with additional sera from patients may lead to the identification of major and minor *Alternaria* allergens and determination of their molecular weights.

The isolation of an allergenic fraction from *Alternaria*, "Alt-I," has been reported by Yunginger et al.¹³ Alt-I is eluted from a gel filtration column in the molecular weight range between 25,000 and 50,000. This is somewhat lower than the apparent molecular weights of most of our allergens as determined by SDS-PAGE and Western blotting. Because different methods were used to estimate the molecular weights of Alt-I and the allergens in our sample, we cannot dismiss the possibility that one of the allergens we have identified and Alt-I have similar molecular weights. In the future we plan to determine if any of the allergens that we have identified by Western blotting demonstrate immunologic identity with Alt-I.

We chose to use SDS-PAGE because it resulted in better resolution of *Alternaria* proteins (approximately 30 bands in a 12.5% gel) than other polyacrylamide gel electrophoresis techniques that we tested, and it also elicited information about the molecular weights of the different antigens. Fewer bands and smearing of proteins were observed with discontinuous native gels (7.7% or 10% acrylamide gels) and in an isoelectric focusing gel of pH 3.5 to 5.5 (data not presented).

One disadvantage of SDS-PAGE is that proteins are denatured before electrophoresis. Some *Alternaria* allergens might not be detected if they do not renature sufficiently. Nevertheless, our results demonstrate that IgE binding to *Alternaria* allergens can be detected despite the denaturing conditions of the sample treatment. Further modifications in sample treatment or renaturing conditions may reveal the presence of additional allergens.

Despite the denaturing effect of SDS and 2-mercaptoethanol, we believe that SDS-PAGE followed by Western blotting is well suited for the study of fungal allergens. Given the complexity and variability of fungi, a technique such as SDS-PAGE that can resolve complex allergen preparations is desirable. Also, since allergen extracts are prepared from different strains of an organism and from different

species and by different culture and harvesting techniques, use of SDS-PAGE, which separates proteins on the basis of their molecular weights, permits comparison of different fungal extracts.

Since we have been able to use Western blotting to detect IgE binding to *Alternaria* allergens, Western blotting may be an important supplement to CRIE for the identification and characterization of fungal allergens and the standardization of fungal allergen preparations.

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