

Antigens and allergens of *Aspergillus fumigatus*

II. Their further identification and partial characterization of a major allergen (Ag 3)

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These further studies demonstrated that most antigens and allergens detected by allergic bronchopulmonary aspergillosis (ABPA) sera were recognized by rabbit antiserum, and a major allergenic component, Ag 3, as previously demonstrated by self-crossed radioimmunoelectrophoresis, was identified in the conventional system. Affinocrossed immunoelectrophoresis with concanavalin A indicated that for the patients with ABPA, although the major antigens contained α -D-manno- or α -D-glucopyranoside terminal residues, the major allergenic components did not. Fractionation of the salt-precipitated, protein-enriched fraction by gel filtration on Sephacryl S-200, monitored by fused-rocket immunoelectrophoresis/fused-rocket radioimmunoelectrophoresis with both the ABPA serum pool and specific rabbit antisera, revealed that most of the antigens were in a molecular weight range >43 kd, whereas the allergens spread from as low as 10 to >100 kd. The major allergen, Ag 3, was identified and demonstrated to have a molecular weight of approximately 24 kd. This component does not bind to concanavalin A and is also very heat labile. (J ALLERGY CLIN IMMUNOL 78:18-24, 1986.)

Recent studies with the use of sera of patients with ABPA in self-XRIE tests^{1,2} demonstrated in vitro formation of IgG/IgE antigen complexes and revealed that the antigenic components of *Aspergillus fumigatus* were of two main types: antigens that produced the strongest precipitin reactions with only weak binding of specific IgE (minor allergenic components) and antigens that appeared to be poorly precipitating but demonstrated strong IgE binding. Of these latter components, three antigens, i.e., Ags 2, 3, and 15, since they reacted with $>50\%$ of sera tested, were regarded as representing major allergenic components.³ Furthermore, one antigen in particular, Ag 3, appeared to be predominant, reacting with 75% of sera and also eliciting the highest degree of radiostaining with most sera.

The purpose of the present study therefore was to

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
XIE:	Crossed immunoelectrophoresis
XRIE:	Crossed radioimmunoelectrophoresis
Con A:	Concanavalin A
FRIE:	Fused-rocket immunoelectrophoresis
FRRIE:	Fused-rocket radioimmunoelectrophoresis
MW:	Molecular weight
kd:	Kilodaltons
Af:	<i>Aspergillus fumigatus</i>
pp:	Protein precipitate
OD:	Optical density

investigate further the physicochemical characteristics of these two types of antigenic components of *Af* and in particular to characterize further this major allergen.

MATERIAL AND METHODS

Antigens and Sera

Af antigens B27, BN63, fraction *Af*-pp, human sera, and rabbit antisera were as described in the previous article.⁴

Quantitative immunoelectrophoretic techniques

XIE and *XRIE* were performed as previously described.⁴ *FRIE* was performed essentially as described by Harboe

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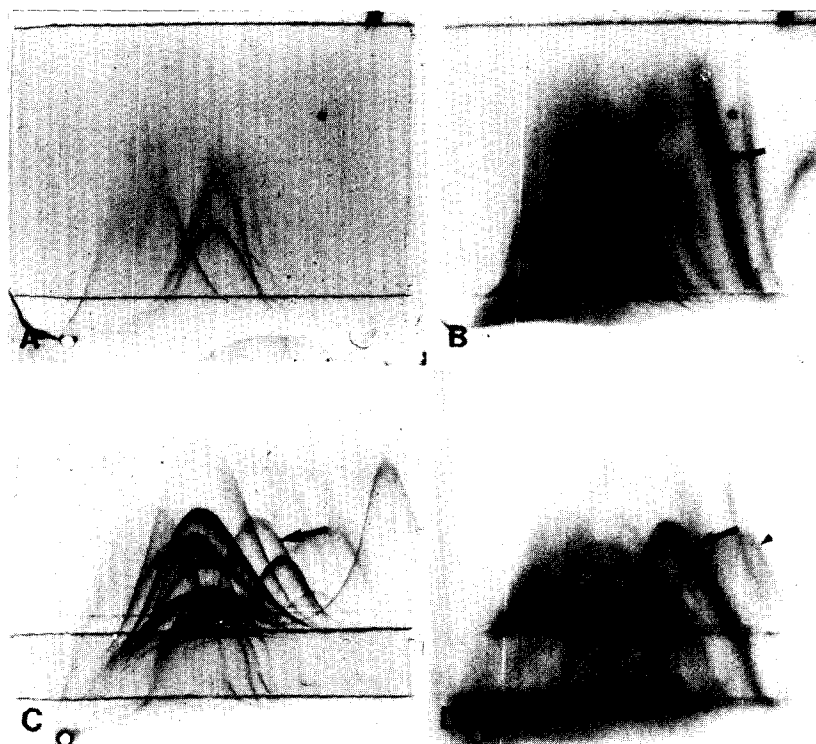


FIG. 1. Self-XRIE test of the reference ABPA serum pool. **A-B** (30 µg *Af*-B27 with 25 µl of serum per square centimeter of gel) compared to conventional XRIE incorporating the ABPA serum in an intermediate gel **C-D** (100 µg of *Af*-B27 with ABPA serum, 35 µl/cm² in the intermediate gel, and rabbit antiserum, 7 µl/cm², in the main antiserum gel. **A** and **C**, Protein stained. **B-D**, Corresponding autoradiographs.). Reactions of partial identity between the human serum and rabbit antiserum are visible in the intermediate gel (**C**). The major allergen Ag 3 is indicated by the arrow, and the three (weakly stained) allergen peaks not recognized by the ABPA serum are indicated by arrowheads.

and Svendsen.⁵ Buffered agarose, as for XIE, was poured onto prewarmed sheets of GelBond (FMC Corp., Bio-Products, Rockland, Me.), 12.5 by 11 cm, to form gels of 1.2 mm thickness. Areas 9.5 by 3.5 cm were then removed and replaced to the same depth with buffered agarose (56° C) containing appropriate serum/antiserum. Antigen wells (1.5 mm in diameter), which were cut in a zigzag fashion into the gel 5 mm below the antibody containing gel (according to a template placed under the GelBond), were filled with alternate fractions from the Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column, allowed to diffuse for 30 minutes, and then electrophoresis was carried out at 1.5 v/cm overnight.

The gels were treated for FRRIE as for XRIE.

Gel filtration

Gel filtration of *Af*-pp (200 mg/3 ml) was carried out at room temperature on a 90 by 2.6 cm column of Sephacryl S-200, equilibrated in NH₄HCO₃ (0.05 mol/L, pH 8) with flow rate 30 ml/hr, and the eluate, monitored at 280 nm (Uvicord II, LKB, Bromma, Sweden), was collected in 4 ml fractions. The column was calibrated with MW standards (Pharmacia Fine Chemicals, Uppsala, Sweden), blue dextran 2000 (BDX), albumin (67 K), ovalbumin (43 K), chymotrypsinogen A (25 K), cytochrome C (12.5 K) and vi-

tamin B₁₂ (1.4 K). The elution profile was also analyzed by FRIE and FRRIE by use of both rabbit antisera and pooled ABPA sera.

RESULTS

Components recognized by human and rabbit antibodies

Incorporation of pooled ABPA serum in the intermediate gel of conventional XIE with rabbit antiserum in the main gel (Fig. 1) identified those components to which both human sera and the rabbit antisera had precipitating antibodies (i.e., comparison of Fig. 1, C with control XIE, Fig. 1, A). For some antigenic peaks, there was not complete identity of reactions between the human and rabbit sera, suggesting that the two species are recognizing different epitopes. However, although the components recognized by ABPA sera were antigenic for the rabbit, there were, as expected, many more components immunogenic for the rabbit that appeared as distinct peaks with no alteration in their height in the main (rabbit antiserum) gel (i.e., not "absorbed" by the ABPA serum pool).

By comparing their associated autoradiographs

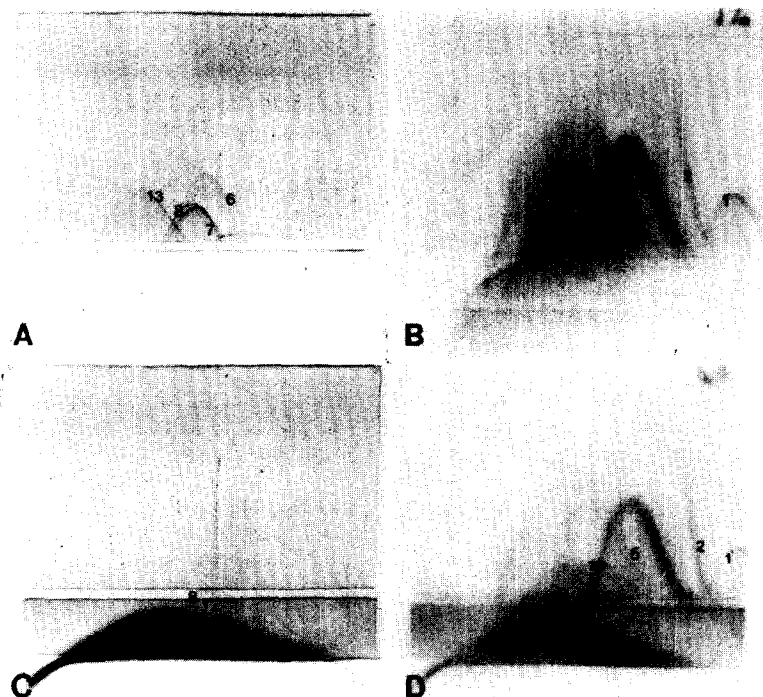


FIG. 2. Self-XRIE tests of the pooled ABPA reference serum. **A-B**, The control test. **C-D**, Incorporating Con-A ($25 \mu\text{g}/\text{cm}^2$) in the intermediate gel, $25 \mu\text{g}$ of *Af*-B27 with ABPA serum, and $25 \mu\text{l}/\text{cm}^2$ in the main gel. **A** and **C**, Protein stained XIE. **B** and **D**, Corresponding autoradiographs. Note removal of antigen peaks, i.e., Ags 6, 7, 13, and, partially, Ag 8, from the main gel after incorporation of Con A, **C** compared to **A**, whereas the allergen peaks, i.e., Ags 1, 2, 3, 5 are not affected, Ag 10, partially, and Ag 11 completely absorbed, **D** compared to **B**. Numbering of the peaks same as in reference 2.

(Fig. 1, *B* and *D*), one peak that demonstrated strong binding of specific IgE antibody in the conventional XRIE (i.e., dense staining in the autoradiograph) could, by its absorption by the ABPA serum pool, be identified as Ag 3, the major allergen of the self-XRIE system.² Although other allergenic peaks were also absorbed by the ABPA pool, three distinct, weakly stained radioactive peaks were detected, indicating additional allergenic (possibly minor) components to which the ABPA pooled sera did not appear to have IgG antibodies.

Physicochemical differences between major antigenic and allergenic activities

With affino-XIE, i.e., with Con A incorporated in an intermediate gel of the "reference" self-XRIE pattern² (Fig. 2), the major precipitating antigens, viz, Ag 6, Ag 7, and Ag 13, were completely absorbed, whereas the remaining one, Ag 8, was partially absorbed. By contrast, the major allergens, i.e., those components not readily visualized by protein staining but demonstrating strong IgE binding, viz, Ags 1, 2, and 5, and in particular, Ag 3, were unaffected, and

only two minor allergens, viz, Ags 11 and 10, demonstrated complete and partial absorption, respectively.

It was not possible here to determine whether the slow migrating major allergen, Ag 15, was affected or not by Con A; however, in the conventional system (not presented) this component did appear to be unaffected.

Gel filtration of *Af*-pp

Sephacryl S-200 fractionation of *Af*-pp yielded multiple peaks, both antigenic and allergenic, over a considerable MW range (Fig. 3, *A* through *C*). From the OD profile (Fig. 3, *A*) an initial sharp peak eluted some tubes after the void volume (blue dextran 2000) with a $\text{MW} \geq 150 \text{ kd}$ (MWs were determined from an elution calibration curve), followed by many overlapping peaks, with the area of highest absorption, 12.5 to 1.4 kd, having a yellowish-brown color.

The FRIE profile (Fig. 3, *B*) with rabbit antiserum demonstrated the complexity of the antigen elution. The initial OD peak consisted of two main components $\geq 150 \text{ kd}$ (fractions 54 to 62), followed by a group of

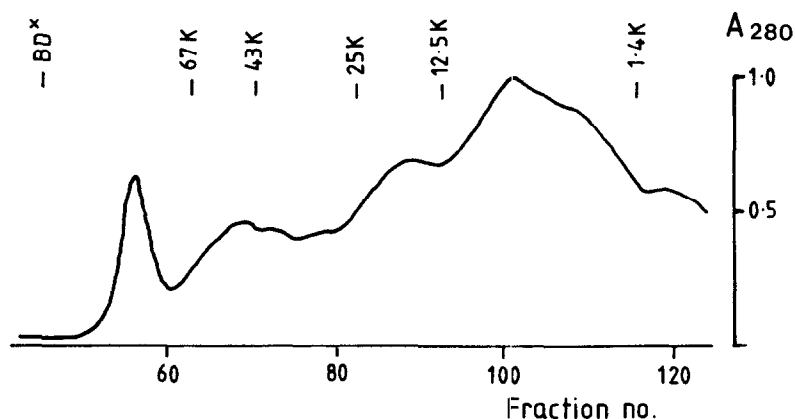


FIG. 3A. Gel filtration of *Af*-pp on Sephacryl S-200. OD profile demonstrating the calibration of the column with the MW protein standards.

at least 16 components between 60 to 35 kd (fractions 64 to 74), and a further approximately 10 antigens in the range 35 to 11 kd (fractions 76 to 96).

The FRRIE profile (Fig. 3, C) also reflected the complexity of allergenic components eluting over the entire MW range, with even the ≥ 150 kd components demonstrating strong uptake of radioactivity as well as an apparent very low MW component contained in the last fractions from the column (fractions 90 to 120). One peak, in particular, eluting in fractions 80 to 88, maximal in fraction 84 with MW 24 kd, merits attention and will be discussed later.

Elution of *Af*-pp was also monitored in a self-FRIE/FRRIE (compare self-XRIE²) in which the pooled ABPA serum was used to precipitate the antigenic components and as the serum overlay before the ¹²⁵I-labeled anti-IgE and autoradiography procedure as usual for FRRIE (Fig. 3, D and E). Most of the visible protein staining antigenic components are apparent in fractions with MWs >43 kd (Fig. 3, D), one, approximately 50 kd, and two, ≥ 150 kd, whereas the corresponding autoradiograph (Fig. 3, E) again demonstrates the wider MW range of allergenic components from the highest to lowest MW, many not visualized by the protein staining. In particular, the component with its maximum peak height fraction 84, i.e., MW 24 kd, demonstrating the strongest radioactive uptake, compare conventional FRRIE.

Identification of the major allergen, Ag 3

XRIE of fraction 84 (Fig. 4, A and B) revealed the presence of a small discrete antigen peak also demonstrating strongly on the autoradiograph and having the electrophoretic mobility of the major Ag 3 allergen. Other more weakly stained and rather heterogeneous peaks with different mobilities were also vis-

ible by protein staining (four weak peaks), and on autoradiography, six weak peaks.

Further confirmation for the presence of the major Ag 3 allergen was obtained from crossed-line immunoelectrophoresis (Fig. 4, C and D) in which fractions 81 to 87 were incorporated in the intermediate gel. This resulted in complete removal of the antigenic peak identified as Ag 3 from the B27 antigenic profile by the process referred to as absorption of antibodies in situ.⁶ Only two other components with quite different electrophoretic mobility to Ag 3 were similarly absorbed. Most of the other components remained with peaks unaltered, although a few did form lines of identity with slightly increased peak heights, indicating no or only trace quantities (respectively) of these components in this combined fraction.

DISCUSSION

A previous study of 32 ABPA sera by self-XRIE² had identified a group of components that appeared poorly precipitating but highly allergenic, of which one, Ag 3, was regarded as representing a major allergen, since 75% of the sera demonstrated strong IgE binding. This major allergen has now been identified in the conventional XRIE system, also as a peak that demonstrated particularly strong binding of radioactivity by use of the ABPA pooled serum and is possibly one of the two major allergens identified in mycelial extracts.⁷

The rabbit antisera, as expected,⁸ identified many more antigenic components than were recognized by ABPA sera. Three of these were also demonstrated to be allergenic. Previous findings of a close correlation ($p < 0.001$) between RAST and self-XRIE grading² had indicated the possibility that patients with ABPA had IgG antibody to most of those components to

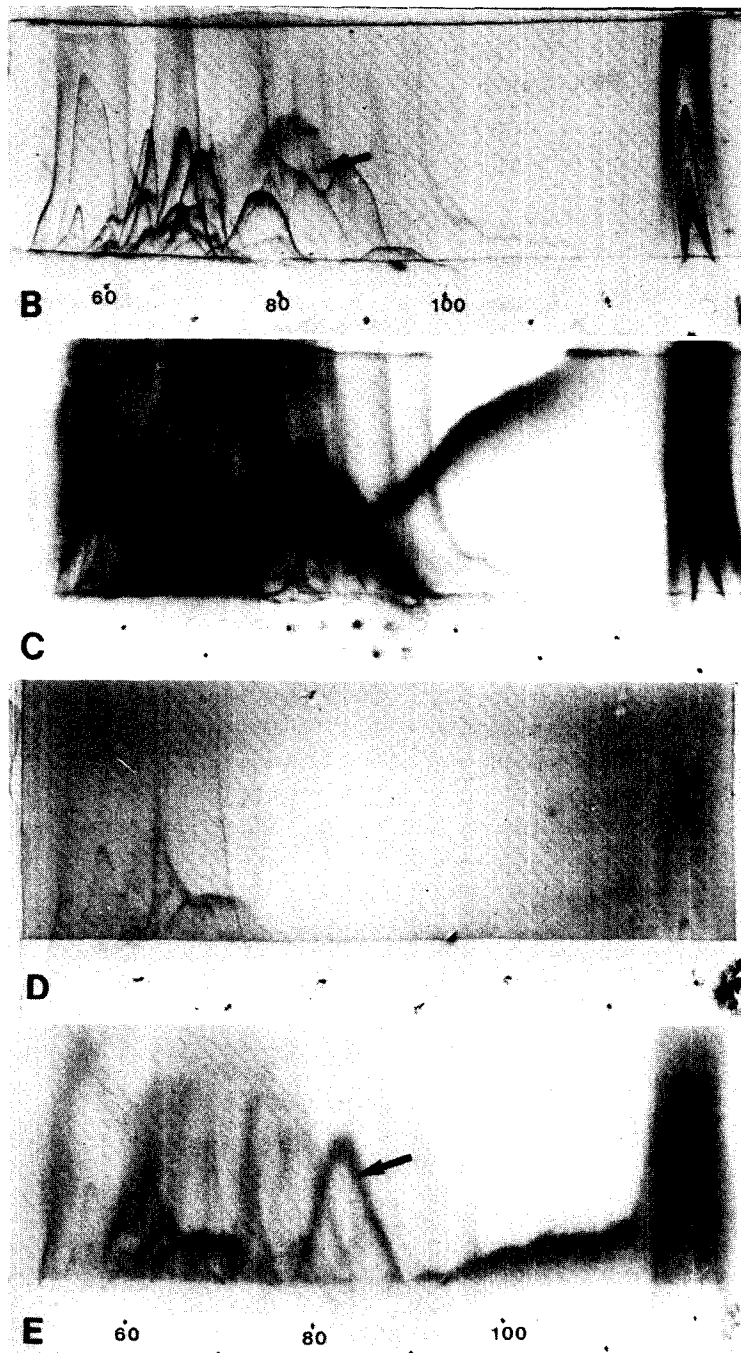


FIG. 3. B-C, FRIE/FRIIE profiles with 4.5 μ l of each fraction as obtained from the column (60 μ g of original Af-pp in the extreme *right hand well*) with rabbit antiserum, 8 μ l/cm², incorporated in the gel. D-E, Self-FRIE/FRIIE profiles with 1.5 μ l of each fraction (15 μ l of original Af-pp in *right hand well*) with the ABPA serum pool, 25 μ l/cm², incorporated in the gel. Ag 3 peak indicated by the arrow.

which they have their IgE antibody. Although this may still be true for most sera, there are obviously at least three components antigenic for rabbits that were not recognized by specific IgG of the ABPA serum pool.

By incorporating the lectin Con A into the intermediate gel of the reference self-XRIE pattern, three of four of the group of antigens producing the strongest precipitin reactions were completely absorbed, and the remaining one was partially absorbed. This

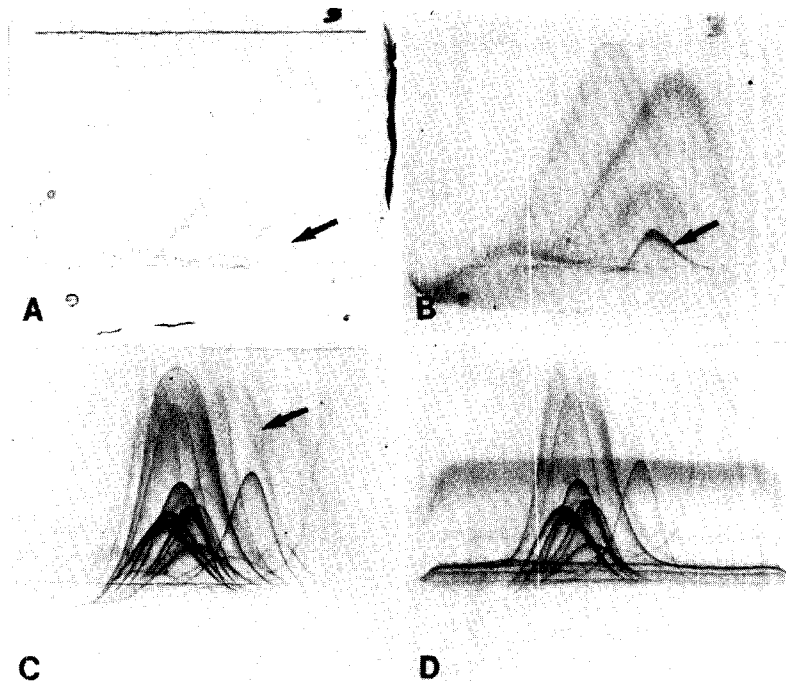


FIG. 4. A-B, XRIE of fraction 84 from gel filtration of *Af*-pp (4.5 μ l fraction with rabbit antiserum, 8 μ l/cm², in the gel). Ag 3 peak indicated by the arrow. C-D, Crossed-line immunoelectrophoresis of B27 (100 μ g with 7.5 μ l/cm² of rabbit antiserum). C, Control. D, Fractions 81 to 87 from gel filtration column (freeze-dried) incorporated in the intermediate gel (120 μ g/cm²). Note "absorption" of the Ag 3 peak.

finding therefore confirms several recent articles of the binding of some antigenic components of *Af* by Con A,⁹⁻¹¹ indicative of their glycoprotein nature and suggesting the presence of either α -D-manno- or α -D-glucopyranoside end residues.

By contrast, at least four, but possibly all five, of the major allergenic components including Ag 3 did not bind to the Con A, indicating the absence of such end residues and thus a different physiochemical nature of these allergenic components compared to the major precipitinogenic components. Only two relatively minor allergenic components demonstrated either complete or partial absorption. In this respect it is of interest that antigens not absorbed by Con A appeared to react in ELISA to detect IgG antibodies more strongly in sera of patients with ABPA than in patients with aspergilloma.¹⁰ This may have been due to detection of the IgG antibodies to the poorly precipitating components that demonstrate high IgE binding and to which patients with aspergilloma possibly do not develop either IgE or IgG antibodies.

Although major allergens of various other allergenic sources have been characterized,¹² no information is available regarding their binding (or otherwise) to Con A. This is the first report therefore indicating that allergens possibly do not possess the

necessary terminal glycosidic linkages to bind to this lectin.

The gel filtration of the protein precipitate on Sephacryl S-200 monitored by FRIE/FRRIE revealed a resolution of its multiple antigenic components over a wide range of MWs and a paralleled spread of allergenic activity, pointing to the molecular complexity of allergenic components. Although most components were eluted as discrete peaks over relatively few fractions, some eluted over a much larger number of fractions, indicative of a considerable heterogeneity in their MWs. No high MW components were detected eluting at the column front (i.e., with blue dextran), as the 470 kd antigen reported for 2-day mycelial extracts.¹³ This is possibly because either it is not liberated into the culture filtrate as used here or it has been enzymatically degraded, which, although it is not proved, is suspected of occurring. The first peak of OD antigenic activity here occurred at approximately 150 kd and consisted of two components that also bound radioactivity, indicating their allergenic nature as well. Thus, there are present in *Af* high MW allergenic components similar to those reported for other allergen sources, e.g., *Dermatophagoides pteronyssinus*¹⁴ and *Cladosporium herbarum*.¹⁵

By parallel self-FRIE monitoring of the column,

those mainly precipitating antigenic components, i.e., visualized by protein staining, were all >43 kd. These fractions would therefore include both previously recognized antigens of Af, i.e., the glycoprotein of 150 to 200 kd, to which precipitins have been reported in 10 of 13 patients with ABPA and 14 of 19 patients with aspergilloma,¹⁶ and the partially purified antigen fraction of approximately 70 kd found to be most reactive by ELISA for 17 patients with "pulmonary aspergillosis" (mostly aspergilloma).¹³

The associated autoradiograph confirmed the wider spread of the allergenic components from high to quite low MW and demonstrated one predominant peak (not protein stained) at MW 24 kd. On XIE of this fraction and by crossed-line immunoelectrophoresis, this component could be identified as the major allergen, Ag 3, that does not bind to Con A and, as demonstrated in the accompanying article, is extremely heat labile. It should also be noted that because of the insensitivity of protein staining of antigenic components <43 kd, discrepancies between ELISA and presence of precipitins are likely to occur in their monitoring of gel-filtration columns.

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