

IMMUNODIAGNOSIS OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS

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Allergic bronchopulmonary aspergillosis (ABPA) is the immunologic response to *Aspergillus* sensitization in immunocompetent patients with atopic asthma. The clinical features of ABPA range from an acute reversible asthma syndrome to a more intractable asthmatic state with transient pulmonary infiltrates, sensitivity to *Aspergillus* antigens, and subsequent irreversible lung diseases (see Varkey's article elsewhere in this issue).^{26, 28, 29} The diagnostic criteria for ABPA have been revised several times since their initial description and are mainly dependent on clinical and immunologic findings.^{35, 87, 88, 94} Criteria include the demonstration of elevated total serum IgE, specific IgE and IgG to relevant *Aspergillus* antigens, and specific antigen-induced in vitro stimulation of peripheral blood mononuclear cells of patients with ABPA and eosinophilia. Immediate skin reactivity and serum precipitins to *Aspergillus* antigens also are present. The predominant organism associated with ABPA is *Aspergillus fumigatus*, but other species including *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus clavatus*, and *Aspergillus nidulans* are sometimes implicated. There are also reports of ABPA-like syndromes caused by *Curvularia*, *Candida*, *Helminthosporium*, and other fungi.⁵⁹

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The aspergilli possess a wide range of antigenic components. Some are metabolic products secreted into the medium (culture filtrate), some are proteins present on the surface of the organism, and others are released either by autolysis or rupture of the organism (mycelial and somatic). Antigens from all these sources have been used either separately or mixed together in diagnosis of ABPA. In immunofluorescence assay, intact organism (germlings or young hyphae) or cross-sections of mycelium have been used as particulate antigens to bind specific antibodies.^{27, 101} Two major difficulties encountered by immunologists in using fungal antigens for immunodiagnosis, however, are the lack of standardized antigens and insufficient knowledge of the molecular nature of the fungal allergens. This article focuses on the recent advancements in characterization of *A. fumigatus* antigens and immunoassays currently in use for the diagnosis of ABPA.

ANTIGENS OF *A. FUMIGATUS* IN IMMUNODIAGNOSIS

A. fumigatus is a eukaryotic filamentous fungus composed of branched tubular hyphae. The segmented branched tubular filaments (hyphae) are collectively called *mycelium*. The spores (conidia) produced externally by various *Aspergillus* strains and species vary in shape, size, texture, color, thickness of the cell wall, and the way they are attached to each other and to the stalks called conidiophores (see Summerbell's article elsewhere in this issue). *A. fumigatus* spores are usually abundant both in indoor and outdoor environments and are considered to be the cause of perennial symptoms in susceptible patients.

Aspergillus growth in culture is affected by temperature, moisture, light, and nutrients of culture medium. Conditions for optimal growth and sporulation may differ within and among strains. Even when a single strain is cultured under standard conditions, marked batch-to-batch variation is detected in the protein composition and allergenic potency of extracts.^{48, 56, 63} The frequent mutation of fungi in culture and also varying growth conditions contribute to substantial variability in the antigens. Moreover, the significant antigenic cross-reactivity between *A. fumigatus* and other aspergilli, as well as between *A. fumigatus* and other fungi and bacteria, may also be responsible for false-positive immunologic test results. This cross-reactivity may be caused by epitope sharing by the antigens and/or to the universal presence of bacterial and fungal antibodies in normal subjects. The cross-reactivity of epitopes in most instances may be caused by the limited number of shared epitopes present in polysaccharides from different fungi.⁵⁹

In addition to immunologic cross-reactivity, some *Aspergillus* antigens react to C-reactive proteins in the sera of patients with a variety of infections and neoplastic and autoimmune diseases. Other factors that interfere adversely with the reproducibility of the test results are the presence of toxins and proteolytic enzymes in *A. fumigatus* antigen preparations.

Antigen Preparation

The chemical nature of the antigens of *Aspergillus* species may be protein, polysaccharide, and glycoprotein.^{33, 56, 59} Several studies indicate that synthetic media, such as Czapek-Dox, glycine, asparagine, or Association of Analytical Chemists (AOAC) Difco broth supplemented with different nutrients, are the suitable media for producing culture filtrate antigens by selectively removing the macromolecular components of the medium that may otherwise interfere with the immunoassay.^{47, 49, 56} The use of synthetic media and incubation of *A. fumigatus* culture at 37°C have been reported to yield more consistent antigens. The available reports suggest that the most reliable cell-associated antigens for immunoassays can be obtained from 4-day-old aerated (shaken) culture, whereas secreted antigens (culture filtrates) can be obtained from 2- to 4-week-old stationary culture of the fungus.^{38, 47, 57, 112, 113} In spite of the difficulties in standardization, crude extracts (commercial) are still in use for routine diagnosis. The results using such antigens must be interpreted with caution because of the variability among the antigen preparations. Likewise, nonreproducibility of the results among different laboratories is also problematic.

Fractionation and Purification

Because of the ease of preparation and the presence of large number of immunoreactive molecules, culture filtrates are frequently used as antigenic sources. The protein profile of culture filtrates prepared from different strains of *A. fumigatus* is shown in Figure 1. The gel electrophoresis pattern clearly demonstrates the variability in protein profiles of different *A. fumigatus* strains both quantitatively and qualitatively. The water-soluble components of *Aspergillus* mycelium have also been reported as a rich source of diagnostic antigens.⁵⁹ Both culture filtrate and mycelial antigens from *A. fumigatus* have been characterized using polyacrylamide gel electrophoresis, isoelectric focusing, and two-dimensional gel electrophoresis.^{62, 64, 68, 89} Immuno-electrophoresis of *Aspergillus* culture filtrate antigens against sera from patients with ABPA demonstrated approximately 35 precipitin arcs, of which 8 to 10 showed specific reactivity with IgE antibodies. In two-dimensional gel electrophoresis, the crude extract of *A. fumigatus* proteins resolved in over 200 components.^{46, 73, 89}

Using crossed immuno-electrophoresis, Kim and Chaparas identified 52 antigens, whereas Kauffman et al identified 22 and 44 antigens respectively from different antigenic preparations of *A. fumigatus*.^{46, 49, 116} On the other hand, water-soluble antigenic preparations from mycelial and spore extracts have been reported to contain over 30 antigens when probed with patients' sera in immunoblotting.^{13, 33, 34} Wilson and associates¹¹⁸ fractionated mycelial extracts by preparative isoelectric focusing and compared the reactivity of three major fractions with that of

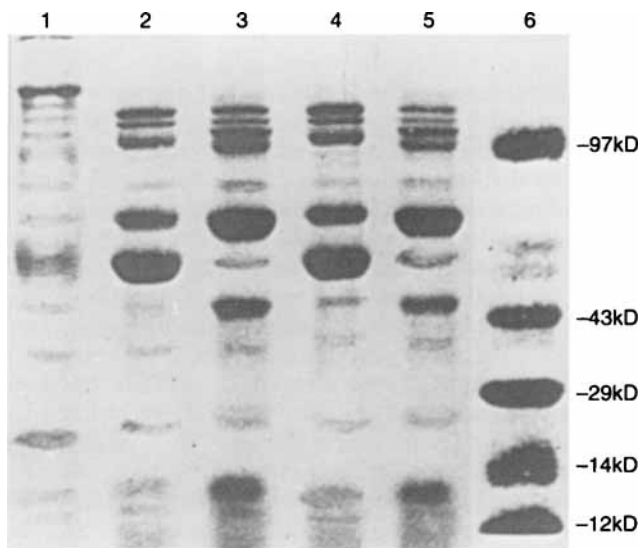


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) pattern of 3WCF proteins of *A. fumigatus* (both soil and clinical isolates of four patients). Lane 1=clinical isolate 1 (isolated from sputum of ABPA patient); Lane 2=clinical isolate 2 (isolated from sputum of ABPA patient); Lane 3=clinical isolate 3 (isolated from sputum of ABPA patient, strain no. 285); Lane 4=clinical isolate 4 (isolated from sputum of aspergilloma patient); Lane 5=soil strain; Lane 6=molecular weight markers.

crude mycelial extracts. All these fractions reacted with sera from patients with aspergillosis, but they also showed cross-reactivity with antibodies against other fungi. By using preparative isoelectric focusing and affinity chromatography, different fractions of *A. fumigatus* have been identified and reported to exhibit strong reactivity with both IgE and IgG antibodies.^{57, 64, 118} Schonheyder et al identified several fractions of *A. fumigatus* antigens with molecular masses of 25 kD to 470 kD.^{99, 103} Some of these fractions were evaluated and found to be specific in the diagnosis of aspergillosis, but none of these components were isolated in pure form. These findings suggest that *A. fumigatus* extracts constitute a very heterogeneous group of antigens.

Kauffman et al⁴⁶ obtained several antigen fractions of *A. fumigatus* using gel filtration, Concanavalin A (ConA) affinity chromatography, isoelectric focusing, and trichloroacetic acid precipitation. They observed that the low molecular weight (5 to 20 kD) fraction usually demonstrated a weak linkage to the polystyrene surface used in enzyme-linked immunosorbent assay (ELISA) and showed weak precipitation. In contrast, the high molecular weight components reacted strongly in the ELISA and precipitin test. On the other hand, Longbottom⁶⁹ found that low molecular weight peptides showed strong IgE binding capacity and were more specific in the diagnosis of aspergillosis.

As already mentioned, the strains of *Aspergillus* used and culture

methods are partially responsible for the antigenic variability of extracts, and even with the use of a defined medium, the results are not sufficiently reproducible. Hence, further attempts to fractionate and purify the antigens and allergens of *A. fumigatus* were carried out using (1) conventional chromatography and the electrophoretic methods, (2) monoclonal antibody-based purification, and (3) molecular cloning of *A. fumigatus* antigens by recombinant DNA technologies.

The recent advancement in bioseparation techniques and recombinant technology has shed new light on our understanding of the entangled antigenic array of *A. fumigatus* antigens. The extensive enzymatic and chemical mapping, as well as immunologic cross-reactivity studies, have shown that *A. fumigatus*-culture filtrate components of 60, 40, and 37 kD sizes have a common peptide core. The recently available primary amino acid sequence data and immunologic evaluation of recombinant proteins and peptide fragments of various allergens also substantiate this observation. The cell sap antigens at 53 kD and 41 kD are also believed to belong to the same peptide family. The differences in the molecular weights and antibody binding profiles of these proteins, however, appear to be caused by the posttranslational modification, such as glycosylation and phosphorylation, which enhance the hydrophilicity of the proteins and are normally found on the surface of the molecules. The differences in glycosylation patterns of the antigens could be the cause for the differences in their antibody-binding profiles. This important finding led researchers to isolate and characterize the glycoprotein antigens of *A. fumigatus* and analyze their carbohydrate moieties, as well as the protein counterparts. Because a majority of these glycoproteins consist of glucose and mannose residues, lectin ConA with specific-binding affinity for these carbohydrate moieties has been used as a tool for the purification of these proteins. Several *A. fumigatus* glycoproteins, purified using ConA sepharose-based affinity chromatography, have been studied extensively. The ConA nonbinding antigens, usually the low molecular weight proteins in the range of 12 to 40 kD, have been reported also to exhibit specific-antibody binding and lymphocyte proliferation in patients with ABPA. On the other hand, proteolytic processing of the antigens and the various enzymes secreted into the medium or expressed on the cell surface appear to contribute to the antigenicity of the fungal preparation. Here we discuss some of the diagnostically relevant enzymes, carbohydrates, glycoproteins, and proteins of *A. fumigatus* isolated and characterized using various purification techniques.

Enzymes of *A. fumigatus*

A. fumigatus antigens include a large number of enzymes, such as chymotrypsin, proteinases, elastase, ribonuclease, catalase, and superoxide dismutases.^{2, 15, 30, 42, 50, 75, 81, 92, 93, 103} The role of these enzymes in immunopathogenesis of ABPA and their usefulness in immunodiagnosis of the disease are yet to be elucidated. Eighteen enzymatically active

components of *Aspergillus* have been detected; out of these, 13 reacted specifically with sera from patients with aspergillosis.¹¹⁰ The antigenic fraction of *A. fumigatus* with chymotryptic activity reacts with sera from patients with aspergilloma but not with ABPA. A high molecular weight antigen fraction of 250 kD, probably a tetramer of catalase, has been characterized by Schonheyder et al.^{100, 103} Patients with cystic fibrosis with the complications of ABPA frequently showed IgE binding antibodies to catalase antigen from *A. fumigatus*.^{34, 102} In a recent report, an immunodominant 90 kD *A. fumigatus* antigen was reported to belong to a catalase subclass that recognized over 90% of serum samples from patients with aspergilloma.⁷⁵ Hamilton et al³⁰ purified a 19 kD superoxide dismutase (SOD) from *A. fumigatus* by isoelectric focusing, gel filtration, and fast-protein liquid chromatography, and this enzyme specifically reacted with the sera from patients with confirmed aspergillosis on Western blots. The proteases from *A. fumigatus* were reported to be involved in the pathogenesis of aspergillosis. Two partially purified proteases of 25 and 50 kD reacted significantly with sera from patients with aspergillosis.¹⁰⁰ In another study, two acid proteases of 73 and 43 kD purified from membrane vesicles of *A. fumigatus* showed reactivity with sera from patients with ABPA in a biotin-avidin linked immunosorbent assay.⁹⁰ A number of *A. fumigatus* proteases have been isolated and characterized either by using a conventional purification method or recombinant DNA technologies.^{42, 81, 92, 93, 106} One of the recombinant alkaline proteases with elastinolytic activity exhibited weak antigenicity when studied in patients with ABPA.⁸⁴ Although *A. fumigatus* proteases are reported to be involved in fungal penetration of the host barriers by catalyzing the hydrolysis of the major structural proteins of the basement membrane of lungs, their involvement in humoral and cellular immune responses in patients with ABPA is yet to be explored.

Carbohydrate Antigens

Polysaccharide fractions from either cell wall or cytoplasm also showed reactivity with antibodies in the sera of patients. These antigens, however, frequently demonstrate cross-reactivity with antigens from other fungi, which is a major limitation in interpreting test results.^{4, 5, 6, 7, 14, 22} Galactomannan present in the cell wall of *A. fumigatus* has structural features common to galactomannan of *A. flavus* and *A. niger*. *A. fumigatus* galactomannan antigen was isolated from mycelial extract using sequential chromatography on anion exchange and ConA affinity columns, and the purity of the antigen was established by gas liquid chromatography. The detection limit for the estimation of galactomannan in serum in patients with invasive aspergillosis was established between 10 and 40 ng/mL. A galactomannan of approximate size of 50 kD was purified by Azuma et al,⁴ showed skin test reactivity, and precipitin reaction in patients. Most of these carbohydrate antigens are useful in immunodiagnosis of invasive aspergillosis.

Glycoprotein Antigens

Partial purification and characterization of glycoprotein antigens of *A. fumigatus* have been reported from different laboratories.^{18, 89, 90} Several glycoproteins isolated from both culture filtrate and mycelial extracts exhibited lectin (ConA)-binding property. The high molecular weight glycoprotein antigens from mycelial or culture filtrate preparations purified using conventional purification technique have been used to detect specific antibodies in the serum of patients suffering from ABPA. The cell sap protein isolated by Calvanico and associates¹⁸ consisted of four polypeptides of 45 kD, linked through disulfide bonds. The isoelectric point (5.2–5.6) and carbohydrate content (approximately 12.5% neutral hexose) indicated that this protein is an acidic glycoprotein. It showed reaction with 75% of sera from patients with ABPA and was not reactive with sera from normal individuals. Four antigens (Ag 3, Ag 5, Ag 7, and Ag 13) were purified by Longbottom using gel filtration, isoelectric focusing, and affinity chromatography.^{71, 73, 74, 108} Ag 7 of 150 to 200 kD and Ag 13, with a molecular mass of 70 kD, bound to ConA and reacted in an ELISA with sera from patients with ABPA and aspergilloma. Kurup et al demonstrated that *A. fumigatus* antigens transferred to polyvinylidene difluoride (PVDF) membrane resolved into 18 bands, and 12 to 14 of these were ConA-binding components of *A. fumigatus*, indicating their glycoprotein nature.⁵⁷ In another study, they isolated a fraction having significant antibody binding with patients that focused at pH 6.5 and showed 20, 40, and 80 kD size components in two-dimensional electrophoresis.⁵⁸ Two other fractions identified and characterized by using preparative isoelectric focusing and affinity chromatography demonstrated reactivity against patient sera.⁶⁴ On the other hand, using hydrophobic interaction chromatography, Schonheyder and Andersen⁹⁹ obtained a component of *A. fumigatus* having molecular mass of 25 to 50 kD. Out of 14 components detected on polyacrylamide gel electrophoresis, three showed ConA-binding property. None of these components were purified or characterized further. An antigenic fraction with two components of 35 and 65 kD were purified and analyzed for IgE and IgG binding with sera from patients with ABPA.⁶²

A major allergenic component (gp 55) of *A. fumigatus* has recently been purified from water-soluble extract of *A. fumigatus* cultures by gel filtration and SDS-gel electrophoresis. This 55 kD protein has N-glycosidically linked oligosaccharides and demonstrated specific IgE binding with the sera from patients with ABPA in Western blot analysis.¹⁰⁹ An immunodominant glycoprotein allergen of 45 kD (gp 45) with specific antibody binding to ABPA patient sera was also purified and characterized.⁸ It was observed that deglycosylation destroys the IgE reactivity, whereas IgG reactivity has been retained. The allergen gp66, a glycoprotein of *A. fumigatus*, was obtained by ammonium sulfate precipitation followed by immuno-affinity column (specific antibody coupled to cyanogen bromide-activated Sepharose) purification.⁶⁷ This allergen showed IgE reactivity on crossed-radioimmuno-electrophoresis

(CRIE) and ELISA with sera from patients with cystic fibrosis with complication of ABPA. In a recent report, a glycoprotein with an apparent size of 93 kD purified from water-soluble extract of *A. fumigatus* (strain NCPF 2109) by a single-step affinity chromatography using mannose-specific lectin coupled to agarose. This antigen also exhibited IgE binding with sera from ABPA.³² Thus, glycoproteins are significant components of *A. fumigatus*, possessing reactivity with both IgE and IgG antibodies in the sera of patients with aspergillosis.

ConA Nonbinding Antigens

Among the four antigens purified by Longbottom et al, Ag 3 and Ag 5 are thermolabile ConA nonbinding antigens of 18 kD and 35 kD, respectively.^{71, 108} These two antigens are also found to be useful in detecting antibodies in the sera of patients with ABPA. The low molecular weight (> 14 kD) ConA nonbinding antigens of *A. fumigatus* were reported to exhibit specific antibody binding with sera from patients with ABPA.⁵⁷

Purification of 18 kD ConA nonbinding protein from different sources and strains of *A. fumigatus* using diverse purification methods has been reported by researchers. These methods include gel filtration and phenyl Sepharose chromatography, ion exchange chromatography, gel permeation chromatography, and Sephacryl S 200 column chromatography followed by phenyl Sepharose chromatography.^{2, 3, 66} In all the preparations, the purified antigen retained immunologic reactivities. In another study, a 20 kD ConA nonbinding antigen was purified to apparent homogeneity using a combination of size-exclusion chromatography on a Sephacryl S 200 column and preparative isoelectric focusing in a pI 2.5 to 6.5 gradient. This antigen showed IgE binding reactivity by radioallergosorbent test (RAST).⁹⁵

In a recent study, a diagnostically relevant protein of molecular weight of 18 kD was purified to homogeneity from the third week culture filtrate (3WCF) of clinical isolate of *A. fumigatus* (strain no. 285) and from a soil strain (ITCC, strain no. 2605) using phenyl Sepharose chromatography followed by preparative high-performance liquid chromatography (HPLC) (Bir Nivedita, unpublished results, 1997). The sequence data of the purified 18 kD protein was found to be homologous to the deduced sequence of Asp f 1.² Immunoreactivity of the fractionated and purified 18 kD antigen from clinical and soil strains of *A. fumigatus* with the monoclonal antibody (MAb) and sera of patients with ABPA and aspergilloma clearly indicates the universal presence of 18 kD antigen in *A. fumigatus* and possible utilization of this protein as a marker for preparation of diagnostic antigens for aspergillosis (Fig. 2).

Raising monoclonal antibodies against diagnostically relevant antigens of *A. fumigatus* was attempted to obtain a permanent source of pure, standardized antigens for immunodiagnosis of the disease. Monoclonal antibodies were raised against culture filtrate and mycelial extracts and against recognized diagnostically relevant antigens of *A. fumigatus*.^{24, 25, 53,}

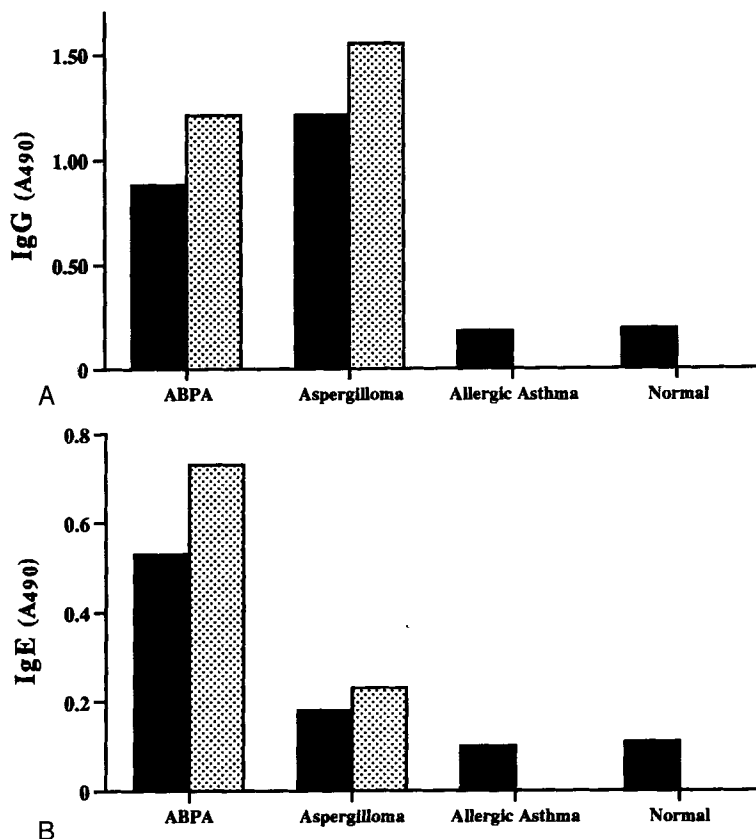


Figure 2. Comparison of IgG (A) and IgE (B) response to the 18 kD antigen (solid bars) and 3WCF antigens (dotted bars) of *A. fumigatus* in sera of patients with ABPA, aspergilloma, allergic asthma, and normal controls.

54, 55, 107 A ConA nonbinding antigen purified using monoclonal antibody affinity column reacted preferentially with the sera from patients with ABPA compared with those sera from patients with aspergilloma and normal controls.⁵³ A monoclonal antibody was also produced against Asp f 1, a protein with ribonuclease activity of 18 kD size.² The monoclonal antibody immunoaffinity column was used to purify this antigen, which is also a major allergen associated with ABPA. The purified allergen Asp f 1 induced specific IgE antibody responses in 85% of patients with ABPA. A 37 kD ConA nonbinding allergen was purified by ConA fractionation followed by monoclonal antibody-based immunoaffinity chromatography.⁹ It reacted strongly with IgE antibodies in Western blots against pooled ABPA sera. The mean ELISA value for IgE antibody against purified allergen was tenfold higher in patients with

ABPA compared with values in normal controls. The high specificity of these antigens facilitates rapid and accurate diagnosis of the disease, as well as the direct comparison of results among different laboratories. Such antigens, however, are not yet available commercially for diagnostic use.

Recombinant Antigens of *A. fumigatus*

To circumvent the problems confronting standardization of allergenic and antigenic preparations and for the better understanding of pathogenesis and immunodiagnosis, biologically and immunologically active recombinant allergens, antigens, and enzymes of *A. fumigatus* are being produced. The genes encoding a number of proteins of *A. fumigatus* have been cloned, characterized, and expressed in prokaryotic systems (see Banerjee's article elsewhere in this issue).^{2, 10, 52, 83}

A 686-bp long *A. fumigatus* cDNA encoding 18 kD ribotoxin of *A. fumigatus* was cloned and expressed in *Escherichia coli* as a fusion protein, and recombinant Asp f I/a (rAsp f I/a) showed IgE reactivity with sera from individuals allergic to *A. fumigatus* by ELISA, Dot blots, and Western blots.⁸³ Recombinant Asp f I/a elicited positive immediate type I skin reactions in individuals allergic to *A. fumigatus* but not in healthy controls, and therefore, has potential diagnostic use in *A. fumigatus*-associated diseases. Moser et al⁸² compared the recombinant *A. fumigatus* allergen I/a (rAsp f I/a) to two commercial *A. fumigatus* extracts by skin-prick tests, intradermal tests, and serologic assays. All patients with ABPA(15) reacted to skin-prick tests with commercial extracts and 8 of the 15 reacted to rAsp f I/a. There was a strong correlation between skin-test reactivity to rAsp f I/a and specific serum IgE as determined by ELISA. Patients with ABPA showed significant elevated levels of rAsp f I/a-specific IgG4 and IgG1 but no significant rAsp f I/a-specific serum IgA levels when compared with the healthy controls. The correlation between rAsp f I/a skin-test results and serologic data demonstrate potential for the use of recombinant allergens in diagnosis.⁸²

A clone from the complementary (cDNA) library was found to express a 65 kD protein of *A. fumigatus*.⁵² The recombinant protein reacted with IgE and IgG class of antibodies in the sera of patients with ABPA. The deduced amino acid sequence of the partially sequenced cDNA of the clone is homologous with the heat shock protein (Hsp) 90 family. Another *A. fumigatus* gene encoding an allergen of 34 kD was cloned and expressed in *E. coli*.¹⁰ The 987 bp long cDNA clone expressed a recombinant allergen Asp f 2. This protein exhibited binding to IgE in the serum samples of ABPA. Moreover, patients with ABPA with central bronchiectasis showed high levels of serum IgE compared with patients without central bronchiectasis, aspergilloma, and patients with asthma and *Aspergillus* skin-prick test reactivity but no evidence of ABPA. The recombinant Asp f 2 has immunologic reactivities comparable to native *A. fumigatus* antigens and could be useful in diagnosis of ABPA, espe-

cially with central bronchiectasis.¹⁰ Recently, a phage display library has been used to express specific proteins from *A. fumigatus*. The expression products have been displayed on the surface of filamentous phage M13 and screened for gene products binding to human serum IgE.¹⁹ The apparent molecular masses of IgE binding proteins selected from the cDNA library were in the range of 20 to 40 kD. A combination of monoclonal antibody affinity purification and molecular cloning and expression of immunodominant antigens may help to obtain standardized and reproducible antigenic preparation for serodiagnosis.

SYNTHETIC PEPTIDE APPROACH

Recent advances in epitope mapping of major antigens resulted in the rapid development of diagnostic technologies and products. The production of large amounts of pure antigens and synthetic epitopes is now possible. These advanced technologies can be extended to fungal antigens. Based on the available amino acid sequences, the secondary and tertiary characters of these allergens/antigens can be determined.^{23, 39, 65, 80, 120} From the algorithm profiles suggested by different groups, the major epitopes of the antigens/allergens can be predicted. Peptides were used successfully to identify specific antibodies in serum of patients with hepatitis C, HIV I, HIV II, and pneumonia using rapid diagnostic technologies.^{37, 40, 51} Recently, the complete amino acid sequencing of a few major *A. fumigatus* allergens/antigens and partial sequencing of several others have been accomplished. This information has widened the scope of the synthetic epitope approach for development of rapid immunodiagnostic technologies for fungal diseases.

IMMUNODIAGNOSIS OF ABPA

Demonstration of circulating antibodies against *A. fumigatus* in the sera of patients is a valuable criterion in the diagnosis of various forms of aspergillosis. A variety of tests with varied sensitivities have been developed to detect circulating antibodies to *Aspergillus* antigens. The common methods used are immunodiffusion test, enzyme-linked immunosorbent assay, and Western blot analysis. These tests show diverse sensitivities depending on the antigen and the type of antibodies investigated.

Immunodiffusion Test

Because of its simplicity and ease of performance, immunodiffusion (ID) is the most common technique used for the serodiagnosis of aspergillosis. Many investigators have shown the usefulness of ID as a diagnostic tool for aspergillosis.^{63, 64, 77} Antigens used in ID should be free of

2 hours or at 4°C overnight. Coating should be followed by blocking the free well sites with bovine serum albumin (BSA) in PBS for 1 hour at room temperature. (2) The antigen-antibody reactions are then carried out by adding suitably diluted sera from various subjects, and the plates are incubated for 2 to 3 hours at room temperature. In between the steps, washing of the plates is carried out by using 0.05% Tween 20 in PBS (PBS-T).³ The enzyme-(such as peroxidase or alkaline phosphatase) linked anti-human antibodies (anti-human IgG for determining IgG antibody level in patients or anti-human IgE for IgE antibody level) are added to the plate, which is incubated for 1 hour.⁴ The color reaction is developed by using appropriate substrate for the enzymes, such as ortho-phenylene diamine (OPD) for peroxidase and p-nitrophenyl phosphate (PNPP) for alkaline phosphatase. The intensity of the color reaction, the measure of antibody titer in patients and in various controls, is read by using an ELISA reader either at wave length 490 nm (peroxidase) or 405 nm (alkaline phosphatase). Each assay is accompanied by appropriate positive and negative controls. The antigen-antibody titration may be expressed in log-log manner (serum dilution versus optical density) to determine the slope of linear titratable region for *A. fumigatus* antigens and specific antibodies in various subjects. The absorbency and dilution values may be expressed in ELISA unit (EU) per milliliter.

For patients with ABPA, a strong correlation was observed between skin-test reactivity to rAsp f I/a and rAsp fl/a specific serum IgE as determined by the antigen-specific ELISA.⁸² In addition, significantly elevated levels of rAsp f I/a specific IgG4, IgG1, but not rAsp f I/a specific serum IgA, were seen when compared with that for healthy controls. The conventional ELISA system has been used successfully to detect *A. fumigatus*-specific IgG in patients with aspergillosis. This assay, however, may not be sensitive enough to detect relatively low levels of antigen-specific IgE or subclasses of IgG. Thus, to overcome the limitations of the conventional ELISA, Brummund et al have developed a modified technique, the BALISA (biotin avidin-linked immunosorbent assay), making use of high-affinity binding between biotin and streptavidin.^{9, 16, 53} BALISA developed for ABPA correlates well with clinical features of the disease and shows sensitivity comparable with that of RAST.

Immunoblot

This method involves the separation of antigens in polyacrylamide gel followed by transfer onto nitrocellulose membranes. The reactivity of such antigens with antibody in the sera of patients can be visualized by the immunoassay on the membrane. Immunoblotting has been used with great success in detecting specific antibodies to *Aspergillus* antigens.^{10, 24, 31, 54, 57, 59, 60, 64}

Analysis of sera from patients suffering from allergic asthma, extrinsic allergic alveolitis caused by *A. fumigatus*, and ABPA by Western blot

showed characteristic IgG subclasses and IgE responses.¹¹¹ The IgG4 reactivities to protein antigens with molecular weights of 14, 20, 29, 34, 38, and 45 kD seemed to be a characteristic pattern of ABPA. IgE antibodies to culture filtrate components of 18, 34, and 38 kD also appeared to be typical of ABPA. The immunologic evaluation of various diagnostic methods using recently purified and characterized recombinant and native allergens of *A. fumigatus* is shown in Table 1.

Lymphocyte Transformation

Studies demonstrate a convincing correlation between proliferative response of lymphocytes from patients with ABPA and the disease activity, particularly when purified antigens are used.^{61, 85, 86, 114, 115} The method is as follows: Peripheral blood mononuclear cells (2×10^5 per well) in a 96 well flat-bottom microtiter plate are cultured in complete RPMI 1640 medium for 7 days. The incubation is carried out at 37°C in 5% CO₂ in an air-humidified incubator. The cultures are stimulated with different concentrations of various antigens and mitogen (ConA, 5 µg/mL). Next, the cells are pulsed with 0.5 µCi of ³H-thymidine per well for 18 hours before harvesting, and the radioactivity is measured (in counts per minute) with a liquid scintillation counter. The results are expressed as a stimulation index (SI = counts per minute in antigen-stimulated cultures/counts per minute in a medium without antigen or mitogen). The significance of the results is determined in comparison with the stimulation index of mitogen-stimulated peripheral blood mononuclear cells of patients and healthy controls.

Radioimmunoassay

Radioimmunoassay (RIA) was first developed to measure antibodies to radiolabeled acid-soluble antigenic fractions of *A. fumigatus*.^{11, 12} More recently, Arruda et al investigated the antigenic and functional relationship between Asp f 1 and mitogillin by using cross-inhibition RIA.² The most commonly used RIA, however, is the RAST, used extensively for the detection of IgE class to various allergens.¹ RAST is a helpful technique to determine the fractions containing allergens on purification. Samuelsen et al have used RAST to demonstrate the allergenicity of a 20 kD antigen of *A. fumigatus*.⁹⁵ Arruda et al have studied the immunoreactivity of the various fractions obtained from purification of *A. fumigatus* antigens using micro-RAST.³ A modified RAST with multiple fungal antigens has been tested with sera of 16 patients meeting the histologic criteria of allergic fungal sinusitis (AFS) and positive fungal cultures. All AFS patients were RAST-positive, indicating the association of type I hypersensitivity in the pathogenesis of AFS (see Nash and Fireman's article elsewhere in this issue).⁷⁶

RAST has several disadvantages for use in a routine diagnostic laboratory. The radioisotopes have a limited storage life to complete the

Table 1. IgE ANTIBODY BINDING OF NATIVE AND RECOMBINANT *A. FUMIGATUS* ALLERGENS WITH SERA FROM VARIOUS SUBJECTS

<i>A. fumigatus</i> Allergens	Subjects	Western Blots	No. of Positives/Total Patients				Reference
			ELISA	Solid-Phase RIA/RAST	ImmunoCAP Assay	Skin Test	
n Asp f 1	ABPA Allergic asthma Nonatopic/normal	- - -	- - -	11/13 0/10	- -	4/5 0/2 0/4	2
r Asp f 1	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	+ ve - - -	+ ve - - -	- - - -	- - - -	5/9 0/6 2/5 0/7	83
r Asp f 1	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	- - - -	- - - -	- - - -	- - - -	8/15 0/6 5/10 0/7	82
n Asp f 1	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	+ ve - - -	15/20 0/10 0/18 24/30	- - - -	- - - -	- - - -	60
r Asp f 1	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	- - - -	0/6 6/10 0/7 15/20	- - - -	- - - -	- - - -	20
r Asp f 2	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	- - - -	5/10 0/10 15/20 1/8	- - - -	24/30 0/9 5/10 0/10	24/30 0/9 5/10 0/10	10
n Asp f 2	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	+ ve - - -	24/25 0/10 0/10 + ve	24/25 0/10 0/10 -	- - - -	- - - -	9
r Asp f 6 (MnSOD)	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	+ ve - - -	- - - -	- - - -	- - - -	- - - -	20
r Asp f 12 (Heat shock protein 90)	ABPA Allergic asthma <i>A. fumigatus</i> skin test positive, asthma Nonatopic/normal	+ ve - - -	- - - -	- - - -	- - - -	+ ve - - -	53

n = native; r = recombinant; - = not done.

assay. In addition to the potential hazard and difficulties involved in the disposal of radioactive wastes, expensive radioactivity detectors are also necessary.

Automated-Specific IgE Assay (Pharmacia ImmunoCAP System)

Pharmacia CAP system is a fully automated system for the measurement of circulating IgE antibodies. This fluoroimmunoassay is calibrated against the World Health Organization (WHO) standard for IgE and allows quantitative expression of total or allergen-specific IgE. The results are expressed as kilo units of allergen-specific IgE per liter (KU/L). In an effort to establish recombinant allergens as standardized reagents for the diagnosis of ABPA, the CAP system being investigated using rAsp f 1. Sera from patients with ABPA, asthmatics with *A. fumigatus* allergy, and control subjects were studied using this system.²⁰ When compared with the rAsp f1-specific ELISA for IgE, the results showed high correlation ($r=0.997$) and were in perfect agreement with the skin-test results (see Table 1). Although rAsp f 1 has shown a great promise as a suitable allergen for the development of a fully automated diagnosis of ABPA, a panel of other recombinant *A. fumigatus* allergens must be individually evaluated using the immunoCAP system to demonstrate that IgE binding is not influenced by the chosen immobilization procedures.

Several other diagnostic procedures that have also been developed for diagnosis of *Aspergillus* include immunoelectrophoresis, passive hemagglutination, indirect immunofluorescence, latex agglutination, complement fixation, enzyme-linked immunofiltration assay, and luminoimmunoassay.^{13, 21, 27, 36, 41, 79, 91, 96, 97, 105} Among different types of immunoelectrophoresis techniques, counter immunoelectrophoresis has been widely tested because of the simplicity but was less attractive because of the lack of specificity.^{55, 72, 79, 119} Crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis have been reported to demonstrate *A. fumigatus*-specific antibodies.^{44, 70} By using an intermediate gel and a reference serum, over 40 specific precipitin arcs could be detected. Using such an immunoelectrophoretic technique, enzymatically active antigens can also be detected.^{4, 102} The lengthy cumbersome procedures and the nonprecipitating nature of antigen-antibody complexes, however, are the major limitations of these methods.

Skin Test

Skin-test reactivity to *A. fumigatus* antigens is widely used to identify patients with suspected ABPA. Two types of skin reactions can be observed in ABPA. Type I (immediate) reactivity consists of the appearance of a "wheal and flare" within 15 to 20 minutes of antigen challenge. The type III reaction (Arthus reaction) is an induration usually devel-

oped within 4 to 8 hours after skin testing. The reaction usually subsides after 24 hours, but occasionally a delayed reaction persists for 24 hours or more. In patients with allergic asthma, only immediate type reaction to *A. fumigatus* is observed. Patients are regarded as being sensitive to a particular antigen preparation if the wheal diameter is greater than 9 mm.^{2, 59}

All the immunodiagnostic assays currently in use must be evaluated for specificity and sensitivity by carefully monitoring the antigen quality, test conditions, consistency of reagents and other materials used, and expertise of the personnel carrying out the tests. The clinical significance of a positive result should be determined by evaluation of the patient's exposure, clinical history, symptoms, and the disease state (exacerbation or relapse). All antigens should be estimated for protein and carbohydrate content and resolved on SDS-PAGE for protein profile for ensuring the quality of the preparation. The reactivity of antigen preparations should be evaluated with standard anti-sera or pooled positive sera by crossed immunoelectrophoresis and Western blots. The tests should be carried out with controls from clinically confirmed patients along with healthy subjects and appropriate blanks.

CONCLUSION

Allergic aspergillosis has been reported worldwide. The immune mechanism involved in the pathogenesis of varying forms of aspergillosis is not well understood. To understand the true disease prevalence and the immune mechanisms responsible for disease development, sensitive and specific serodiagnostic methods must be widely available. This article reviewed the substantial recent progress towards that goal. Achievement includes the molecular immunology of relevant allergens or antigens of *A. fumigatus*. These studies have brought us closer to being able to develop reference standards and obtain universal diagnostic antigens.

References

1. Arbesman CE, Wypych JI, Reisman RE: Evaluation of RAST inhibition as a method for standardization of ragweed pollen extracts. *Int Arch Allergy Appl Immunol* 53:310, 1977
2. Arruda LL, Platts-Mills TA, Fox JW, et al: *Aspergillus fumigatus* allergen 1, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* 172:1529, 1990
3. Arruda LK, Platts-Mills TA, Longbottom JL, et al: *Aspergillus fumigatus*: Identification of 16, 18 and 45 kD antigens recognized by human IgG and IgE antibodies and murine monoclonal antibodies. *J Allergy Clin Immunol* 89:1166, 1992
4. Azuma I, Kimura H, Hirao F, et al: Biochemical and immunological studies of *Aspergillus*, I. Chemical and biological investigations of lipopolysaccharide, protein,

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References

1. Arbesman CE, Wypych JJ, Reisman RE: Evaluation of RAST inhibition as a method for standardization of ragweed pollen extracts. *Int Arch Allergy Appl Immunol* 53:310, 1977
2. Arruda LL, Platts-Mills TA, Fox JW, et al: *Aspergillus fumigatus* allergen 1, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* 172:1529, 1990
3. Arruda LK, Platts-Mills TA, Longbottom JL, et al: *Aspergillus fumigatus*: Identification of 16, 18 and 45 kD antigens recognized by human IgG and IgE antibodies and murine monoclonal antibodies. *J Allergy Clin Immunol* 89:1166, 1992
4. Azuma I, Kimura H, Hirao F, et al: Biochemical and immunological studies of *Aspergillus*, I. Chemical and biological investigations of lipopolysaccharide, protein,

24. Fratafico PM, Buckley HR: Identification and characterization of an immunodominant 58 kDa antigen of *Aspergillus fumigatus* recognized by sera of patients with invasive aspergillosis. *Infect Immun* 59:309, 1991
25. Fratafico PM, Long WK, Buckley HR: Production and characterization of monoclonal antibodies to a 58 kDa antigen of *Aspergillus fumigatus*. *Infect Immun* 59:316, 1991
26. Glimp RA, Bayer AS: Fungal pneumonias (Part 3): Allergic bronchopulmonary aspergillosis. *Chest* 80:85, 1981
27. Gordon MA, Lapa EW, Kane J: Modified indirect fluorescent-antibody test for aspergillosis. *J Clin Microbiol* 6:161, 1977
28. Greenberger PA: Allergic bronchopulmonary aspergillosis and fungoses. *Clin Chest Med* 9:599, 1988
29. Greenberger PA, Patterson R: Application of enzyme-linked immunosorbent assay (ELISA) in diagnosis of allergic bronchopulmonary aspergillosis. *J Lab Clin Med* 99:288, 1982
30. Hamilton AJ, Holdom MD, Hay RJ: Specific recognition of purified Cu, Zn superoxide dismutase from *Aspergillus fumigatus* by immune human sera. *J Clin Microbiol* 33:495, 1995
31. Harvey C, Longbottom JL: Characterization of a major antigenic component of *Aspergillus fumigatus*. *Clin Exp Immunol* 65:206, 1986
32. Haynes KA: Purification and characterization of 93 kDa *Aspergillus fumigatus* antigen with diagnostic potential. *J Med Vet Mycol* 34:421, 1996
33. Hearn VM: Antigenicity of *Aspergillus* species. *J Med Vet Mycol* 30:11, 1992
34. Hearn VM, Wilson EV, Latge JP, et al: Immunochemical studies of *Aspergillus fumigatus* mycelial antigens by polyacrylamide gel electrophoresis and Western blotting techniques. *J Gen Microbiol* 136:1525, 1990
35. Hinson KFW, Moon AJ, Plummer NS: Bronchopulmonary aspergillosis: A review and report of eight new cases. *Thorax* 7:317, 1952
36. Hipp SS, Berns DS, Tompkins V, et al: Latex slide agglutination for *Aspergillus* antibodies. *Sabouraudia* 8:237, 1970
37. Hirschberg L, Holme T, Krook A: Human antibody response to the major adhesion of mycoplasma pneumoniae: Increase in titres against synthetic peptides in patients with pneumonia. *APMIS* 99:515, 1991
38. Hoehne JH, Reed CE, Dickie HA: Allergic bronchopulmonary aspergillosis is not rare: With a note on preparation of antigens for immunologic tests. *Chest* 63:177, 1973
39. Hopp PT, Woods KR: Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* 78:3824, 1981
40. Hosein B, Fang CT, Popovsky MA, et al: Improved serodiagnosis of hepatitis C virus infection with synthetic peptide antigen from capsid protein. *Proc Natl Acad Sci USA* 88:3647, 1991
41. Ikemoto H, Shibata S: Indirect haemagglutination in pulmonary aspergilloma diagnosis. *Sabouraudia* 11:167, 1973
42. Jaton-Ogay K, Suter M, Cramer R, et al: Nucleotide sequence of a genomic and cDNA clone encoding an extracellular alkaline protease of *Aspergillus fumigatus*. *FEMS Microbiol Lett* 71:163, 1992
43. Kauffman HF, Beaumont F, Sluiter MJ, et al: Immunologic studies in bronchoalveolar fluid in a patient with allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* 74:835, 1984
44. Kauffman HF, de Vries K: Antibodies against *Aspergillus fumigatus*, II. Identification and quantification by means of crossed immunoelectrophoresis. *Int Arch Allergy Appl Immunol* 62:265, 1980
45. Kauffman HF, van der Heiden PJ, van der Laan S, et al: Antibody determination against *Aspergillus fumigatus* by means of the enzyme-linked immunosorbent assay, II. Physico- and immunochemical properties of the polystyrene-binding components. *Int Arch Allergy Appl Immunol* 78:174, 1985
46. Kauffman HF, van der Heide S, van der Laan S, et al: Standardization of allergenic extracts of *Aspergillus fumigatus*. Liberation of IgE-binding components during cultivation. *Int Arch Allergy Appl Immunol* 76:168, 1985
47. Kim SJ, Chaparas SD: Characterization of antigens from *Aspergillus fumigatus*, I.

- Preparation of antigens from organisms grown in completely synthetic medium. *American Review of Respiratory Disease* 118:547, 1978
48. Kim SJ, Chaparas SD: Characterization of antigens from *Aspergillus fumigatus*, III. Comparison of antigenic relationships of clinically important aspergilli. *American Review of Respiratory Disease* 120:1297, 1979
 49. Kim SJ, Chaparas SD, Brown TM, et al: Characterization of antigens from *Aspergillus fumigatus*, II. Fractionation and electrophoretic, immunologic, and biologic activity. *American Review of Respiratory Disease* 118:553, 1978
 50. Kolattukudy PE, Lee JD, Rogers LM, et al: Evidence for possible involvement of an elastolytic serine protease in aspergillosis. *Infect Immun* 61:2357, 1993
 51. Kovac J: Recombinant proteins derived from immunodominant regions of the gag and env genes of HIV-1 and their potential for use in serologic diagnosis. *Acta Virol* 35:383, 1991
 52. Kumar A, Kurup VP, Greenberger PA, et al: Production and characterization of a monoclonal antibody to a major Concanavalin A-nonbinding antigen of *Aspergillus fumigatus*. *J Lab Clin Med* 121:431, 1993
 53. Kumar A, Reddy LV, Sochanik A, et al: Isolation and characterization of a recombinant heat shock protein of *A. fumigatus*. *J Allergy Clin Immunol* 91:1024, 1993
 54. Kurup VP: Production and characterization of a murine monoclonal antibody to *Aspergillus fumigatus* antigen having IgG and IgE antibody binding activity. *Int Arch Allergy Appl Immunol* 86:400, 1988
 55. Kurup VP, Elms N, Fink JN: Characterization of a monoclonal antibody against Concanavalin A binding antigen of *Aspergillus fumigatus*. *Hybridoma* 10:387, 1991
 56. Kurup VP, Fink JN, Scribner GH, et al: Antigenic variability of *Aspergillus fumigatus* strains. *Microbios* 19:191, 1977
 57. Kurup VP, Greenberger PA, Fink JN: Antibody response to low-molecular-weight antigens of *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *J Clin Microbiol* 27:1312, 1989
 58. Kurup VP, John KV, Resnick A, et al: A partially purified antigen from *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 79:263, 1986
 59. Kurup VP, Kumar A: Immunodiagnosis of aspergillosis. *Clin Microbiol Rev* 4:439, 1991
 60. Kurup VP, Kumar A, Kenealy WR, et al: *Aspergillus* ribotoxins react with IgE and IgG antibodies of patients with allergic bronchopulmonary aspergillosis. *J Lab Clin Med* 123:749, 1994
 61. Kurup VP, Nair MP, Schwartz SA, et al: Serum antibodies and their role in antibody-dependent cell-mediated cytotoxicity in aspergillosis. *Immunobiology* 169:362, 1985
 62. Kurup VP, Ramasamy M, Greenberger PA, et al: Isolation and characterization of a relevant *Aspergillus fumigatus* antigen with IgG and IgE binding activity. *Int Arch Allergy Appl Immunol* 86:176, 1988
 63. Kurup VP, Resnick A, Scribner GH, et al: Comparison of antigens and serological methods in *Aspergillus fumigatus* antibody detection. *Mykosen* 27:43, 1984
 64. Kurup VP, Ting EY, Fink JN: Immunochemical characterization of *Aspergillus fumigatus* antigens. *Infect Immun* 41:698, 1983
 65. Kyte J, Doolittle RF: A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157:105, 1982
 66. Latge JP, Moutaouakil M, Debeaupuis JP, et al: The 18 kilodalton antigen secreted by *Aspergillus fumigatus*. *Infect Immun* 59:2586, 1991
 67. Little SA, Warner JO: Improved diagnosis of allergic bronchopulmonary aspergillosis with gp66 (formerly antigen 7) of *Aspergillus fumigatus* for specific IgE detection. *J Allergy Clin Immunol* 98:55, 1996
 68. Longbottom JL: Allergens of *Aspergillus fumigatus*: Identification and characterization of components combining with specific IgE antibody. *Zentralbl Bakteriol Mikrobiol Hyg Reihe A* 261:503, 1986
 69. Longbottom JL: Allergic bronchopulmonary aspergillosis: Reactivity of IgE and IgG antibodies with antigenic components of *Aspergillus fumigatus* (IgE/IgG antigen complexes). *J Allergy Clin Immunol* 72:668, 1983
 70. Longbottom JL: Antigens/allergens of *Aspergillus fumigatus*. Identification of antigenic

- components reacting with both IgG and IgE antibodies of patients with allergic bronchopulmonary aspergillosis. *Clin Exp Immunol* 53:354, 1983
71. Longbottom JL: Antigens and allergens of *Aspergillus fumigatus*, II. Their further identification and partial characterization of a major allergen (Ag 3). *J Allergy Clin Immunol* 78:18, 1986
 72. Longbottom JL: Immunological aspects of infection and allergy due to *Aspergillus* species. *Mykosen Supplement* 1:207, 1978
 73. Longbottom JL, Austwick PK: Antigens and allergens of *Aspergillus fumigatus*. Characterization by quantitative immunoelectrophoretic techniques. *J Allergy Clin Immunol* 78:9, 1986
 74. Longbottom JL, Harvey C, Taylor ML, et al: Characterization of immunologically important antigens and allergens of *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 88:185, 1989
 75. Lopez-Medrano R, Ovejero MC, Calera JA, et al: An immunodominant 90-kilodalton *Aspergillus fumigatus* antigen is the subunit of a catalase. *Infect Immun* 63:4774, 1995
 76. MacMillan RH, Copper PH, Body BA, et al: Allergic fungal sinusitis due to *Curvularia lunata*. *Hum Pathol* 18:960, 1987
 77. Malo JL, Paquin R, Longbottom JL: Prevalence of precipitating antibodies to different extracts of *Aspergillus fumigatus* in a North American asthmatic population. *Clin Allergy* 11:333, 1981
 78. Manning SC, Mabry RL, Schaefer SD, et al: Evidence of IgE-mediated hypersensitivity in allergic fungal sinusitis. *Laryngoscope* 103:717, 1993
 79. Mehta SK, Sandhu RS: Efficacy of counter immunoelectrophoresis in the detection of fungal antibodies in allergic bronchopulmonary mycoses. *Zentralblatt für Bakteriologie* 247:537, 1980
 80. Miller S, Janin J, Lesk-AM, et al: Interior and surface of monomeric proteins. *J Mol Biol* 196:641, 1987
 81. Monod M, Togni G, Rahalison L, et al: Isolation and characterization of an extracellular alkaline protease of *Aspergillus fumigatus*. *J Med Microbiol* 35:23, 1991
 82. Moser M, Crameri R, Brust E, et al: Diagnostic value of recombinant *Aspergillus fumigatus* allergen I/a for skin testing and serology. *J Allergy Clin Immunol* 93:1, 1994
 83. Moser M, Crameri R, Menz G, et al: Cloning and expression of recombinant *Aspergillus fumigatus* allergen I/a (rAspfI/a) with IgE binding and type I skin test activity. *J Immunol* 149:454, 1992
 84. Moser M, Menz G, Blaser K, et al: Recombinant expression and antigenic properties of a 32 kilodalton extracellular alkaline protease representing a possible virulence factor from *Aspergillus fumigatus*. *Infect Immun* 62:936, 1994
 85. Murali PS, Kurup VP, Greenberger PA, et al: Concanavalin-A nonbinding *Aspergillus fumigatus* antigen: A major immunogen in allergic bronchopulmonary aspergillosis. *J Lab Clin Med* 119:377, 1992
 86. Murali PS, Pathial K, Saff RH, et al: Immune responses to *Aspergillus fumigatus* and *Pseudomonas aeruginosa* antigens in cystic fibrosis and allergic bronchopulmonary aspergillosis. *Chest* 106:513, 1994
 87. Patterson R, Greenberger PA, Halwig JM, et al: Allergic bronchopulmonary aspergillosis, natural history, and classification of early disease by serologic and roentgenographic studies. *Arch Intern Med* 146:916, 1986
 88. Patterson R, Greenberger PA, Radin RA, et al: Allergic pulmonary aspergillosis: Staging as an aid to management. *Ann Intern Med* 96:286, 1982
 89. Piechura JE, Huang CJ, Cohen SH, et al: Antigens of *Aspergillus fumigatus*, II. Electrophoretic and clinical studies. *Immunology* 49:657, 1983
 90. Piechura JE, Kurup VP, Daft LJ: Isolation and immunochemical characterization of fractions from membranes of *Aspergillus fumigatus* with protease activity. *Can J Microbiol* 36:33, 1990
 91. Pinon JM, Thoannes H, Poirriez J, et al: Enzyme-linked immunofiltration assay (ELIFA) for the detection of IgG, IgM, IgA, and IgE antibodies against *Aspergillus fumigatus*. *J Med Vet Mycol* 25:77, 1987
 92. Reichard U, Buttner S, Eifffert H, et al: Purification and characterization of an extracel-

- lular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *J Med Microbiol* 33:243, 1990
93. Rhodes JC, Amlung TW, Miller MS: Isolation and characterization of an elastinolytic protease from *Aspergillus flavus*. *Infect Immun* 58:2529, 1990
 94. Rosenberg M, Patterson R, Mintzer R, et al: Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med* 86:405, 1977
 95. Samuelsen H, Karlsson-Borga A, Paulsen BS, et al: Purification of a 20 kD allergen from *Aspergillus fumigatus*. *Allergy* 46:115, 1991
 96. Schonheyder H, Andersen P: An indirect immunofluorescence study of antibodies to *Aspergillus fumigatus* in sera from children and adults without aspergillosis. *Sabouraudia* 20:41, 1982
 97. Schonheyder H, Andersen P: Complement-binding antibodies to *Aspergillus fumigatus* in patients with pulmonary aspergillosis. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica Section B, Microbiology* 91:1, 1983
 98. Schonheyder H, Andersen P: Determination of antibodies to partially purified *Aspergillus* antigens by an enzyme-linked immunosorbent assay. *Int Arch Allergy Appl Immunol* 70:108, 1983
 99. Schonheyder H, Andersen P: Fractionation of *Aspergillus fumigatus* antigens by hydrophobic interaction chromatography and gel filtration. *Int Arch Allergy Appl Immunol* 73:231, 1984
 100. Schonheyder H, Andersen P: IgG antibodies to purified *Aspergillus fumigatus* antigens determined by enzyme-linked immunosorbent assay. *Int Arch Allergy Appl Immunol* 74:262, 1984
 101. Schonheyder H, Andersen P, Stenderup A: Serum antibodies to *Aspergillus fumigatus* in patients with pulmonary aspergillosis detected by immunofluorescence. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica Section B, Microbiology* 90:273, 1982
 102. Schonheyder H, Jensen T, Laessoe IH, et al: Serum antibodies to *Aspergillus fumigatus* catalase in patients with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* 7:40, 1988
 103. Schonheyder H, Storgaard L, Andersen P: Variation of a 470,000 daltons antigen complex and catalase antigen in clinical isolates of *Aspergillus fumigatus*. *Sabouraudia* 23:339, 1984
 104. Sepulveda R, Longbottom JL, Pepys J: Enzyme-linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of *Aspergillus fumigatus*, 1979. *Clin Allergy* 9:359, 1979
 105. Sharma GL, Sarma PU: Luminoimmunoassay for detection of specific antibodies in allergic bronchopulmonary aspergillosis. *Serodiagnosis and Immunotherapy in Infectious Disease* 5:186, 1993
 106. Sirakova TD, Markaryan A, Kolattukudy PE: Molecular cloning and sequencing of the cDNA and gene for a novel elastinolytic metalloproteinase from *Aspergillus fumigatus* and its expression in *Escherichia coli*. *Infection and Immunity* 62:4208, 1994
 107. Ste. Marie L, Senechal S, Boushira M, et al: Production and characterization of monoclonal antibodies to cell wall antigens of *A. fumigatus*. *Infect Immun* 58:2105, 1990
 108. Taylor ML, Longbottom JL: Partial characterization of a rapidly released antigenic/allergenic component (Ag5) of *Aspergillus fumigatus*. *J Allergy Clin Immunol* 81:548, 1988
 109. Teshima R, Ikebuchi H, Sawada J, et al: Isolation and characterization of a major allergenic component (gp55) of *Aspergillus fumigatus*. *J Allergy Clin Immunol* 92:698, 1993
 110. Tran-van Ky P, Torck C, Vaucelle T, et al: Comparative study on immunoelectrophoregrams of enzymes of the antigenic extract of *Aspergillus fumigatus* revealed by experimental serums and serums of patients with aspergillosis. *Sabouraudia* 7:73, 1969
 111. Trompelt J, Becker WM, Schlaak M: Analysis of IgG subclass and IgE response in allergic disease caused by *Aspergillus fumigatus* by immunoblotting techniques. *Int Arch Allergy Immunol* 104:390, 1994
 112. van der Heide S, Kauffman HF, de Vries K: Cultivation of fungi in synthetic and

- semi-synthetic liquid medium, I. Growth characteristics of the fungi and biochemical properties of the isolated antigenic material. *Allergy* 40:586, 1985
113. van der Heide S, Kauffman HF, de Vries K: Cultivation of fungi in synthetic and semi-synthetic liquid medium, II. Immunochemical properties of the antigenic and allergenic extracts. *Allergy* 40:592, 1985
 114. Walker CA, Fitzharris P, Longbottom JL, et al: Lymphocyte sensitization to *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *Clin Exp Immunol* 76:34, 1989
 115. Walker CA, Fitzharris P, Longbottom JL, et al: T and B cell responses to *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* 81:184, 1988
 116. Wallenbeck I, Aukrust L, Einarsson R: Antigenic variability of different strains of *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 73:166, 1984
 117. Wang JL, Patterson R, Rosenberg M, et al: Serum IgE and IgG antibody activity against *Aspergillus fumigatus* as a diagnostic aid in allergic bronchopulmonary aspergillosis. *American Review of Respiratory Disease* 117:917, 1978
 118. Wilson EV, De Magaldi SW, Hearn VM: Preparative isoelectric focusing of immunologically reactive components of *Aspergillus fumigatus* mycelium. *J Gen Microbiol* 130:919, 1984
 119. Wilson EV, Hearn VM: Use of *Aspergillus fumigatus* mycelial antigens in enzyme-linked immunosorbent assay and counter-immunoelectrophoresis. *J Med Microbiol* 16:97, 1983
 120. Wolfenden R, Andersson L, Cullis PM, et al: Affinities of amino acid side chains for solvent water. *Biochemistry* 20:849, 1981

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