

Studies of the dual antibody response in allergic bronchopulmonary aspergillosis

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Bronchopulmonary aspergillosis was diagnosed in a 22-year-old man with pulmonary infiltrates and peripheral eosinophilia on the basis of precipitating antibody to A. fumigatus in serum and characteristic type I and type III responses following intradermal skin testing with the mold. The antibody was eluted from a DEAE-cellulose column, and the activity was shown to be IgG as demonstrated by gel diffusion analysis. These antibodies could be specifically absorbed from the serum by A. fumigatus but not by other members of the Aspergillus genus. Serum reagins were found in the IgE class of immunoglobulins and were developed against determinants common to several Aspergillus molds. However, both in vivo and in vitro absorption experiments established the majority of skin-sensitizing activity to be specific for determinants peculiar to A. fumigatus. Attempts were made to fractionate the allergenic material, and three peaks were obtained by application to a Sephadex G-200 column. The fractions obtained varied in capacity to precipitate with the patient's serum in gel diffusion and to evoke wheal and flare responses at P-K sites sensitized with the patient's serum.

Bronchopulmonary hypersensitivity aspergillosis is an allergic syndrome characterized by episodic bronchospasm associated with transient pulmonary infiltrates and peripheral blood eosinophilia. The disease is produced by an immunological response to the mold, *Aspergillus fumigatus*, present within the respiratory tree. Diagnosis can be made by culturing the mold from the sputum and demonstrating precipitating antibody to *Aspergillus fumigatus* in the patient's serum by gel diffusion. In addition, intradermal challenge with the allergen elicits a characteristic immediate wheal and flare (type I) response followed several hours later by an Arthus (type III) phenomenon. Both reaginic and precipitating antibodies are required for expression of this dual skin reaction and are felt to play major pathogenetic roles in production of the systemic response as well.

Most cases of hypersensitivity bronchopulmonary aspergillosis have been described in England¹⁻³, but recent papers indicate increasing recognition of the syndrome in North America.⁴⁻⁷ The first case of this unusual pulmonary dis-

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order documented in Hawaii is reported below, followed by immunochemical studies of the two antibody types present in the patient's serum.

CASE REPORT

A 22-year-old Caucasian airman began to have symptoms of cough, fever, and general malaise shortly after being stationed on Maui, Hawaiian Islands. A local physician noted diffuse bronchospasm and treated the patient with antibiotics, but symptoms failed to improve. A chest film revealed a large infiltrate in the anterior segment of the right upper lobe. Cough persisted after one week of antibiotic therapy, and the size of the right upper lobe infiltrate increased. The patient was hospitalized on October 10, 1969, with the diagnosis of unresolved pneumonia.

On admission the patient did not appear acutely ill. Blood pressure was 120/90, pulse 104 per minute, temperature 98° F. Pertinent physical findings were confined to the chest where a few scattered rhonchi and wheezes were heard over both lung fields but without evidence of localized consolidation.

The hematocrit was 49 per cent and the white blood cell count was 9,250. A differential count revealed neutrophils 39 per cent, stabs 4 per cent, lymphocytes 36 per cent, monocytes 2 per cent, basophils 1 per cent, and eosinophils 18 per cent. The urine was negative for albumin and sugar and the specific gravity was 1.017. Sputum smears for acid-fast bacilli were negative on two occasions, and pathogens failed to grow on routine sputum cultures. An intermediate purified protein derivative was negative. The previously described right upper lobe infiltrate was present on the chest film.

Treatment was instituted with ampicillin, and, over the course of one week, the patient became asymptomatic. Minimal improvement was noted in the x-ray, and the patient was discharged to continue antibiotic therapy at home. As soon as he left the hospital, severe cough, sputum production, and wheezing recurred, and he was readmitted one week after discharge for treatment of worsening respiratory symptoms.

Physical examination was unchanged from the previous admission. He was afebrile, and diffuse rhonchi and occasional wheezes were heard over both lung fields.

The white blood cell count was 15,300 with 18 per cent eosinophils present on differential count. Many *Neisseria* and moderate *Aerobacter* were cultured from the sputum. The right upper lobe abnormality was still present on the chest x-ray, and an additional infiltrate was present anterior and superior to the original one.

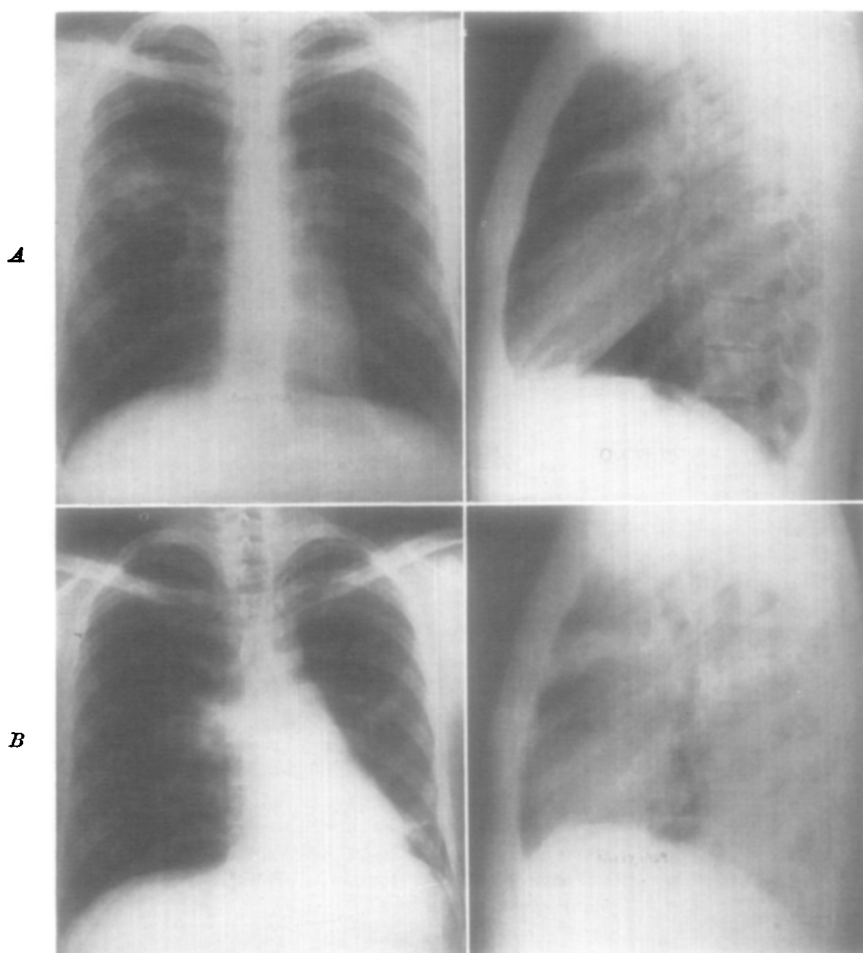
Bronchoscopy did not disclose a cause for the persistent pneumonia, and a diagnosis of Loeffler's pneumonia was made. Prednisone was started at a dose of 60 mg. per day and was tapered to 10 mg. over the course of a week. The patient improved and was discharged without evidence of respiratory distress.

The cough recurred after discharge despite steroid therapy. Repeat x-rays showed persistence of the old infiltrates with additional atelectasis of the left lower lobe (Fig. 1, A and B). At this time the patient was referred to Tripler Army Hospital for further diagnostic studies.

At the time of admission he was asymptomatic, and the physical findings were minimal except for the presence of enlarged, wet, inferior turbinates on nasal examination. Negative laboratory studies included blood count, urinalysis, serology, electrolytes, sputum culture, stool for ova and parasites, protein electrophoresis, and serum immunoglobulins.

Inquiry into the allergic history revealed perennial nasal stuffiness for many years, most prominent in the morning on arising. Episodes of rhinorrhea, sneezing, and lacrimation were also documented during August and September, 1969, while the patient was on the United States mainland.

Skin testing was carried out with a standard set of inhalant allergens, and responses were obtained to many molds as well as to ragweed. A history of pulmonary infiltrates with eosinophilia suggested the diagnosis of bronchopulmonary aspergillosis. Thus an intradermal challenge with *Aspergillus fumigatus* (23 µg per milliliter) was performed. Fifteen minutes after injection, a 15 mm.² wheal and 100 mm.² area of erythema was observed. The same

**Fig. 1**

Representative chest x-rays from patient's clinical course. *A*, Posteroanterior and lateral views showing infiltrate in anterior segment of right upper lobe. *B*, Films taken one month later demonstrating collapse of left lower lobe (heart shifted to left, elevated left hemidiaphragm, and increased density of lower thoracic vertebrae). Right upper lobe infiltrate has not completely resolved.

site examined 15 hr. later disclosed an area of edema, erythema, and tenderness measuring 6 × 6 cm. A 3 mm. punch biopsy taken from the site revealed histologic changes shown in Fig. 2.

MATERIALS AND METHODS

Allergens

Aspergillus mold extracts preserved with 50 per cent glycerine* were diluted to the appropriate concentration with 0.15M NaCl. Concentrates used in the study were as follows: *A. fumigatus* (0.23 mg. per milliliter), *A. niger* (0.52 mg. per milliliter), *A. terreus* (0.53 mg. per milliliter), *A. glaucus* (0.25 mg. per milliliter), and *A. nidulans* (0.44 mg. per milliliter).

*Hollister-Stier Laboratories, Spokane, Wash.

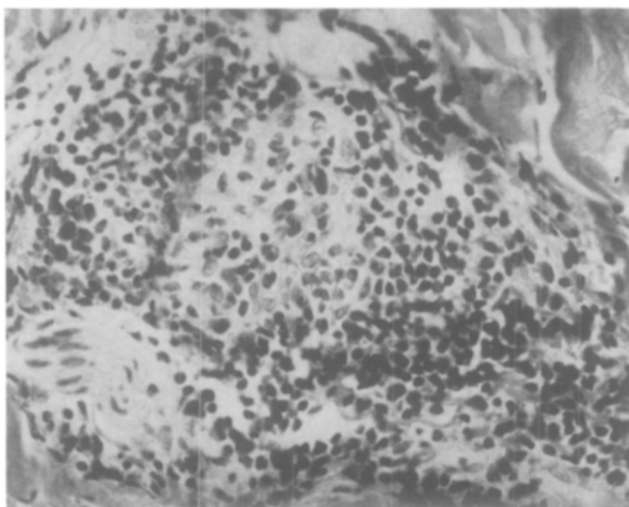


Fig. 2

Skin biopsy of local Arthus reaction at 15 hr. following challenge by *A. fumigatus*. The dermis is infiltrated primarily with mononuclear cells, and perivascular cuffing is noted. Several eosinophils are also present in the field. Original magnification $\times 400$.

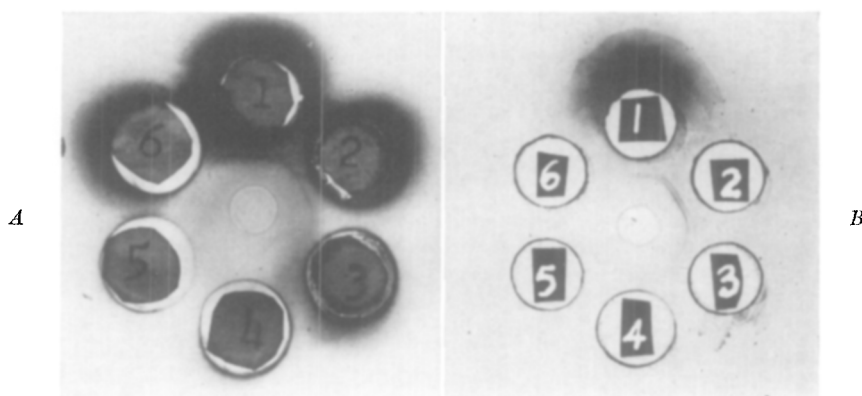


Fig. 3

A, Gel diffusion study demonstrating precipitins to *A. fumigatus* in an allergic aspergillosis serum. The central well contains *A. fumigatus*, 57.5 μg per milliliter, and peripheral wells are filled as follows: 1, undiluted allergic serum; 2 to 5, serial twofold dilutions of allergic serum; 6, normal serum control. *B*, Gel diffusion study demonstrating precipitins to *A. fumigatus* in allergic aspergillosis serum fractions eluted from DEAE-cellulose. The central well contains *A. fumigatus*, 57.5 μg per milliliter, and peripheral wells are filled as follows: 1, undiluted whole serum; 2 to 3, fractions eluted with 0.01 and 0.02M buffer; 4 to 6, fractions eluted with 0.04M buffer.

Fractionation of allergen on Sephadex G-200 gel filtration

Aspergillus fumigatus allergen (0.23 mg. per milliliter) was concentrated approximately 8 times with polyethylene glycol (Carbowax) and a 3 ml. sample applied to a 34×2 cm. glass column of Sephadex G-200 equilibrated with 1M NaCl in 0.1M sodium phosphate, pH 7.8.⁸ Two-milliliter fractions were collected and protein concentration read at 280 m μ .

Pools made from the protein peaks were concentrated to 3 ml. by negative pressure dialysis.

In another study the fraction containing IgM was isolated from the patient's serum by application of 10 ml. of serum to a similar column of Sephadex G-200 equilibrated with 0.15M NaCl. The initial protein peak containing the macroglobulins was pooled and concentrated with Carbowax to the original volume of serum applied.

Serum fractionation by DEAE-cellulose column chromatography

Ten milliliters of the patient's serum was fractionated by diethylaminoethyl (DEAE)-cellulose column chromatography.⁹ Stepwise elution was carried out with sodium phosphate buffer, pH 8.0, of increasing molarity: 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1. The peaks were concentrated to the original serum volume by dialysis against Carbowax and then analyzed by immunoelectrophoresis¹⁰ and quantitated for immunoglobulin levels by means of radial immunodiffusion.¹¹

Immunodiffusion test

A 1.25 per cent special Noble agar* prepared in 0.05M barbital buffer, pH 8.6 was used for Ouchterlony¹² double diffusion studies. The template was designed with peripheral antibody wells 8 mm. in diameter and the central antigen well 4 mm. The distance between antigen and antibody wells was 10 mm. center to center.

In vivo and in vitro absorption of serum with *Aspergillus* allergens

Two sites sensitized with 1:10 serum were used for an in vivo neutralization experiment. One site was challenged with *A. fumigatus* until there was no further reaction, and the other site was challenged with *A. niger* until there was no further reaction. The allergen challenges were then reversed.

Aliquots of reaginic serum were mixed in a 1:1 ratio with various concentrations of *A. fumigatus*, *A. niger*, and *A. glaucus*. Samples were allowed to stand for 1 hr. at 20° C. and then overnight at 4° C. Absorbed sera were then diluted with 0.15M NaCl to a final concentration of 1:20 and centrifuged at 2,300 r.p.m. P-K sites were sensitized with the supernatants obtained, and the presence of reaginic antibody determined by challenge with 0.4 µg of *A. fumigatus*.

Various concentrations of *A. fumigatus*, *A. niger*, *A. glaucus*, and *A. terreus* were mixed in a 1:1 ratio with undiluted serum aliquots to test for absorption of precipitating antibody. In this study the final concentration of serum was 1:2 and absorbed samples were tested by gel diffusion for precipitins to *A. fumigatus* (57.5 µg per milliliter).

Passive transfer (P-K) studies

Diluted whole serum or absorbed serum was injected intradermally and used for P-K studies.¹³ Sites were challenged with 0.4 µg of *Aspergillus* allergen, and 15 min. later the mean diameter of both the wheal and flare were measured and recorded as a P-K score,¹⁴ defined as ½ the erythema plus the wheal in centimeters ($E/2 + W$).

Effect of heat upon skin-fixing property

A 1:5 aliquot of reaginic serum was placed in a constant-temperature water bath at 56° C. for 4 hr. After this period of exposure the sample was diluted to a final concentration of 1:20 and used to sensitize a P-K site.

Effect of IgE absorption upon skin-fixing property

Reaginic serum (1:5) was mixed with various concentrations (1:400 to 1:25,600) of sheep antiserum to IgE (Lot No. 8930 kindly supplied by World Health Organization) in a

*Difco Laboratories, Detroit, Mich.

manner similar to that already described for the previous absorption experiments. Absorbed samples used to sensitize P-K sites were diluted to a final serum concentration of 1:20 and challenged with 0.4 μ g of *A. fumigatus*.

RESULTS

Precipitating antibody to *A. fumigatus*

The patient's serum diluted 1:2 demonstrated precipitins with *A. fumigatus* by double diffusion in agar gel (Fig. 3, A). The first two fractions eluted from the DEAE-cellulose column by 0.01M and 0.02M buffers formed precipitins with *A. fumigatus* (Fig. 3, B), and these fractions contained IgG concentrations of 1,370 and 600 mg. per cent, respectively. These two fractions showed slow γ mobility by immunoelectrophoresis. Attempts to demonstrate precipitating antibodies in fractions eluted by buffers of higher ionic strength were unsuccessful even after the fractions were pooled and concentrated. A macroglobulin fraction obtained by gel filtration of the patient's serum and containing IgM failed to show a precipitin line in agar gel diffusion against *A. fumigatus*.

Absorption studies established the specificity of serum antibody for antigenic determinants present only in *A. fumigatus* mold mixtures. Precipitins were completely removed from a 0.5 ml. serum aliquot by 11.5 μ g of *A. fumigatus*, whereas serum absorbed with 13.6 μ g of *A. terreus* still retained the ability to form a line of precipitation with *A. fumigatus* in gel diffusion (Fig. 4). In a subsequent study, *A. terreus*, *A. niger*, and *A. glaucus* were used in a fivefold higher concentration and still failed to absorb precipitins to *A. fumigatus*.

Reaginic antibody to *A. fumigatus*

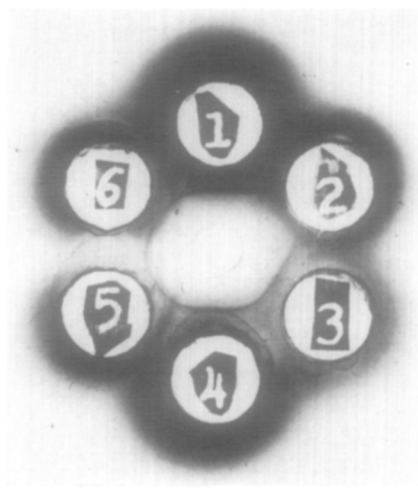
By P-K testing, a positive wheal and flare response was obtained at sites challenged with each of several *Aspergillus* allergens used. However, the largest P-K score was obtained at the site challenged by *A. fumigatus*. These results indicate reagins have been developed to allergenic determinants common to all the molds.

No P-K reaction with *A. fumigatus* was observed at the site sensitized with heated reaginic serum.

In vivo and in vitro neutralization of reagins

The site that had been exhausted to challenge by *A. niger* gave a 4.3 cm. P-K score following injection of 0.4 μ g of *A. fumigatus*. The site previously exhausted to *A. fumigatus* failed to respond to challenge with *A. niger*, indicating reagins had been formed to allergenic determinants peculiar to *A. fumigatus* and not present in *A. niger* mixtures.

Aliquots of 1:5 serum were absorbed with various quantities of *A. fumigatus*, *A. niger*, and *A. glaucus*. Skin sites sensitized with these samples were challenged with 0.4 μ g of *A. fumigatus* to determine if skin-sensitizing activity had been absorbed. As shown in Fig. 5, reaginic activity could be completely removed from 1 ml. of whole serum by 2.3 μ g of *A. fumigatus*, whereas almost five times the amount of either *A. niger* or *A. glaucus* failed to suppress the wheal and flare reaction as shown by P-K scores of 1.75 and 2.8 cm., respectively.

**Fig. 4**

Gel diffusion study demonstrating in vitro absorption of precipitins from allergic aspergillosis serum by *A. fumigatus*. The central well is filled with *A. fumigatus*, 57.5 μg per milliliter, and peripheral wells are filled as follows: 1 and 4, undiluted allergic aspergillosis serum; 2, serum absorbed with 11.5 μg of *A. fumigatus*; 3, serum absorbed with 13.6 μg of *A. terreus*; 5 and 6, allergic aspergillosis serum diluted 1:2.

Serum absorption by anti-IgE antibody

All serum samples absorbed with various concentrations of anti-IgE gave immediate wheal and flare responses indicating either antibody excess-induced reversed PCA¹⁵ or the presence of soluble antigen-antibody complexes.¹⁶ The aliquot absorbed with anti-IgE, 1:3,200, failed to respond 24 hr. later to challenge by 0.4 μg of *A. fumigatus*, whereas all samples absorbed with higher antiserum dilutions gave positive P-K responses.

Study of Aspergillus allergen by gel filtration

The three allergen fractions obtained were assayed for relative potency by P-K and gel diffusion studies (Fig. 6). All three contained determinants reactive at P-K sites. However, the first peak with the lowest optical density could be diluted only 1:100 and still retain activity. On the other hand, the initial peak formed a line in gel diffusion in a 1:16 dilution, while the intermediate peak contained less activity and the last peak failed to precipitate with the allergic aspergillosis serum.

DISCUSSION

Spores of the ubiquitous mold, *Aspergillus fumigatus*, are frequently inhaled and expectorated in the sputum,¹⁷ and their presence in the respiratory tree is generally benign. However, in certain clinical settings their presence may indicate disease. Administration of corticosteroids or other immunosuppressive agents may create a state of impaired immunological response in which *A. fumigatus* becomes an opportunistic invader¹⁸ causing localized or disseminated

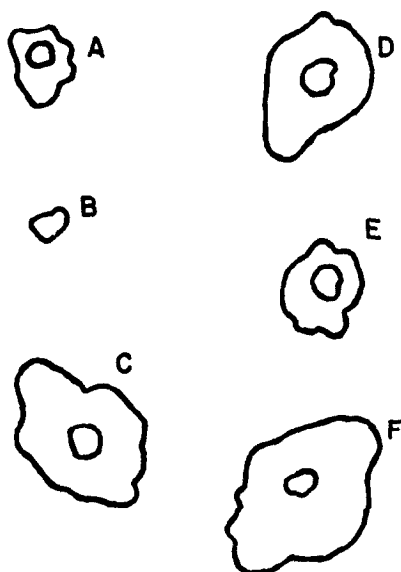
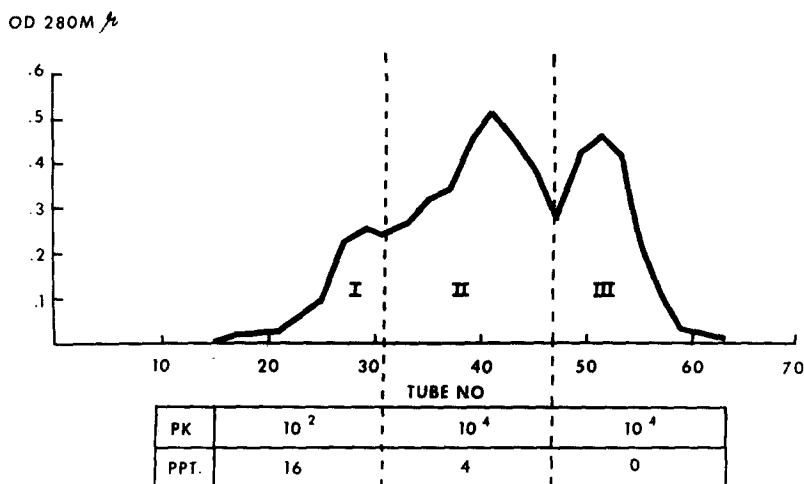


Fig. 5

In vitro neutralization of reagins in allergic aspergillosis serum by *A. fumigatus* allergen. Serum aliquots absorbed with various aspergillus molds were injected into P-K sites and challenged 24 hr. later by 0.4 μ g of *A. fumigatus*. Samples were absorbed as follows: *A* and *B*, *A. fumigatus* 1.6 and 2.3 μ g; *C*, unabsorbed serum diluted 1:20; *D* and *E*, *A. niger* 5.2 and 10.4 μ g; *F*, *A. glaucus* 10 μ g.

pulmonary infection. The mold may flourish by growth in a tuberculous or a bronchiectatic cavity to form a mycetoma, and in this case precipitins to *Aspergillus* are usually developed by the host in response to the local growth of the mold. A more familiar role played by *A. fumigatus* is its place as an allergen in atopic bronchial asthma, in which local growth of the mold is perpetuated by inflammation and edema caused by the release of pharmacologic mediators from cells sensitized by reagins.¹⁹

The present case represents an unusual example of atopic disease in which both precipitins and skin-sensitizing antibodies have developed against *A. fumigatus*. The dual skin response is integral to the diagnosis of allergic aspergillosis and provides some insight into the immunopathology of the disease. A wheal and flare appearing shortly after intradermal challenge is a type I reaction mediated by reaginic antibody. Several hours later at the same site a type III Arthus phenomenon appears, presumably requiring the presence of precipitating antibody and serum complement. In the present study, a skin biopsy taken at 15 hr. showed a preponderance of round cells rather than infiltration by polymorphonuclear cells, as classically described. However, Pepys²⁰ studied lesions 4 hr. following challenge with *Aspergillus fumigatus* allergen and found mostly mononuclear cells and only a scattering of polymorphonuclear cells. He suggested that only a few of the latter cells may be required for initiating a type III response and that predominance of polymorphonuclear cells in experimentally induced Arthus lesions reflects a more intense inflam-

**Fig. 6**

Separation of concentrated *A. fumigatus* allergen by Sephadex G-200 gel filtration. Each of the 3 peaks obtained was assayed for allergen potency by gel diffusion and P-K studies, and the reciprocal of the titers obtained for each fraction are shown.

matory response than generally is present in the clinical situation. A response analogous to the skin reaction follows bronchial challenge by the allergen and is characterized by both an immediate and a late decrease in FEV₁.²¹ This suggests that a similar pathogenetic response may be operative in the lungs. This view has recently been substantiated further by animal studies in which a monkey was sensitized with an allergic aspergillosis serum. Following intrabronchial challenge with the allergen, a pneumonitis developed characterized by peribronchial nodular infiltrates comprised of polymorphonuclear cells, round cells, and eosinophils.⁷

In the present study, precipitating antibody was demonstrated by gel diffusion. The antibody was eluted from DEAE-cellulose with low molarity buffer, had a slow electrophoretic mobility, and was shown to be IgG by gel diffusion analysis. Immunoglobulins present in serum fractions eluted by buffer of higher ionic strength did not precipitate with *A. fumigatus* in gel diffusion, but this may reflect the relative insensitivity of the technique. Immunofluorescence has been used by others to show the presence of immunoglobulins in Arthus (type III) skin reactions.²² By means of this technique, positive staining for IgG, IgA, IgM, and β_2C was demonstrated, whereas tuberculin reactions and manganese butyrate-induced inflammatory lesions studied in the same manner were all negative. However, the significance of these findings with respect to Arthus immunopathology can be only speculative, and no definite conclusion is warranted until these immunoglobulins are shown to possess antibody activity. In the present study precipitins in agar gel were specific for *A. fumigatus* and could not be absorbed out by other species of *Aspergillus*. The low precipitin titer (1:2) is not unusual and does not necessarily connote a benign clinical picture. Vigorous Arthus reactions have been reported in individuals whose sera had to be con-

centrated to demonstrate a line in gel diffusion,²³ perhaps reflecting the need for relative antigen excess for expression of a type III phenomenon.

P-K sites sensitized with the patient's serum responded to several members of the *Aspergillus* genus, as has been the experience of others.² However, the majority of the skin-sensitizing activity (P-K titer = 1:80) was directed toward allergens present only in the *A. fumigatus* mixture, as established by both in vivo and in vitro neutralization studies. In the former experiment, a P-K site exhausted by challenge with *A. niger* responded with a large wheal and flare when subsequently challenged with *A. fumigatus*. On the other hand, a site exhausted in similar fashion by *A. fumigatus* failed to respond to challenge by *A. niger*. By in vitro neutralization, it was shown that *A. fumigatus* could completely absorb reaginic activity from serum, whereas other *Aspergillus* molds were unable to do so. Thus, a small portion of serum reagins was developed against allergens common to most of the *Aspergillus* molds, but the majority of skin-sensitizing activity was formed against determinants present only in *A. fumigatus* mixtures.

It was of interest to determine the antibody class to which reagins in this serum belonged. Most skin-sensitizing activity has now been established as being associated with the IgE class of immunoglobulins,^{24, 25} although reaginic activity has been detected previously in serum fractions containing only IgG.^{26, 27} Precipitating activity in the present study was found to be due to IgG, and the importance of these antibodies in the clinical setting of allergic aspergillosis suggested that the reagins could also be IgG. However, absorption of whole serum by antibodies specific for IgE heavy chain completely removed skin-sensitizing activity and established these reagins as belonging to the IgE class.

Three peaks were obtained by gel filtration of *A. fumigatus* allergen on Sephadex G-200, and the first two were able to form lines in gel diffusion with precipitins present in the allergic aspergillosis serum. Of interest was the fact that the initial peak diluted 1:16 still formed a line, while the intermediate peak containing fractions of much higher optical density could be diluted only 1:4. All three peaks contained allergens able to elicit a wheal and flare response at P-K sites, but in this case the more potent allergenic activity was contained in fractions with high optical density. In an earlier study, Bonilla-Soto²⁸ found that results of skin testing with *Aspergillus* fractions varied from patient to patient and that fractions with lower optical density were the more potent. Differences in preparation and extraction of molds used in the two studies may explain the disparity in the findings.

The dual antibody response present in hypersensitivity bronchopulmonary aspergillosis is rather unusual in clinical immunology, although pulmonary disease with systemic manifestations has been reported in response to inhaled dusts of animal origin. Alveolitis accompanied by the production of reagins and precipitins has been reported in pigeon fanciers' disease²⁹ and in those taking pituitary snuff of bovine or porcine origin for diabetes insipidus.³⁰ Symptoms from exposure to other organic dusts may prove to have similar immunopathologic relationships. In the present study, skin-sensitizing and precipitating antibodies have been developed specifically against allergens present in *Aspergillus fumigatus* mixtures. However, certain *A. fumigatus* allergens separated by gel

diffusion reacted with reagin at P-K sites but failed to precipitate with antibody in gel diffusion. Studies to characterize these allergens further would certainly be of interest and, in addition, may be of value in explaining why some individuals develop the dual response characteristic of bronchopulmonary aspergillosis.

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