Immunodiagnosis of Aspergillosis

VISWANATH P. KURUP* AND ANOOPA KUMAR
Allergy-Immunology Division, Department of Medicine, Medical College of Wisconsin, and Research Service, Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin 53295-1000

INTRODUCTION.................................................................439
CLASSIFICATION OF DISEASES CAUSED BY ASPERGILLUS SPECIES .................................................................439
CHARACTERISTIC FEATURES OF ASPERGILLUS-INDUCED DISEASES .................................................................440
Saprophytic Colonization .................................................................440
Bronchopulmonary colonization .................................................................440
Aspergillosis .................................................................440
Allergic Diseases .................................................................440
Allergic asthma .................................................................440
Allergic rhinitis .................................................................440
Allergic sinusitis .................................................................441
HP .................................................................441
ABPA ........................................................................441
(i) Clinical presentation of ABPA .................................................................441
(ii) Diagnostic criteria of ABPA .................................................................441
Systemic Disease .................................................................442
IA ................................................................................442
IMMUNOLOGY OF ASPERGILLOSA S SPECIES ................................................................................443
Humoral Immune Response .................................................................443
Cellular Immune Response .................................................................443
ETIOLOGY AND EPIDEMIOLOGY ................................................................................443
Etiology of Aspergillosis ........................................................................443
Epidemiology of Aspergillosis .................................................................444
IMMUNODIAGNOSIS ........................................................................444
Antigens ................................................................................444
Antigen preparation ........................................................................444
Fractionation and purification .................................................................445
Serological Tests ........................................................................447
ID ................................................................................447
RIA ................................................................................447
ELISA and BALISA ........................................................................448
Immunoblotting ........................................................................448
Skin Tests ........................................................................449
Detection of Circulating Antigens for Diagnosis of IA .................................................................449
CONCLUSION ........................................................................450
ACKNOWLEDGMENTS ........................................................................450
REFERENCES ........................................................................450

INTRODUCTION

Members of the genus Aspergillus are fungi ubiquitously distributed from the Arctic region to the tropics sparing no materials or objects. The term aspergillus was first described by Micheli in 1729 (149), and the first description of a human disease caused by aspergilli was made in 1847 (212). The aspergilli have become increasingly important since then and have been implicated as the causative agents of a number of diseases in humans and other animals. The aspergilli are considered opportunistic pathogens, and a variety of underlying conditions including impaired immune status contribute to disease production. In addition, however, the duration and quantity of exposure to the organism are important determinants of host response, and selected forms of aspergilloses have been detected in apparently healthy subjects. The number of Aspergillus conidia in the air and on various substrates apparently influences the development of various clinical forms of aspergillosis.

CLASSIFICATION OF DISEASES CAUSED BY ASPERGILLUS SPECIES

Aspergillus species have been associated with a wide spectrum of diseases in humans and other animals. Colonization and exposure to antigens in normal hosts can lead to allergic diseases ranging from asthma to allergic bronchopulmonary aspergillosis (ABPA). Aspergillomas may develop in paranasal sinuses or in preexisting pulmonary cavities. Invasive aspergillosis (IA) is primarily an infection of immunocompromised patients, but severe infection can also occur in patients with chronic diseases such as diabetes, alcoholism, and cancer. IA may also develop in patients as a

* Corresponding author.
postoperative or posttraumatic complication or as a superinfection following antibiotic therapy or the insertion of prosthetic devices or indwelling catheters. Although neutropenia is the most common predisposing factor, infections have been reported in completely normal individuals, in some instances, following exposure to exceptionally high concentrations of Aspergillus conidia (1, 93, 151).

In most patients, the clinical entities are distinct; in a few patients, however, two or more entities may coexist, or there may be progression from one to the other. Hence, no single classification of Aspergillus-induced diseases is entirely satisfactory. However, these diseases can be broadly classified according to the following clinical conditions: saprophytic colonization, including saprophytic bronchopulmonary aspergillosis and pulmonary aspergillosis; allergic diseases, including allergic asthma, allergic rhinitis, allergic sinusitis, hypersensitivity pneumonitis (HP), and ABPA, and systemic disease, i.e., IA.

CHARACTERISTIC FEATURES OF ASPERGILLUS-
INDUCED DISEASES

Saprophytic Colonization

Bronchopulmonary colonization. Bronchopulmonary colonization refers to organism infestation of airways without evidence of direct tissue damage. The most important factors predisposing to colonization of the respiratory tract with aspergilli or other fungi include chronic destructive pulmonary disease, prolonged use of broad-spectrum antibiotics, malnutrition, and debilitation. A high incidence of saprophytic colonization has also been demonstrated in patients with bronchial asthma, bronchiectasis, chronic bronchitis, cystic fibrosis, and primary ciliary dyskinesia syndrome (19, 20). Some of these patients mount an immunologic response manifested by serum precipitins, immediate skin reactivity, and an Arthus skin reaction to Aspergillus antigen. Although aspergilli have been isolated from the respiratory tracts of such patients, no direct correlation with underlying pulmonary disease could be made. Thus, saprophytic colonization in its usual and uncomplicated form is not an infection in the strict sense. However, its presence could adversely affect the immune responsiveness in a host and may interfere with the diagnosis of various disease entities (21).

Aspergillum. Aspergillum, also known as mycetoma or fungus ball, is a saprophytic manifestation of a fungus growing in a preformed and poorly drained lung space. In 1938, Deve coined the term aspergillum to describe the mycetomalike lesion caused by Aspergillus species (48). An intrathoracic cavity, whether bronchial, parenchymatous, or pleural, can become colonized by Aspergillus species if it communicates with the bronchial tree. Aspergillus species may grow in the preformed spaces in the lung for months or years without invading the wall or other tissues. Many pulmonary diseases favor the development of aspergillomas. The most common antecedent diseases are pulmonary tuberculosis with healed cavities and chronic pulmonary sarcoidosis with cystic spaces (31, 90). Fungal balls may also develop in the presence of active pulmonary tuberculosis or in ectatic bronchi with accompanying ABPA (187, 232). Other antecedent diseases include ankylosing spondylitis, histoplasmosis, lung abscess, bronchiectasis, lung tumors, radiation fibrosis, pneumoconiosis, and pulmonary infarction. Diagnosis usually occurs in the fifth or sixth decade of life. The most common symptom associated with aspergillum is recurrent hemoptysis. The cause is uncertain, but hemoptysis has been ascribed to intracavitary growth, mechanical friction due to mycetomal movement, and a trypsinlike proteolytic enzyme produced by aspergilli (39, 232). A chronic cough with the production of purulent sputum and generalized symptoms of fever, weight loss, and malaise are common in patients who are atopic. Patients with aspergillum and concomitant ABPA may also demonstrate wheezing and tightness of chest.

Pulmonary aspergillomas can often be diagnosed by their characteristic appearance in roentgenographs (167). The characteristic sign is an opacity occupying some or most of a round or ovoid cavity that is partially surrounded by a crescent-shaped patch of air. Aspergillomas most often are located in the upper lobes or the superior segment of lower lobes. Although they tend to be solitary, bilateral and multiple aspergillomas have been described (35, 165). In the early stage of the disease, the presence of pleural thickening overlying the involved pulmonary parenchyma may suggest the development of Aspergillus superinfection. Additional radiographic findings may include proximal lobar shrinkage, bronchiectasis, and extensive patchy infiltrate contiguous to the cavity or enlargement of the cavity by the fungus ball.

Allergic Diseases

Allergic asthma. Asthma is a chronic disease characterized by increased responsiveness of the tracheobronchial tree to various stimuli, particularly following exposure to specific allergens. Fungal spore-induced asthma may cause chronic and even severe respiratory symptoms such that patients require inhaled or alternate-day oral corticosteroids. Mold-associated asthma may be more prevalent in children than in adults in some geographical areas. A number of fungi, including Aspergillus species, have been implicated in allergic asthma. Exposure to fungal conidia results in nasal symptoms like those of a cold followed by coughing and wheezing. The development of asthma is slow and persistent in mold-sensitive patients. The symptoms due to mold asthma can be differentiated from those attributable to other allergen-induced asthm by a history of exposure, skin test reactivity, or in vitro demonstration of specific immunoglobulin (immunoglobulin E [IgE]) antibodies in patient sera. Many patients with allergic asthma exhibit a biphasic response to inhalation challenge with appropriate antigens (166). However, the lack of sufficiently pure and reliable allergenic extracts poses a major problem in the development of in vivo and in vitro tests for diagnosis (256).

Allergic rhinitis. The classical forms of nasal allergy, hay fever and allergic rhinitis, may develop in susceptible individuals on exposure to mold spores and mycelia (34). Allergic rhinitis develops as a result of interaction of allergens and IgE fixed to specific mast cells in the epithelial surface of the nasal mucosa. This results in the release of a number of chemical mediators, including histamine, eosinophil and other chemotactic factors, leukotrienes, and prostaglandins, which contribute to the pathogenesis of the disease (53, 65, 94, 210). Allergic rhinitis can occur as a seasonal or a nonseasonal disease. In addition to Aspergillus species, a variety of other fungi can also act as causative agents of allergic rhinitis. The symptoms and signs of fungus-induced allergic rhinitis are usually indistinguishable from those caused by the inhalation of pollen, dust, danders, and insect allergens. The clinical symptoms may include persistent or intermittent nasal obstruction, nasal discharge, pruritis, and eye irritation. On subsequent exposures to fungal spores, sneezing and nocturnal cough may develop. The diagnosis of
allergic rhinitis is based on clinical history and IgE-mediated skin reactivity to appropriate Aeroallergens. In vitro tests such as the radioallergosorbent test and paper radioimmunosorbent test are useful in the diagnosis (34, 53, 65, 94, 210, 218, 256).

Allergic sinusitis. Sinus disease due to fungi includes several clinicopathological syndromes, including fungal ball production and allergic fungal sinusitis (32, 94, 137). The pathophysiology of allergic sinusitis differs considerably from that of fungal mycetoma. Allergic fungal sinusitis, which occurs in atopic individuals, is chronic and affects multiple sinuses without tissue invasion. Several fungi have been implicated in allergic fungal sinusitis. The most important ones are Aspergillus, Curvularia, Alternaria, and Bipolaris species (32, 63, 94, 137). Most affected individuals are young and atopic, and nasal polyposis, asthma, and rhinitis are the frequently noted symptoms (63). Mucoid impaction ("allergic mucin"), resulting in plugs containing fragments of the fungi similar to those seen in the sputum plugs of patients with ABPA, is a consistently noticed feature in patients with ABPA, is a consistently noticed feature in

VOL.

VOL.

VOL.

VOL.

VOL.

immunodiagnosis of aspergillosis. Immunodiagnosis of aspergillosis 441

ABPA. Early reports of Aspergillus disorders, possibly ABPA and characterized by severe bronchitis accompanied by asthmatic symptoms, were given by Popoff in 1887 (175) and Hamman in 1927 (73). The first definitive case of ABPA was reported by Hinson et al. from the United Kingdom in 1952 (84). Originally considered a rarity, ABPA is currently recognized with much greater frequency. Since the report of Hinson et al., many cases have been reported from all over the world, and considerable information on the clinicopathologic features has been obtained. ABPA with varied clinical presentations has been reported to occur in 20% of asthmatic patients admitted to hospitals and in 5% of all rhinitis cases, while the incidence in patients with cystic fibrosis may vary from 10 to 25% (21, 208). Nevertheless, the onset of ABPA can be traced to early childhood or even infancy, and the disease may remain undiagnosed for years or decades (67). Thus, the actual incidence of ABPA seems to be much higher than is indicated from the reported number of cases.

ABPA is the result of hypersensitivity to Aspergillus antigens in patients with long-standing atopic asthma. ABPA usually presents as an acute, easily reversible asthmatic syndrome that progresses to a more intractable asthmatic state with transient pulmonary infiltrates and a striking sensitivity to Aspergillus antigen. Subsequently, a chronic stage with admixture of reversible and irreversible obstructive lung diseases emerges (138).

(i) Clinical presentation of ABPA. With few exceptions, patients with ABPA are atopic and have a history of bronchial asthma (26, 209, 226). Although there is no special age or sex distribution for ABPA, the disease tends to affect younger people, with most cases occurring before the age of 40. The disease has been reported even in children of <2 years of age (89). The clinical symptoms of an acute episode of ABPA should be differentiated from those of more chronic forms. The acute onset is characterized by severe asthma and wheezing, fever, malaise, weight loss, chest pain, and productive cough with blood-streaked sputum. As the disease becomes chronic, the patient develops bronchiectasis with production of copious purulent sputum. Patients may cough up green, brown, or often blood-tinged mucous plugs containing eosinophils, Charcot-Leyden crystals, and fungal elements.

The radiological findings are quite varied. The commonest finding is homogeneous or nonhomogeneous consolidation, with or without atelectasis, involving a segment or a lobe. Generally, the upper lobes are involved (184). The upper lobe infiltrates shift from one place to another as the disease progresses. Repeated episodes of ABPA result in edema of bronchial walls, their dilation, and their eventual destruction. Radiographically, these changes are manifested as thickening of the walls and saccular dilation. With multiple episodes, bronchiectasis, cavitation, atelectasis, and subsequent fibrosis may also develop. Because the chest X-ray and symptoms may not exclude the possibility of ABPA in some patients, and some patients with ABPA show no remarkable symptoms, procedures such as tomography or computerized tomography scanning may be essential for demonstrating bronchiectasis. These procedures usually reveal cylindrical or saccular bronchiectasis with distal sparing, which suggests a pattern of localized obstruction and inflammation resulting from mucus and mycelial plugs in the larger air passages (53, 152). The administration of oral corticosteroids clears the mucoid impactions which are responsible for much of the radiological involvement in patients with ABPA.

(ii) Diagnostic criteria of ABPA. ABPA has been described as an immunological disease that ranges from asthma to fatal destructive lung disease with defined clinical, serological, radiological, and pathological features (64, 66, 156, 157, 159,
TABLE 1. Criteria for diagnosis of ABPA<sup>a</sup>

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Occurrence of symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRI&lt;sup&gt;b&lt;/sup&gt; present</td>
</tr>
<tr>
<td>Asthma</td>
<td>+</td>
</tr>
<tr>
<td>Peripheral blood eosinophilia (&gt;1,000/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>+</td>
</tr>
<tr>
<td>Immediate cutaneous reactivity to &lt;i&gt;A. fumigatus&lt;/i&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Serum precipitins to &lt;i&gt;A. fumigatus&lt;/i&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Elevated level of serum IgE (&gt;1,000 ng/ml)</td>
<td>+</td>
</tr>
<tr>
<td>Proximal bronchiectasis</td>
<td>+</td>
</tr>
<tr>
<td>Elevated level of serum IgE and IgG to &lt;i&gt;A. fumigatus&lt;/i&gt;</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adapted from Greenberger (65).

<sup>b</sup> CRI, chest roentgenographic infiltrate.

<sup>c</sup> Compared with serum from patients with asthma and immediate cutaneous reactivity to <i>A. fumigatus</i> but in whom ABPA has been ruled out. This assay is not widely used and is not absolutely necessary in the diagnosis of classic cases. However, it is valuable for early diagnosis when all of the features of ABPA are not present.

160, 163, 164). Hinson et al. originally described ABPA as an allergic response to <i>Aspergillus fumigatus</i> in bronchial secretions. However, they could not demonstrate skin test reactivity to an <i>Aspergillus</i> extract in their patients (84). Since then, a number of additional features of ABPA have been recognized (49). The diagnostic criteria have been revised several times and elaborated upon since their initial description (21, 65, 145, 158, 185). The diagnosis of ABPA depends on central bronchiectasis and the presence or absence of chest roentgenographic infiltrate. The criteria are listed in Table 1. Patients in whom proximal bronchiectasis is not detected by radiological procedures are designated ABPA-S (for seropositive), and those with central bronchiectasis are designated ABPA-CB. These designations combined with currently available serodiagnostic methods aid in the early diagnosis of ABPA (65).

According to Greenberger and Patterson, ABPA should be ruled out in all patients with chronic asthma (67). Although the classic patients meet the criteria for ABPA, other suspected patients should be evaluated over a period of time (65, 72, 125, 181). After being tested with <i>A. fumigatus</i> extracts, patients usually manifest dual cutaneous reactivity that consists of a wheal and erythema reaction within 10 to 20 min and erythema and induration (without a wheal) in 4 to 8 h. A dual response following bronchial provocation with <i>A. fumigatus</i> and production of sputum plugs containing hyphae are the characteristic features of a classic patient with ABPA (146). A different set of diagnostic criteria was used by Sandhu et al. in their study (190). They considered asthma, dual cutaneous reactions to <i>A. fumigatus</i>, recurrent roentgenographic infiltrates, elevated peripheral blood eosinophilia, positive sputum culture, and precipitins to <i>A. fumigatus</i> as diagnostic. These criteria would identify only the classic cases and miss the early cases of ABPA and cases of ABPA in remission.

ABPA should be ruled out in patients with chronic asthma exhibiting immediate reactivity to <i>A. fumigatus</i> or other <i>Aspergillus</i> antigens. Most of these patients demonstrate a 3+ to 4+ skin prick test response and will not require an intracutaneous injection. Although an elevated level of total serum IgE is suggestive, it is not specific for ABPA. The roentgenographic consolidation resulting from bacterial pneumonia can be differentiated from that of pulmonary infiltrates of ABPA (66). Posterior segments of upper lobes are often affected, simulating tuberculosis, but middle and lower lobes have also been involved. The possibility of ABPA should also be considered in patients with a history of surgical resection for bronchiectasis (66). There are also reports of ABPA-like syndromes caused by <i>Curvularia</i>, <i>Candida</i>, and <i>Helminthosporium</i> species and other fungi (5, 65, 71, 83). Skin tests and tests for precipitins to <i>A. fumigatus</i> may be negative in these cases, whereas the culture of sputum or the presence of precipitins to other fungi may aid in specific diagnosis.

### Systemic disease

IA. Although IA has been considered an infection exclusively of immunocompromised patients, sporadic cases have been reported even in normal hosts (1, 93, 151). Infection is common in patients with leukemia and other hematological malignancies, in patients undergoing organ transplantation, and in chronic granulomatous disease of childhood (17, 29, 148, 176, 177). The association of IA with AIDS is still controversial in spite of the few reported cases (43, 62). An increased prevalence of IA has been noted in bone marrow transplant recipients (42, 168, 231). Predisposing factors for the development of IA in immunocompromised patients are manifold. They include neutropenia, granulocytopenia, underlying chronic diseases, and treatments such as those with corticosteroids, broad-spectrum antibiotics, and cytotoxic drugs (3, 54, 59, 70). The IA detected in apparently healthy individuals may be due to unrecognized subtle immunological defects, tissue damage, viral infections, or antibiotic therapy for a bacterial infection (30). Several recent nosocomial outbreaks of IA that occurred among leukemic patients and renal transplant recipients have been attributed to a variety of environmental sources. The potential sources of <i>Aspergillus</i> conidia from contaminated environments included hospital renovations, road constructions, and fire-proofing materials. <i>A. fumigatus</i> conidia from contaminated air conditioners have been implicated in a number of cases of IA (4, 11, 126, 191).

The pathogenesis of IA is complex. The lung serves as the portal of entry for the fungus, and colonization of the respiratory tract is followed by endobronchial proliferation and bronchial ulceration. The fungus may spread to the lung parenchyma, resulting in necrotizing bronchopneumonia, lobar pneumonia, or hemorrhagic infarction. An alternative route of colonization is through necrotic lung tissue following pulmonary emboli or bacterial infection or from the cavitory growth. As a result, single or multiple abscesses may be formed, in which case the diagnosis is made only at autopsy. Hematogenous spread from sites such as the gastrointestinal tract or intravenous catheters results in multiple miliary microabscesses throughout all organs (154, 155, 253).

The symptoms of IA are mostly nonspecific. They include cough, wheezing, dyspnea, and fever. In <i>Aspergillus</i>-induced bronchitis, the chest radiograph may be normal, but single or multifocal nodules can be seen in established invasive disease. These nodules may undergo infarction or cavitation and appear often as bilateral consolidations (155). The widespread radiological infiltrates give an indication of how extensively the disease has spread. The differential diagnosis of IA includes infections such as those due to <i>Pneumocystis carinii</i> or <i>Nocardia</i>, <i>Candida</i>, <i>Mycobacterium</i>, or <i>Mycoplasma</i> species as well as some of the common bacterial infections.
IMMUNOLOGY OF ASPERGILLOSIS

Because of the universal distribution of aspergilli, all individuals are exposed to these organisms, but the normal defense mechanisms act as a barrier to infection from inhaled Aspergillus conidia. The aerosolized conidia are easily inhaled, and they encounter alveolar macrophages as the first line of defense. Studies in mice have shown that alveolar macrophages are efficient scavengers of inhaled Aspergillus conidia (51). Macrophages do not readily kill the conidia, but they prevent their germination (107). The mucociliary cells efficiently clear conidia from the lung into the gastrointestinal tract during swallowing (44). The different responses to pergilloma, very little is known about the cellular immune mechanisms in Aspergillus conidia in and around the dwellings of two patients with ABPA (24). They found heavy spore contamination in the

Humoral Immune Response

Humoral immune responses in patients with ABPA and aspergilloma have been well studied. Sera from patients with ABPA consistently demonstrate high levels of A. fumigatus-specific circulating antibodies of the IgG and IgE isotypes (130, 185). Specific antibodies belonging to other immunoglobulin classes have also been reported in these patients. According to Brummund et al., in addition to elevated levels of IgE and IgG antibodies, there was evidence of polyclonal elevation of levels of all immunoglobulin isotypes in patients with Aspergillus-induced diseases (33). In a recent study, Kurup et al. showed that, along with all immunoglobulin isotypes, levels of subclasses of IgG, particularly IgG1 and IgG2, are also elevated in ABPA patients (120). Similarly, A. fumigatus-specific antibodies belonging to the IgG class of immunoglobulins have been reported to be the predominant antibody isotype in patients with aspergilloma (240). Recently, we have demonstrated that A. fumigatus-specific IgG1 is the predominant subclass of IgG in patients with aspergilloma (120).

Specific humoral responses differ considerably in patients with different clinical forms of aspergillosis because of the participation of diverse antigens of A. fumigatus (21, 65, 240). Serum antibodies in patients with ABPA frequently react with protein antigens of A. fumigatus. Identical levels of A. fumigatus-specific antibodies belonging to the IgG1 and IgG2 subclasses are usually found in the sera of these patients (120). Although aspergilloma patients frequently react to carbohydrate and glycoprotein antigens of A. fumigatus, their sera show more IgG1 than IgG2 subclasses. The characteristic expression of A. fumigatus-specific antibodies in the sera of patients with ABPA and aspergilloma suggests a difference in the fundamental immunoregulatory response to Aspergillus antigens in these disorders.

Cellular Immune Response

Lymphocytic infiltration and granuloma formation in the lung clearly demonstrate the presence of a cell-mediated immune response in ABPA patients (208). In some studies of A. fumigatus antigen-induced lymphocyte proliferation in ABPA patients, no correlation with disease activity was detected. In addition, proliferation was not significantly higher in ABPA patients than in patients with a positive immediate skin test reaction to A. fumigatus (77, 211). These findings suggest that T-lymphocyte sensitization is not an important feature of ABPA. The lack of lymphocyte stimulation reported in these studies may be due to the use of less relevant antigens or to the treatment of the patients with prednisonsone. In contrast, other reports indicate that antigen-induced lymphocyte transformation can be demonstrated in patients with ABPA (57, 104, 234, 235). An antigen-induced proliferative response of lymphocytes from these ABPA patients has been correlated with disease activity. During the exacerbation of disease, the proliferative response of peripheral blood lymphocytes was undetectable. Analysis of peripheral blood by lymphocyte phenotyping demonstrated a significant increase in T-helper or inducer cells, whereas the suppressor cell population was normal (104). Antibody-dependent, cell-mediated cytotoxicity has also been reported in both ABPA and aspergilloma (117).

The pathogenesis of ABPA is not completely understood. The host immunological response presumably is exaggerated in terms of specific antibody isotype, total serum IgE production, sensitized lymphocytes, cellular hyperreactivity, and complement activation in the bronchoalveolar compartment. Studies of cell-mediated immunity in Aspergillus-induced diseases are conflicting. The limited knowledge and conflicting results of studies on cell-mediated immune responses in patients with ABPA may be due partly to the lack of pure, dependable, and reproducible antigens. Studies with purified antigens would be of value in understanding both humoral and cell-mediated arms of immune response in aspergillosis. In contrast, the lymphokines and the cell-mediated mechanisms by which T cells participate in the regulation of IgE synthesis and eosinophil activation and differentiation in certain other allergic diseases have been well documented (99, 231). Similar mechanisms may play a major role in the pathogenesis of ABPA.

ETIOLOGY AND EPIDEMIOLOGY

Etiology of Aspergillosis

A. fumigatus is the species of Aspergillus most frequently isolated from human beings with saprophytic, allergic, or invasive disease manifestations. It is also associated with food spoilage and with diseases of plants and animals (2, 12, 28, 37, 92, 182). Although A. fumigatus is the principal etiological agent of ABPA, other species such as A. terreus, A. flavus, A. nidulans, A. oryzae, and A. niger have been associated with the disease (6, 124, 190). Most aspergillomas are caused by A. fumigatus. Other species, such as A. niger, have also been incriminated as causative agents (207). The predominant species involved in IA is A. fumigatus, but other species including A. flavus, A. glaucus, A. niger, A. nidulans, and A. terreus have also been implicated (253).

Opinions vary on the importance of the number of Aspergillus conidia in the immediate environment of patients. ABPA has resulted from smoking moldy marijuana and from exposure to A. fumigatus-contaminated dump sites and bird droppings (106, 119, 128, 145). In addition, closed environments, pets, house plants, and carpets often contribute to the excessive concentration of A. fumigatus conidia inside houses or other buildings (213, 214, 230, 233). Beaumont et al. measured airborne concentrations of Aspergillus conidia in and around the dwellings of two patients with ABPA (24). They found heavy spore contamination in the...
environment of one patient, with lower spore concentrations in the other. Both patients had increased bronchial obstruction during periods when the Aspergillus spore content in the outside air was high. The results of the study suggest that avoiding an Aspergillus spore-contaminated environment and limiting overall exposure may play a major role in preventing exacerbation of the disease. Such aerobiological studies in the microenvironments of patients with ABPA may aid in detecting a particular mold source and in monitoring the intensity of exposure. These parameters may be vital in immunosuppressed patients at risk of contracting IA.

**Epidemiology of Aspergillosis**

Better epidemiological tools for the characterization of aspergilli associated with IA in immunosuppressed patients are needed. Although a detailed epidemiological investigation may not be necessary when the causative agent is readily identifiable from a particular source, it may be of utmost importance in hospital-acquired infections (87). Identification of aspergilli is based mainly on morphological characteristics, but variants within a species are known to occur, and strain recognition is then a problem. In a study on the morphological variation of a series of A. fumigatus isolates from aspergilloma patients, Leslie and co-workers found strains exhibiting progressively more abnormal characteristics during the course of the infection (127).

Recently, typing schemes for the identification of aspergilli have been explored. A biotyping scheme known as killer typing, based on the toxins released from the fungus, has been reported to be an effective epidemiological marker in the differentiation of A. fumigatus strains (174). Burnie and associates used immunoblot fingerprinting for the examination of strains of A. fumigatus (36). They found 11 immunoblot types among 21 isolates obtained from eight aspergilloma patients. However, it is not known whether this delineation of types was due to quantitative or qualitative differences in proteins. Restriction enzyme analysis of mitochondrial DNA has been used as an aid in determining the taxonomy of the genus Aspergillus (105). A recent study has shown that restriction fragment length polymorphism analysis of total cellular DNA can be used to distinguish or match diverse or related A. fumigatus isolates (46). The analysis of 31 epidemiologically characterized isolates from three continents revealed 24 patterns (DNA types). Among these, three DNA types were represented by three isolates each and one DNA type was represented by two isolates. Twenty types were unique. This kind of a typing system might help answer questions concerning the genetics, pathogenicity, epidemiology, and ecology of A. fumigatus. An exploration of the clonality of A. fumigatus by using this typing method might throw light on the epidemiological aspects of aspergillosis.

**IMMUNODIAGNOSIS**

**Antigens**

The diagnosis of ABPA is dependent on clinical and immunological findings. Circulating antibodies to specific antigens have been detected in patients with most of the Aspergillus-induced diseases. Extracts from the whole mycelium or metabolic products secreted into the medium during the growth of A. fumigatus have been used as antigens to detect antibodies in the sera of patients. In addition, a few semipurified antigens with more predictable reactivity with patient sera have been isolated in recent years (80, 82, 115, 132, 172, 194, 197, 249). However, standardized antigens are still not available for dependable use in the serological diagnosis of ABPA and other Aspergillus-induced diseases. Pure antigens of known physicochemical properties would be invaluable in defining the different immunological mechanisms involved in these diseases.

Demonstration of circulating antibodies is considered an important criterion in the diagnosis of Aspergillus-induced diseases. However, currently available antigen and antibody detection methods are not altogether satisfactory and need to be improved for dependable results. The major reasons for the nonavailability of standardized antigens are as follows. (i) Antigen preparations are highly variable. Culture filtrate and mycelial extracts show considerable differences in their antigenic components. The variation is further aggravated by the use of different strains of A. fumigatus and different cultural conditions and extraction procedures (114). (ii) Antigens show cross-reactivity. Significant cross-reactivity exists between A. fumigatus strains and other aspergilli as well as between A. fumigatus and other fungi and bacteria (19, 22). This cross-reactivity may be due to epitope sharing between the antigens or to the universal presence of bacterial and fungal antigens in the sera of normal subjects. In addition to immunological cross-reactivity, due to shared antigens, some Aspergillus antigens frequently show reactivity to C-reactive protein in the sera of patients with a variety of infectious, neoplastic, and autoimmune diseases. The cross-reactivity of epitopes in most cases is due to the limited number of shared epitopes, mostly between polysaccharides from different fungi. (iii) Dependable serological methods are lacking. Not all available serological assays are sensitive and specific when crude antigens are used. Sensitive methods such as the enzyme-linked immunosorbent assay (ELISA) and radioimunoassay (RIA) need purified antigens for reliable and reproducible results (22, 98, 122, 123). (iv) Toxins and enzymes may be present. Antigen preparations frequently contain toxins such as endotoxins, hemolytic toxins, and, rarely, aflatoxins (121). In addition, several proteolytic enzymes from A. fumigatus antigens may cause interference and adversely affect the reproducibility of test results.

In light of these problems, purified or semipurified and standardized antigens are essential for the development of reliable and sensitive immunossays. For rapid progress in the standardization of antigens for laboratory and clinical use, identification of relevant components of crude antigenic extracts is of primary importance. Once such components are identified, it is possible to obtain fractions with fewer or homogeneous components. Such preparations can be standardized with less difficulty and can be successfully used in immunodiagnosis as well as in understanding the immunopathogenesis of the disease.

The chemical nature of antigens of Aspergillus species may be polysaccharide, protein, or glycoprotein. In many early studies, carbohydrate antigens (14–16, 188, 189, 216) were used in immunossays, but the cross-reactivities of these antigens with those of other fungi was a major drawback in interpreting test results. Most later investigators used the protein and glycoprotein antigens to detect antibodies in patient serum (74, 115, 122, 197, 248, 250). On the other hand, in IA, galactomannan and glycoproteins are the major circulating antigens (25, 50, 179).

**Antigen preparation.** A. fumigatus strains vary considerably in their antigenic makeup. Kurup et al. studied 11 strains of A. fumigatus from different sources and tested
their culture filtrate antigens against sera from 33 patients with aspergillosis (114). Individual antigens reacted differently with these sera, and the detection of specific antibodies in these 33 sera varied from 40 to 76% (114). Similar results have been reported by other investigators (102, 220, 221, 223, 226). Variation in the antigenic components was also noted with different batches of antigens prepared from the same strain. On the other hand, Odds et al. reported qualitative similarity of all batches of antigens from A. fumigatus and considered that the variability was only quantitative in nature (153). Our studies as well as the results reported by other investigators indicate that use of synthetic medium and incubation of A. fumigatus at 37°C resulted in more consistently reactive antigens (101, 114, 228, 229). From the available reports it can be concluded that the most reliable cell-associated antigens for immunoassay can be obtained from 4-day-old growth of A. fumigatus in aerated cultures. Reliable culture filtrate antigen for immunoassays can be obtained from 2- to 4-week-old stationary cultures of the organism. The number of antigenic components is higher obtained from 2- to 4-week-old stationary cultures of the organism. The number of antigenic components is higher.

Fractionation and purification. In recent years several investigators have attempted to characterize and purify relevant antigens of A. fumigatus (Table 2). Most of the earlier attempts involved harsh physical and chemical treatments, which had an adverse effect on antigenicity. Both culture filtrate and mycelial extracts contain a number of antigenic components capable of reacting with patient sera. These antigens include heterogeneous proteins, carbohydrates, and glycoproteins (38, 60, 78, 79, 81, 96, 103, 115, 171, 200, 247). Kim et al. demonstrated up to 28 bands by polyacrylamide gel electrophoresis (103), while Piechura et al. showed about 200 demonstrable components by two-dimensional electrophoresis (171). Immunoblots from one- and two-dimensional electrophoreses have shown that a number of these components react with sera from patients with aspergillosis. Crossed immunoelectrophoresis of Aspergillus culture filtrate antigen against sera from patients with ABPA demonstrated approximately 35 precipitin arcs, of which 8 to 10 showed specific reactivity with IgE antibodies (118, 133). Using crossed isoelectric focusing, Kim and associates demonstrated 52 precipitin arcs (103). These findings indicate that the A. fumigatus antigens constitute a very heterogeneous group.

Aspergillus antigens demonstrating a variety of enzyme activities have been reported by several investigators. By using the enzyme staining method of Uriel et al., Tran van Ky et al. detected 18 enzymatically active components from Aspergillus species (222–225). Of the 18 antigens showing enzyme activity, 13 reacted specifically with sera from patients with aspergillosis. Biguet et al. demonstrated antigen fractions having chymotryptic activity that specifically reacted with sera from patients with aspergillosis but not with sera from patients with ABPA (27). A fraction having a molecular weight of 250,000, probably a tetramer demonstrating catalase activity, has been reported by Schonheyder et al. (200). This fraction had sugar subunits with affinity to concanavalin A (ConA) and was detected in 13 of 14 clinical isolates of A. fumigatus. However, this component with catalase activity was also shared by other Aspergillus species and has not been clinically evaluated. Antibodies to catalase antigen have been found in patients with cystic fibrosis having ABPA (199). Fractions with protease activity have been reported from both pathogenic and nonpathogenic species of Aspergillus. Schonheyder and Andersen partially purified a 25- to 30-kDa protease fraction having reactivity with patient sera (197). We have isolated two acid proteases from membrane vesicles of A. fumigatus. The molecular weights of these fractions were 73,000 and 43,000. Both fractions bound to ConA and showed reactivity with sera from patients with ABPA in a biotin-avidin-linked immuno sorbent assay (BALISA) (172).

Polysaccharide fractions from either cell wall or cytoplasm show reactivity with specific antibodies. However, these antigens frequently demonstrate cross-reactivity with other fungal antigens. Galactomannan, with a molecular weight of 50,000 or more, has been purified by Azuma et al. It showed both skin test reactivity and a precipitin reaction (13–16). These authors also characterized and purified a galactomannan peptide complex by using a Sephadex G-200 column. This galactomannan has been identified as the significant circulating antigen in patients with IA (25, 50, 179).

Several investigators identified ConA-binding components of A. fumigatus as the major antigens consistently showing reactivity with patient sera (74, 76, 123, 196, 248). These antigens have been isolated from both culture filtrate and mycelial extracts of A. fumigatus (100, 204, 246, 249, 256). Wilson and Hearn fractionated mycelial extracts by preparative isoelectric focusing and compared the reactivity of the three major fractions with that of crude water-soluble extracts (248). They found that all fractions reacted with sera from patients with aspergillosis but also showed cross-reactivity with antibodies against other fungi. By using preparative isoelectric focusing and affinity chromatography, we have isolated two fractions, one a glycoprotein and the other a protein (123). In an ELISA, both fractions demonstrated reactivity against patient sera. We have also isolated another fraction which focused at pH 6.5 and showed 20-, 40-, and 80-kDa components in two-dimensional electrophoresis. This fraction also showed good reactivity with both IgE and IgG antibodies (116). Longbottom and associates characterized four antigens (Ag 3, Ag 5, Ag 7, and Ag 13) by using gel filtration, isoelectric focusing, and affinity chromatography (74–76, 132, 134, 135, 219). Two of these fractions, Ag 7 and Ag 13 with molecular masses of 150,000 to 200,000 and 70,000, respectively, showed ConA binding. Both of these antigens reacted in an ELISA with sera from patients with ABPA and aspergillosis. Two other fractions, Ag 5 and Ag 3, which were thermolabile peptides with molecular masses of 35 and 18 kDa (24 kDa by gel filtration), respectively, were also isolated from A. fumigatus and found to be useful in detecting antibodies in patients with ABPA. Schonheyder and associates identified several fractions of A. fumigatus antigens with molecular masses of 25,000 to >470,000 Da (196, 200). Some of these fractions were evaluated and found to be very specific in the diagnosis of aspergillosis. Using hydrophobic interaction chromatography, Schonheyder and Andersen obtained a component having a molecular mass distribution of 25 to 50 kDa (196). In polyacrylamide gel electrophoresis this fraction showed 14 components, of which 3 had ConA-binding ability. None of these components, however, were isolated in pure form. By using hydrophobic interaction and affinity chromatography,
<table>
<thead>
<tr>
<th>Antigen extract</th>
<th>Disease category</th>
<th>Antibody assay</th>
<th>Antibodies detected</th>
<th>Results and conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma</td>
<td>RIST, RAST, XIEF</td>
<td>IgE, precipitins</td>
<td>Individual components and reactivity with sera varied</td>
<td>95</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma, allergic asthma, aspergilloma with ABPA</td>
<td>ID, EAST, RAST, RIEF</td>
<td>IgE, IgG</td>
<td>IgE binding components found in early logarithmic phase; precipitating antigenic components low in early phase, increased during late phase</td>
<td>194</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma</td>
<td>2-DIEF, ID, PAGE</td>
<td>Precipitins</td>
<td>Concentrated ABPA sera showed precipitins similar to aspergilloma</td>
<td>82</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma, IA, other mycoses</td>
<td>ELISA, IIF, ID</td>
<td>Precipitins</td>
<td>Aspergillosis patients reacted with wide range of antigens of A. fumigatus; some antigens cross-reacted with sera from other mycotic diseases</td>
<td>45</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma, bronchial asthma</td>
<td>ID, ELISA</td>
<td>IgE, precipitins</td>
<td>Polysaccharides cross-reacted with other Aspergillus species and fungi</td>
<td>204</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA</td>
<td>RAST, self-XIEF, ELISA, PRIST</td>
<td>IgE</td>
<td>Two components: major antigens with strong IgE binding and poor precipitating activity and minor antigens with strong precipitating activity but weak IgE binding</td>
<td>131</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA</td>
<td>ELISA, XIEF, XIEF, fused RIEF</td>
<td>IgE</td>
<td>Ag 3, low molecular wt heat labile, non-ConA binding, reacted with 75% of ABPA sera</td>
<td>132</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, A. fumigatus STP</td>
<td>ELISA, XIEF</td>
<td>IgG, IgG1, IgG4</td>
<td>Elevated IgG to Ag 7 detected in 97% of ABPA sera</td>
<td>75</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma, farmer’s lung, A. fumigatus STP</td>
<td>ELISA, XIEF, fused RIEF</td>
<td>IgE, IgG</td>
<td>Ag 13, heat labile, ConA binding, with chymotryptic activity, poor binding to IgE</td>
<td>76</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma</td>
<td>ELISA, XIEF, ID</td>
<td>IgE, IgG</td>
<td>Glycoprotein antigen reacted with aspergillosis</td>
<td>123</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma</td>
<td>BALISA</td>
<td>IgE, IgG</td>
<td>Fractions 18, 19, 20 were ConA binding, reacted with ABPA and aspergilloma</td>
<td>116</td>
</tr>
<tr>
<td>CF</td>
<td>ABPA, aspergilloma</td>
<td>FAST, BALISA, Western blot</td>
<td>IgG, IgE</td>
<td>Low-molecular-wt, non-ConA-binding antigens reacted with ABPA</td>
<td>115</td>
</tr>
<tr>
<td>Mycelial</td>
<td>ABPA, aspergilloma, EAA, persistent lung infiltration, COPD</td>
<td>ELISA</td>
<td>IgG</td>
<td>70,000-Da antigen was reactive</td>
<td>194</td>
</tr>
<tr>
<td>Mycelial</td>
<td>ABPA, aspergilloma</td>
<td>ID, CIEP</td>
<td>Precipitins</td>
<td>Triton X fraction superior to water-soluble fraction; cross-reactivity between other aspergilli</td>
<td>80</td>
</tr>
<tr>
<td>Mycelial</td>
<td>ABPA, aspergilloma, lung disorders with A. fumigatus colonization</td>
<td>ID, CIEP, ELISA, XIEF</td>
<td>Precipitins</td>
<td>ConA-bound fraction superior to non-ConA-bound fraction</td>
<td>246</td>
</tr>
<tr>
<td>Mycelial</td>
<td>ABPA, aspergilloma, asthma, cystic fibrosis with A. fumigatus precipitins</td>
<td>BALISA</td>
<td>IgE, IgG</td>
<td>ConA-binding glycoprotein reacted with IgG and IgE compared with non-ConA-binding antigen</td>
<td>118</td>
</tr>
<tr>
<td>Mycelial and CF</td>
<td>ABPA, aspergilloma</td>
<td>ELISA, XIEF, ID</td>
<td>IgG</td>
<td>470,000-Da fraction reacted with normal controls and patients; 250,00-Da antigen had catalase activity</td>
<td>196</td>
</tr>
<tr>
<td>Mycelial and CF</td>
<td>ABPA, aspergilloma, bronchial asthma, bronchiectasis, tuberculosis</td>
<td>ELISA, ID</td>
<td>IgG and IgM</td>
<td>Cytoplasmic extracts bound more weakly to IgG than mycelial fractions</td>
<td>247</td>
</tr>
</tbody>
</table>

*Continued on following page*
we have isolated a fraction with two components of 35 and 65 kDa. This fraction consistently showed binding to IgG and IgE antibodies in sera from patients with ABPA (118). Using gel filtration, ConA affinity chromatography, iso-electric focusing, and trichloroacetic acid precipitation, Kauffman et al. obtained several antigen fractions from A. fumigatus (97). They observed that the low-molecular-weight (5,000 to 28,000) fraction usually demonstrated weak linkage to the polystyrene surface used in ELISA and gave weak precipitins. In contrast, high-molecular-weight components reacted strongly in the ELISA and precipitin tests. Longbottom found that low-molecular-weight peptides showed strong IgE-binding capacity and were more specific in the diagnosis of allergic aspergillosis (131).

Monoclonal antibodies produced against Aspergillus species will be of value in purifying the relevant Aspergillus antigens (55, 109, 110, 215). Using several such monoclonal antibodies, we purified antigens by employing monoclonal antibody affinity chromatography. Some of these antigens showed both IgE- and IgG-binding activities and will be of use in immunodiagnosis (109, 110). Thus, it can be seen that a number of homogeneous fractions that are reactive with patient sera can be purified. However, such antigens are not yet available commercially or for general use.

Serological Tests

Serological tests serve as important aids in the effective diagnosis of various types of aspergillosis. Circulating antibodies against A. fumigatus or other species of Aspergillus can be readily detected in immunocompetent patients. However, a different approach may be necessary in immunosuppressed patients with no demonstrable antibodies against the invading fungus. In these instances antigen detection systems may provide information helpful in diagnosing the disease. A variety of tests with diverse sensitivities have been developed to detect circulating antibodies to aspergilli.

ID. Because of its simplicity and ease of performance, immunodiffusion (ID) is still the most widely used technique for the serodiagnosis of aspergillosis. Several investigators have highlighted its usefulness in demonstrating precipitin arcs in agar gels as a diagnostic tool for aspergillosis (18, 19, 40, 113, 122, 123, 136, 140, 170, 239). Antigens used in ID may be extracts from mycelia or culture filtrates, which should be free of C-reactive substance (136). Concentrating the serum specimens before testing may yield better results without reduction in specificity (137). However, the ID test lacks sensitivity and gives no quantitative information on antibody concentrations. A micromodification of Wadsworth’s agar gel diffusion technique has been used in our laboratory for testing antibodies in the sera of patients against a panel of antigens (52). This method yielded reproducible results and has correlated well with other diagnostic methods, although the sensitivity is much lower than that of ELISA or RIA.

Immunogold assay. An immunogold assay was developed by Gugnani et al. for the detection of A. fumigatus-specific IgG and IgE antibodies (69). This method is similar to indirect immunofluorescence (IIF) but more sensitive and does not need any specialized equipment. Slide cultures of A. fumigatus, fixed in methanol, are treated with different dilutions of patient sera, then with goat anti-human IgG or IgE, and finally with rabbit anti-goat IgG conjugated with colloidal gold. After being dehydrated with alcohol and xylol, the slides are mounted and observed under light and phase-contrast microscopes. A positive reaction is indicated by a brownish color on the surface of hyphae caused by binding of gold particles. Gugnani et al. found a positive correlation between the degree of reactivity detected by the immunogold assay, the BALISA titers, and the precipitins detected by agar gel diffusion. To make the sensitivity of the immunogold assay comparable to that of the BALISA or RIA, silver enhancement can be used (141). Recent studies using immunogold electron microscopy with thin sections of Aspergillus hyphae and conidia have shown that both spores

---

**TABLE 2—Continued**

<table>
<thead>
<tr>
<th>Antigen extract</th>
<th>Disease category</th>
<th>Antibody assay</th>
<th>Antibodies detected</th>
<th>Results and conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial and CF</td>
<td>ABPA, aspergilloma, other fungal diseases</td>
<td>ID, PAGE, XRIEF</td>
<td>Precipitins</td>
<td>CS2 reacted with 75% of ABPA and aspergilloma</td>
<td>38</td>
</tr>
<tr>
<td>Mycelial and CF</td>
<td>ABPA, aspergilloma, other fungal diseases</td>
<td>ID, IEF, PAGE, XRIEF, ELISA</td>
<td>IgG, IgA, IgM</td>
<td>CS2 fraction reacted with aspergillosis sera</td>
<td>171</td>
</tr>
<tr>
<td>Mycelial, CF, spore extract</td>
<td>ABPA, aspergilloma, Aspergillus asthma</td>
<td>ELISA, ID, RAST, EAST</td>
<td>IgE, IgG</td>
<td>Spore cytoplasm is similar to CF and mycelial antigen, but showed low ELISA titers; spore surface antigen had low immunological activity</td>
<td>96</td>
</tr>
<tr>
<td>CF, germinated conidia, conidial surface antigens</td>
<td>ABPA</td>
<td>IF, XRIEF, RIEF, fused RIEF, ELISA</td>
<td>IgE, IgG</td>
<td>Ag.5, heat labile, non-ConA binding, reacted with IgG and IgE</td>
<td>219</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td>ABPA, aspergilloma</td>
<td>ID, XRIEF, BALISA</td>
<td>IgE, IgG</td>
<td>Two fractions with protease activity reacted with ABPA and aspergilloma</td>
<td>172</td>
</tr>
</tbody>
</table>

a. CF, culture filtrate; EAA, extrinsic allergic alveolitis; STP, skin test positive; COPD, chronic obstructive pulmonary disease; IEF, immunoelectrophoresis; RIST, radioimmunosorbent test; RAST, radioallergosorbent test; XRIEF, crossed immunoelectrophoresis; EAST, enzyme allergosorbent test; RIEF, rocket immunoelectrophoresis; 2-DIEF, two-dimensional immunoelectrophoresis; PAGE, polyacrylamide gel electrophoresis; self-XRIEF, self-crossed radioimmuno-electrophoreses; PRIST, paper radioimmunosorbent test; FAST, fluorescent allergosorbent test; CIEP, counterimmunoelectrophoresis.
and hyphae react identically with IgG and IgE antibodies of serum from aspergillosis patients (178). Sera from patients with ABPA and aspergilloma reacted with cell envelope antigens, whereas sera from patients with IA also bound to cell sap.

RIA. A RIA for the measurement of Aspergillus antibody was first described by Bardana et al. (22). The antigen was prepared by ultrasonic disruption of A. fumigatus mycelia followed by ammonium sulfate precipitation. The radiolabeled trichloroacetic acid-soluble antigen fraction was used to detect antibodies to A. fumigatus. Several investigators have developed and utilized RIA as a reliable tool for detecting antibody in patients with aspergillosis (142, 161, 162). A radio Allergosorbent test developed by Arbesman et al. used the culture filtrate antigen covalently bound to cellulose disks for the detection of IgE antibodies to A. fumigatus (10). RIA has several disadvantages including limited storage life of the radioisotopes, length of time required to complete the assay, need for expensive radioactivity detectors, and potential hazards and difficulties connected with exposure to and safe disposal of radioactive wastes.

ELISA and BALISA. Because of its high sensitivity, reliability, and versatility, the ELISA is being widely used in the serodiagnosis of various forms of aspergillosis. Although the sensitivity of the ELISA system depends on many variables, the nature and type of antigens used and their abilities to bind to solid surfaces are of prime importance. The complexity of A. fumigatus antigens used in the techniques and the inherent variations in antigen preparations may lead to major discrepancies in results from different laboratories. Both mycelial and culture filtrate antigens of A. fumigatus have been used in ELISA (100, 204, 249). Wilson and Hearn used partially purified mycelial and culture filtrate antigens of A. fumigatus, rich in protein and carbohydrate, to compare the efficacy of these antigens in ELISA (249). They found that fractionation of mycelial extract yielded components that proved to be more sensitive in detecting specific antibodies in patient sera. However, they observed batch-to-batch variation in the antigen preparations. Khan et al. used a fraction from a culture filtrate of A. fumigatus to detect specific IgG in patients with allergic aspergillosis (100). They noticed rapid binding of IgG antibodies to both whole and fractionated antigens. Mohan et al. compared different A. fumigatus antigens and found the metabolic antigen prepared from 24-h-old culture to be specifically reactive against patient sera (150). Schonheyder and Andersen studied the reactivities of three antigen fractions of molecular weights 470,000, 250,000, and 25,000 to 50,000 (197). The 470,000-molecular-weight fraction reacted with most sera from normal controls and patients with nonfungal lung diseases. The antibody response in aspergillosis patients was much stronger against this fraction than against control sera. When the 250,000-molecular-weight antigen was used in ELISA to test the sera from patients with aspergilloma, 13 of 14 patients gave positive results, but only one of nine serum samples from patients with ABPA showed antibodies to this antigen. Schonheyder and Andersen concluded that these three fractions may all be of value in the immunodiagnosis of patients with A. fumigatus-induced diseases, particularly when other methods fail to demonstrate specific antibodies.

We have compared antigens from three strains of A. fumigatus by using the serological methods ID, IIF, and ELISA, all with patient sera (122). The results showed wide differences in the reactivity of antigens by different methods. It has been concluded from the results that simultaneous use of several different A. fumigatus antigen preparations and serological methods might help eliminate both false-positive and -negative results, although such procedures are cumbersome and time-consuming.

The studies described above reveal that ELISA results vary widely depending on the type of antigen used for detection of antibodies to A. fumigatus. The conventional ELISA system has been used successfully to detect A. fumigatus-specific IgG in patients with aspergillosis. This assay detected and quantitated antigen-specific IgG when it was present in relatively high concentrations in the sera. However, the test was not sensitive enough to detect relatively low levels of antigen-specific IgE or subclasses of IgG.

To overcome this disadvantage of the conventional ELISA (108), Brummond et al. have developed a modified technique, the BALISA (33). This method has a sensitivity comparable to that of RIA. The technique makes use of high-affinity binding between biotin and avidin, which can be covalently coupled to other molecules such as proteins and polysaccharides. We have been using the BALISA technique routinely in our laboratory for the detection of A. fumigatus-specific antibodies and have obtained reproducible results. BALISA correlates well with clinical features of disease and has sensitivity comparable to that of RIA.

Immunoblotting. Several studies have shown that glycoprotein antigens, a major constituent of both the mycelium and the culture filtrate of A. fumigatus, have high immunological reactivity (23, 74, 115, 197, 246, 250). These antigens can be characterized by their molecular size, isoelectric points, ability to bind to ConA or other lectins, and enzymatic activity. The reactivity of specific components of A. fumigatus antigens to specific antibodies in the sera of patients can be studied by immunoblotting. This method involves the separation of denatured 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 color reaction. Immunoblotting has been used with great success in detecting specific antibodies to Aspergillus species. The detailed methods of immunoblotting for the demonstration of anti-A. fumigatus IgG and IgE in the sera of patients with aspergillosis have been reported (115).

Several other diagnostic procedures that have also been developed for detecting aspergillosis include immunoelectrophoresis, passive hemagglutination, IIF, latex agglutination, complement fixation, and enzyme-linked immunofiltration assays (7, 22, 45, 58, 60, 61, 85, 87, 88, 95, 112, 129, 130, 139, 147, 173, 193, 195, 198, 201–203, 237, 238, 252).

Several types of immunoelectrophoretic techniques have been used in diagnosing aspergillosis. Among these, counterimmunoelectrophoresis has been widely used, mainly because of the short time required to obtain results with this test. By using counterimmunoelectrophoresis, Longbottom could detect precipitins in 78% of patients with allergic aspergillosis; the ID test was positive in only 64% of the same cases (129). However, compared with that for ID, the improved efficacy of immunoelectrophoresis has not been demonstrated because of the lack of specificity due to an increased number of false-positive results (112, 139, 147).

Crossed immunoelectrophoresis and crossed radioimmunoelectrophoresis have been reported to have added significance in the demonstration of A. fumigatus-specific antibodies. By using an intermediate gel and a reference serum, over 40 specific precipitin arcs have been detected (95, 130). With such an immunoelectrophoretic technique, enzymatically
active antigens can also be detected (95, 200), but these techniques are not often used because of the lengthy and cumbersome procedures. The occasional failures of these methods have been related to either a shortcoming in the techniques or the nonprecipitating nature of antigen-antibody complexes (95, 241).

Apart from the use of immunoelectrophoresis in the characterization of antigens, the use of other tests is fairly limited because of factors such as false positivity, instability of antigen-treated erythrocytes, and the recent availability of more versatile and more sensitive detection methods. While the ID test and ELISA are the most widely used serodiagnostic methods in diagnosing aspergillosis, immunoblotting has the advantage of revealing additional characteristics of the relevant and reactive antigens.

**Skin Tests**

Skin test reactivity to *Aspergillus* antigen extracts is widely used to screen patients with suspected ABPA, in whom at least two types of skin reactions can be observed. The type I (immediate) reaction consists of cutaneous "wheat and flare" within 15 to 20 min of skin test challenge. A type III reaction, or the Arthus reaction, can be seen as an induration usually developing by 4 to 10 h after skin testing. This reaction subsides after 24 h, but occasionally a delayed reaction, which might persist for 72 h or more, can also be seen. In patients with allergic asthma, only the immediate reaction is visible, whereas some aspergilloma patients having concomitant ABPA demonstrate both immediate and late skin test reactivities. A delayed-type skin test reactivity may also be present in patients with HP.

**Detection of Circulating Antigens for Diagnosis of IA**

Aspergillosis is a major infection in patients with underlying diseases and other predisposing factors such as organ transplantation. The mortality of IA is very high and can be substantially reduced if an early diagnosis is made. Such a diagnosis is often difficult because major clinical symptoms are nonspecific and blood cultures are usually negative. Bronchoscopy and biopsy of the lung and involved organs are essential for diagnosis, but these invasive procedures have their own associated risks and high rates of false-negative results (91). Interest in developing rapid serological methods for the diagnosis of this disease has therefore increased.

The usual serological studies have not been useful in the diagnosis of IA. Because of the unusual predisposition of patients with IA, namely, lack of a functionally normal immune system because of malignancy or intentional immunosuppression as in organ transplantation, there is very little or no antibody response in these patients. Even so, all previous reports have dealt with the detection of Aspergillus-specific antibodies in the sera and body fluids of IA patients. Young and Bennett could not detect any significant antibodies against *A. fumigatus* in 15 patients tested by ID, complement fixation, and IIF (252). All of their patients had underlying hematological malignancies. Coleman and Kaufman reported precipitins in the sera of 14 of 16 patients studied (41). Other investigators have also reported various degrees of success in detecting specific antibodies against *A. fumigatus* in the sera of patients with IA (60, 68, 86, 143, 144, 148, 192). When success in demonstrating specific antibodies against *Aspergillus* spp. has been reported, it has not received widespread recognition because of the lack of reliability and sensitivity of the tests. Since patients develop the antibody only at the terminal stage of the disease, when treatment is no longer effective, it is essential to look for rapid and more reliable assays for the early diagnosis of IA. Hence, in recent years considerable attention has been paid to the early detection of *A. fumigatus* antigens in the blood and urine of patients suspected of having aspergillosis.

Several investigators have detected *A. fumigatus* antigens in animals with experimentally induced IA (47, 50, 169, 179, 180, 206, 217, 243). The methods used were ID, ELISA, and RIA. The sensitivity of these earlier methods was not sufficient to allow an early diagnosis. However, they were instrumental in the development of more refined assays for demonstrating antigenemia in patients. Recently, Western blotting (immunoblotting) has been used to detect circulating antigens in the sera and urine of rats with experimental IA (254). Van Cutsem et al. used a commercially available latex agglutination test in experimental invasive aspergillosis in guinea pigs. The test had a 97.6% sensitivity in detecting circulating galactomannan in the plasma samples from those animals. The kit was tested with human sera and was positive in 13 of 15 proven cases and 17 of 18 patients with a high probability of invasive aspergillosis (227).

A number of investigators have evaluated methods for detecting *A. fumigatus* antigens in the bronchoalveolar lavage fluid, sera, and urine of patients with aspergillosis (9, 50, 58, 78, 91, 186, 205, 244, 245, 250, 255). The recent reports on the detection of antigenemia are summarized in Table 3. Weiner and associates used competitive binding RIA for detecting cell wall carbohydrates and found antigenemia in 78% of the patients with aspergillosis and leukemia (244). Other investigators used inhibition ELISA to quantitate specific antigens in the sera of patients with aspergillosis and of those predisposed to aspergillosis (50, 56, 183, 186, 250). The circulating antigen detected in these instances is a major cell wall polysaccharide, galactomannan. The sensitivity is still not satisfactory or comparable to that of RIA. Sabetta et al. demonstrated antigenemia in 11 of 19 patients (186), while Dupont et al. could detect galactomannan in only 2 of 12 patients studied (50). On the other hand, Wilson et al., using a sonicated mycelial extract, were able to detect antigenemia in 78% of their patients (250). They considered an antigen concentration of 100 ng or more diagnostic. Using a BALISA method, Fujita and associates demonstrated antigenemia in 16 of 18 patients studied (56). Our study, in which we used a double-sandwich inhibition BALISA, dem-

### Table 3. Summary of recent published reports on antigen detection in IA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Antigen</th>
<th>Specimen</th>
<th>No. positive/totals patients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>3/3</td>
<td>205</td>
</tr>
<tr>
<td>RIA, ELISA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>7/9</td>
<td>244</td>
</tr>
<tr>
<td>Inhibition ELISA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>11/19</td>
<td>186</td>
</tr>
<tr>
<td>RIA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>4/7</td>
<td>245</td>
</tr>
<tr>
<td>ELISA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>8/9</td>
<td>250</td>
</tr>
<tr>
<td>RIA, ELISA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>2/12</td>
<td>56</td>
</tr>
<tr>
<td>RIA</td>
<td>Carbohydrate</td>
<td>Urine</td>
<td>7/13</td>
<td></td>
</tr>
<tr>
<td>Inhibition BALISA</td>
<td>Glycoprotein</td>
<td>Serum</td>
<td>4/5</td>
<td>91</td>
</tr>
<tr>
<td>Inhibition BALISA</td>
<td>Glycoprotein</td>
<td>Urine</td>
<td>18/19</td>
<td>183</td>
</tr>
<tr>
<td>Western blotting</td>
<td>Galactomannan</td>
<td>Urine</td>
<td>10/10</td>
<td>78</td>
</tr>
</tbody>
</table>
onstrated the presence of heat-stable *A. fumigatus* antigens in sera from all five bone marrow recipients with aspergillosis (91). In this assay, the antigens detected were mostly heat-resistant glycoproteins. Using polyclonal antibodies, Haynes and associates detected antigens in the urine of all 10 of their patients with aspergillosis and in 23 neutropenic patients with no evidence of aspergillosis. However, no reaction was found when monoclonal antibody against galactomannan was used (78).

The success in detecting antigenemia in a patient’s serum mainly depends on the frequent monitoring of samples (78, 242, 244, 250). The importance of serial sampling and adequate storage conditions has been emphasized. When single serum samples were tested, Wilson et al. found a frequency of detection of only 3.5%, which was increased to 19.2%, however, when two or more serum samples were obtained per patient (250). Weiner et al. reported a frequency of 70 to 76% antigenemia in serum samples from proven cases of invasive disease (242, 244). The false-negative results often obtained when detecting antigenemia may be ascribed to antifungal drugs administered to the patient, rapid clearance of circulating antigen, the presence of different antigenic components in the serum or urine at different time periods.

Details of the three methods commonly used for detecting *A. fumigatus* antigenemia, RIA, inhibition BALISA, and immunoblotting, may be found in the literature (8, 78, 91, 108).

**CONCLUSION**

IA, with its wide spectrum of clinical manifestations, has become a major fungal infection. Similarly, *A. fumigatus*-induced allergic diseases are increasingly being diagnosed. With the increasing incidence reported in recent years and the paucity of appropriate treatment, there is a need for an early diagnosis of these diseases for effective management. There has been significant progress towards the characterization and purification of antigens of *A. fumigatus*, but the common antigen(s) that can be used universally for reliable diagnosis has not yet been identified. In this context, use of monoclonal antibodies in serodiagnostic methods may yield improved results. *A. fumigatus*-specific antigens can be purified with monoclonal antibodies and standardized to give reliable results in immunodiagnosis. Rapid and reliable immunodiagnosis may also be achieved by the use of molecular biological methods to obtain purified antigens. Studies with these reagents may then enhance our understanding of the immune mechanisms of these diseases.

**ACKNOWLEDGMENTS**

This work was supported in part by a Public Health Service grant from the National Institutes of Health (AI-23071) and by the Department of Veterans Affairs.

**REFERENCES**


IIMUNODIAGNOSIS OF ASPERGILLOSIS 453
454 KURUP AND KUMAR

antigen and antibody relevance to infection. Mycopathologia 70:37-41.


152. Need, D. A., L. R. Goodman, J. W. Gurney, P. A. Green-


162. Patterson, R., M. Roberts, A. C. Ghory, and P. A. Green-


179. Reiss, E., and P. F. Lehmann. 1979. Galactomannan antigene-


189. Sakaguchi, O., K. Yokota, and M. Suzuki. 1969. Immunochemo-


IMMUNODIAGNOSIS OF ASPERGILLOSIS


Vol. 4, 1991


Letter to the Editor

Antigen Detection in Invasive Aspergillosis

In the article on immunodiagnosis of aspergillosis by Kurup and Kumar (2) some of our work was incorrectly cited, and we wish to bring this to the attention of the readers who may have been misled. We refer specifically to Table 3, page 449, and to text on pages 449 and 450.

With regard to Table 3 and the information summarized from our paper (3), we wish to point out that two inhibition enzyme-linked immunosorbent assays (ELISAs), not the biotin-avidin-linked immunosorbent assay (BALISA), were used; in one case a polyclonal antiserum derived from a human patient with aspergillosis was employed, and in the other, a monoclonal antibody to galactomannan was used. Because of the way our data were presented in that paper, it is somewhat difficult to ascertain values for serum versus urine specimens on a per patient basis. Therefore, we provide the following information. The sera of 17 of 19 patients and 16 of 19 patients, all with invasive aspergillosis (IA), were positive with the polyclonal antiserum and the monoclonal antibodies, respectively. Urine specimens from six of eight patients and eight of eight patients were positive with the polyclonal and monoclonal ELISAs, respectively.

With regard to the information presented in Table 3 on Western blotting of urine samples (1), again, two separate antibody preparations were employed for antigen detection. In the first instance, a polyclonal antiserum specific for unfractionated cell walls was used, and 10 of 10 IA patients tested were positive. Only three urine specimens were available for blotting with the second antibody preparation, viz., the monoclonal antigalactomannan antibody, but all three specimens were positive. The band patterns obtained with the two antibody preparations were completely different. The anti-cell wall antiserum revealed seven bands, the major ones corresponding to molecular masses of 11, 18, and 29 kDa, whereas the antigalactomannan monoclonal antibody revealed bands corresponding to molecular masses greater than 45 kDa plus a single band at 21 kDa. We concluded that none of the antigens detected by the polyclonal antiserum were galactomannan.

In the text on page 450, again with respect to the work of Haynes et al. (1), Kurup and Kumar state that “... antigens (were detected) in the urine of all 10... patients with aspergillosis and in 23 neutropenic patients with no evidence of aspergillosis.” Technically, that is correct, but the information is misleading. Urine samples from 23 neutropenic patients without fungal infection were pooled and tested with both a polyclonal anti-cell wall antiserum and the monoclonal antigalactomannan antibody. The polyclonal antiserum did react with the pooled urine specimen, but the bands that appeared were diffuse, lightly stained, and of different molecular masses from the distinct bands of 11, 18, and 29 kDa detected in IA patients. No bands appeared when the pooled urine from neutropenic patients was tested with the monoclonal antibody.

We appreciate the opportunity to clarify these issues.

REFERENCES

Author’s Reply

We would like to thank Drs. Haynes, Rogers, Latge, and Barnes for drawing our attention to some errors in Table 3 and in the text with regard to their work on antigen detection. As they indicated, it was difficult to ascertain data on a per patient basis in one of their papers, and in an effort to be as concise as possible, we might have misinterpreted some of the information. In our review we selectively incorporated results from different studies for comparison of similar procedures and methods. Our objective was not to discuss any particular paper in detail. In addition, as we were examining Table 3 with the goal of revising it, we noted a mistake with regard to reference 56 in that a capture BALISA was used instead of an ELISA and 16 of 18 serum samples studied were positive. In an effort to correct any misconceptions these errors may have caused, we have revised Table 3 (see below). Our apologies to all concerned.

TABLE 3. Summary of recently published reports in antigen detection in IA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Antigen detected</th>
<th>Specimen</th>
<th>No. of patients</th>
<th>No. positive</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>3</td>
<td>3</td>
<td>205</td>
</tr>
<tr>
<td>RIA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>9</td>
<td>7</td>
<td>244</td>
</tr>
<tr>
<td>Inhibition ELISA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>19</td>
<td>11</td>
<td>186</td>
</tr>
<tr>
<td>RIA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>9</td>
<td>4</td>
<td>245</td>
</tr>
<tr>
<td>ELISA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>9</td>
<td>8</td>
<td>250</td>
</tr>
<tr>
<td>Capture BALISA</td>
<td>Galactomannan</td>
<td>Serum</td>
<td>18</td>
<td>16</td>
<td>56</td>
</tr>
<tr>
<td>RIA</td>
<td>Carbohydrate</td>
<td>Serum</td>
<td>22</td>
<td>16</td>
<td>217</td>
</tr>
<tr>
<td>Inhibition BALISA</td>
<td>Glycoprotein</td>
<td>Serum</td>
<td>5</td>
<td>4</td>
<td>91</td>
</tr>
<tr>
<td>Inhibition ELISA</td>
<td>Unknowna</td>
<td>Serum</td>
<td>19</td>
<td>17</td>
<td>183</td>
</tr>
</tbody>
</table>

* Polyclonal antibody derived from a human patient was employed.

Western blotting

<table>
<thead>
<tr>
<th>Cell wall derivedb</th>
<th>Urine</th>
<th>10</th>
<th>10</th>
<th>78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactomannan</td>
<td>Urine</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* Polyclonal antiserum raised against Aspergillus cell walls was used, and it contained antibodies capable of reacting with compounds in a pool of urine specimens taken from 23 neutropenic patients without fungal infection. The antigens detected, however, had different molecular masses than the major antigens of 11, 18, and 29 kDa detected in patients with IA.

V. Kurup
A. Kumar
Medical College of Wisconsin
Milwaukee, Wisconsin 53205-1000