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Diagnostics for Aspergillosis : Emerging Trends

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Fungal diseases like aspergillosis have emerged as a public health threat in recent years. This is due to an increase in the number of patients suffering from Acquired immunodeficiency syndrome, various malignancies, multi drug resistant tuberculosis and cases of bone marrow, lung and liver transplant, which are susceptible to opportunistic fungal infections leading to increased morbidity and mortality. Candidiasis and aspergillosis are the two major opportunistic fungal diseases often encountered. *Aspergillus fumigatus* is the main causative agent for different forms of *Aspergillus* induced diseases major being allergic bronchopulmonary aspergillosis and invasive aspergillosis. Allergic and infectious aspergillosis are investigated in detail with respect to pathogen related factors such as antigenic structure and the immunological functions, host factors such as immune mechanisms and host-pathogen interactions. These studies provide a basis for diagnosis and the therapy. Recombinant allergens of *A. fumigatus* have now opened up possibilities for preparation of international reference standards for immunodiagnosis of aspergillosis. Epitopic analysis of immunodominant allergens and antigens resulted in preparation of synthetic epitopes based on diagnostic antigens. An insight into the host pathogen interactions and the role of lung surfactant proteins A and D as innate immune molecules in antifungal defense offers scope for several applications. Full genome sequencing of *A. fumigatus* has been initiated recently. Information on *A. fumigatus* genome is anticipated to result in formulation of new therapeutic strategies and effective antifungal drugs by rational drug designing. Advances in *Aspergillus* research also provide basis for development of molecular probes for predictive diagnosis of allergic and infectious aspergillosis.

Key Words: Fungi, Diagnosis, *Aspergillus fumigatus*, Allergic bronchopulmonary aspergillosis, Invasive aspergillosis, Therapy, Allergens/antigens, Epitopes, Recombinant, Host-factors

Introduction

An upsurge in the incidence of life threatening fungal diseases (mycoses) has renewed the interest of clinical and biomedical researchers in mycotic diseases which lead to increased ability to diagnose and treat such mycotic infections. World Health Organisation (WHO) reported that acute respiratory infections including respiratory mycoses, are among the leading killer diseases of the year 1999 (approximately 5.5 million deaths). On a world-wide basis, the estimated annual cost of combating fungal diseases is US$ 30 billion. Three major forms of fungal diseases are often encountered in humans: 1, infections with fungal species; 2, allergies due to fungi; and 3, mycotoxicosis due to ingestion of toxic fungal metabolites in that order of importance. These forms of the fungal diseases have been recognised for centuries. However, they have been posing greater problems during the last few decades. Infections induced by various pathogenic fungi such as *Candida*, *Aspergillus*, *Cryptococcus*, and *Histoplasma* are reported to be leading causes of morbidity and mortality (Jadhav et al. 1990, Saral 1991, Andrews et al. 1996, Gupta et al. 1996, Sternberg 1994, Toscano & Jarvis 1999, Kaur et al. 2000). High incidence of the devastating human

Abbreviations: *ABPA, Allergic bronchopulmonary aspergillosis; A. fumigatus, Aspergillus fumigatus; kDa,* Kilo Dalton; *rAsp f (n),* Recombinant *A. fumigatus allergen* (number); *ELISA,* Enzyme-linked immunosorbent assay; *PCR,* Polymerase chain reaction

immuno-deficiency cases and the emergence of multi drug resistant tuberculosis, has necessitated intensification of biomedical research efforts for understanding the immune mechanisms operative in the opportunistic fungal and other infections, for development of sensitive and specific diagnostic methods and effective therapeutics. Recent advances in our understanding of the biology and pathogenesis of few infectious diseases and their relationship to the immune system have provided basis to the developments of immunodiagnostics and DNA based diagnostics and possibly predictive diagnostics for a number of infectious diseases soon. Among the various medically important fungi, filamentous pathogenic fungi such as *Aspergillus* are studied in some detail with respect to their biology and immunology. This article is focussed on aspergillosis as a case study, enumerating few emerging trends applicable to the diagnosis of the allergic and invasive forms.

Aspergillosis

Aspergillosis is a group of fungal diseases which include allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, chronic necrotising aspergillosis, hypersensitivity pneumonitis and invasive aspergillosis. *Aspergillus fumigatus*, along with other less frequently reported species of *Aspergillus* such as *A. flavus* and *A. niger*, is the major causative fungus (Kurup & Kumar 1991). Majority of the fungal allergies are due to *Aspergillus* species, with spores (conidia) and mycelia of the fungus as the infectious forms. An *Aspergillus fumigatus* (*A. fumigatus*) conidiophore bearing conidia (spores) is shown in figure 1. *A. fumigatus* is an ubiquitous microbe and universal in distribution. *Aspergillus* species grow at temperatures of 15 to 53°C in contrast to many fungi that do not grow above 35°C. Two major *A. fumigatus* induced diseases are an allergic form, Allergic bronchopulmonary aspergillosis (ABPA) and an invasive form, Invasive pulmonary aspergillosis.

Figure 1 An *Aspergillus fumigatus* conidiophore bearing conidia (spous).

Allergic Bronchopulmonary Aspergillosis (ABPA)

The allergic form of the *Aspergillus* induced disease is named as Allergic bronchopulmonary aspergillosis (ABPA), which is an immunological disease and depicts the immune mechanisms similar to that of asthma. ABPA is often encountered in patients of bronchial asthma (15%, 16 out of 107), cystic fibrosis (7.8%, 191 out of 12,447), sinusitis (13%, 3 out of 23), rhinitis (5%, 3 out of 62) (Chetty et al. 1985, Grammer et al. 1986, Panchal et al. 1997, Mastella et al. 2000). A study with 35 patients of ABPA showed that 12 (33%) of them were misdiagnosed as patients of pulmonary tuberculosis and were treated with various antitubercular drugs (Behera et al. 1994). A conglomeration of intertwined *Aspergillus* hyphae matted together with fibrin, mucus and cellular debris, within a pulmonary cavity or ectatic bronchus, is termed as Aspergilloma. Patients of aspergilloma usually have an underlying pulmonary disease such as fibrocystic sarcoidosis, cavitary tuberculosis or histoplasmosis, bullous emphysema, or fibrotic lung disease (Kauffman 1996).

Diagnosis: Aspergillosis of the lung does not show characteristic clinico-radiological features to permit the diagnosis and should be considered in the differential diagnosis of tuberculosis, pneumonia, bronchiectasis, lung abscess and bronchial asthma (Kothari et al. 2000). Early diagnosis of ABPA is important for the following reasons :

- Firstly, to prevent irreversible damage of the bronchi and the lungs. Bronchiectasis and

bronchiolitis are known sequelae of the disease and if undiagnosed in early stages, may lead to pulmonary fibrosis and respiratory failure. Progression of ABPA has been classified clinically into five stages viz. Acute, Remission, Exacerbation, Corticosteroid dependent asthma and Pulmonary fibrosis (Rosenberg et al. 1978).

- ABPA may be the cause of recurrent pneumonias in children and may increase the severity of asthma in some patients.
- Association of ABPA in cystic fibrosis may worsen the course and prognosis.
- ABPA has also been found in multiple members of a family and a need has been felt for screening all the family members of a newly diagnosed case.
- Occurrence of ABPA is also known to be associated with pulmonary tuberculosis. It has been observed that both diseases show similar clinical symptoms that cause a diagnostic dilemma.

Elevated levels of *A. fumigatus* specific IgE and IgG antibodies detectable by various serodiagnostic techniques is one of the important diagnostic criteria for ABPA (Rosenberg et al. 1978). However, unfamiliarity with the diagnostic tests and nonavailability of certain serologic tests at clinical laboratories compounds the difficulty for the clinician. Reference standards of *Aspergillus* antigens for immunodiagnosis are not available till today either with the world health organisation (WHO) or any international agencies. This is mainly due to the complex nature of antigens of *Aspergillus* species, which require multiple purification processes.

The diagnosis of Aspergilloma is usually made clinically without a lung biopsy, and the chest radiographic features along with positive serum precpitins are of utmost importance.

Therapy: Treatment of allergic aspergillosis comprises suppression of the immunological response to the fungus by corticosteroids (Stevens et al. 2000). However, steroid treatment is an important risk factor for invasive pulmonary aspergillosis as steroids impair the conidicidal activity of human macrophages (Palmer et al. 1991, Lake et al. 1983, Taramelli et al. 1996). Exposure to a pharmacological dose of hydrocortisone increases the growth rate of *A. fumigatus* and *A. flavus* by 30-40% (Ng et al. 1994). Combined drug-therapy such as with antifungal drugs and steroids has now been initiated as a new therapeutic strategy for ABPA patients (Stevens et al. 2000). Hyposensitisation therapy is contraindicated in *Aspergillus* induced allergic disorders as the specific IgG antibodies were implicated in pathogenesis rather than in protection. Utility of liposomal and polymer matrix formulations of *A. fumigatus* allergens / antigens for hyposensitisation therapy is being explored (Madan et al. 1997e). In aspergilloma, the therapeutic options range from medical management without direct intervention to surgical resection with or without concomitant antifungal therapy (Stevens et al. 2000). Systemic therapy with Amphotericin B or itraconazole or local treatment via the bronchial tree are the recommended therapies for aspergilloma patients(Yamada et al. 1993).

Invasive Aspergillosis

In the light of increasing i) incidence of human immunodeficiency virus (HIV), ii) use of cytotoxic drugs in malignancies and use of immuno-suppressive drugs, the frequency of invasive aspergillosis is reported to have risen approximately 14-fold over the period 1980 to 1992 (Saral 1991). The cumulative incidence of invasive mycotic infections was 178.3 per million per year with *Candida* (72.8 per million per year), *Cryptococcus* (65.5), *Coccidioides* (15.3), *Aspergillus* (12.4), and *Histoplasma* (7.1) being the most common fungal pathogen in a population-based active laboratory surveillance conducted during 1992 and 1993 in three California counties: Alameda, Contra Costa, and San Francisco (population, 2.94 million) (Rees et al. 1998). A review of 1187 autopsies performed during the 4-year period 1993-1996 showed invasive aspergillosis as the cause of death in 48 (4%) cases (Vogeser et al. 1999). The mortality from invasive aspergillosis is around 85% under untreated conditions which could be reduced to 50% on treatment (Sternberg 1994). The incidence of invasive aspergillosis has been reported in patients using chemotherapeutic and immunosuppressive drugs such as lung transplant cases (40%, 53 out of 130), bone marrow transplant cases (6%, 8 out of 148 in a study by Hovi

et al. 2000 and 11%, 15 out of 142 in a study by Jantunen et al. 1997), and in patients of leukemia (36%, 31 out of 87) etc. (Jantunen et al. 1997, Hovi et al. 2000, Mehrad et al. 2001, Oren et al. 2001).

Diagnosis: Serodiagnosis of inavsive aspergillosis is generally based on detection of circulating antigens by immunodiagnostic or gene detection methods. Screening the patients susceptible to invasive aspergillosis in the early stages by sensitive diagnostic methods and monitoring them with effective antifungal drugs is needed to reduce the risk of morbidity and mortality associated with the disease.

Therapy: Invasive aspergillosis patients are treated with antifungal drugs such as Amphotericin B, Nystatin, Azole group, and a new group of drugs, Pneumocandins (Sternberg 1994, Kurtz et al. 1995, Stevens et al. 2000). Some of these drugs exhibit hepatotoxicity and nephrotoxicity in humans. The liposomal formulations of thes e drugs have shown better efficacy with minimal toxicity and also provided prophylactic advantage over parental formulations (Lopez-Berestein 1989, Allen et al. 1994, Wallace et al. 1997, Saxena et al. 1999). Some more new drugs, such as voriconazole, posaconazole, caspofungin and FK-463, are under various stages of evaluation (Stevens et al. 2000). Restoration of host immune defences by tapering of immunosuppressive therapy in transplant patients or correction of granulocytopenia in haematological disease by adjuvant modalities, such as granulocyte transfusions, administration of colony-stimulating factors (G-CSF, M-CSF, GM-CSF) or cytokines (IFN-g), are the cornerstones of modern treatment of aspergillosis in immunocompromised patients (Pun et al. 1997, Nemunaitis 1998, Bernhisel-Broadbent et al. 1991, Vose et al. 1991, Bhatia et al. 1994, Rowe 1998).

Pathogen Related Factors

Colonization

Inhaled *Aspergillus* spores are known to colonise in the bronchial mucus in the pulmonary tract of susceptible individuals including some patients with asthma and cystic fibrosis. The spores are capable of germinating at body temperatures. The large hyphae establish themselves in airways and release antigens. *A. fumigatus* colonisation causes injury to the bronchial epithelium by IgE antibody mediated mast cell degranulation and release of mediators which increase epithelial permeability and chemotaxis of eosinophils This causes continuous release of histamine from basophils and mast cells. Molecular mechanisms of colonisation of the fungal pathogen in the host are not well understood, as the molecular nature of virulent factors of the fungus and the host factors involved in immunopathogenesis of aspergillosis are not studied in detail till recently. The mechanism of pathogenesis is based on interplay of a large number of proteins of human host and the *Aspergillus* allergens and antigens. Recent initiative on *A. fumigatus* genome sequencing will facilitate identification of several functionally important proteins, which also include colonising factors of the fungus.

Features of Allergenic Proteins

One of the important features of any allergen is its ability to activate T helper cells that induce Th2 responses in the allergic asthmatic patients. The induction of allergic response in genetically predisposed individuals is based on the interactions of a number of functional and effector cells in recognising the allergen stimulated synthesis of IgE antibodies. Existence of any intrinsic property in the proteins that possess the characteristic feature of allergenicity is not yet known. However, the allergenic proteins that trigger type I and type III hypersensitivity reactions may possess multiple epitopes that cross-link IgE antibodies on mast cells leading to the release of mediators. It appears allergens may have somewhat similar molecular conformations, which is yet to be proved.

Allergens/Antigens

Aspergillus fumigatus secretes a plethora of somatic and metabolic proteins that are biologically and immunologically active complex macromolecules (table 1). These proteins can be categorised into metabolic products (secreted into the medium), and surface expressed proteins which can be subclassified as mycelial and somatic (released either by autolysis or rupture of the organism). Some of them have relevance in immunodiagnosis of aspergillosis. Some of them are potential virulent

Table 1 *Pathogen factors secreted by Aspergillus fumigatus and their diagnostic relevance*

Source of the pathogenic factor	Characterisation	Clinical importance
Piechurra et al. 1987 Pathogenic strain	CS2 composed of 81, 53, and 41 kDa on SDS-PAGE	Reacted with specific IgG of 75% of aspergilloma patients
Banerjee et al. 1990 Soil strain no. 2605, & strain isolated from sputum of ABPA patient Three week culture filtrate	70, 55, 34, 28,18 and 14 kDa	Reacted with specific IgG and IgE antibodies in ABPA patients
Latge et al. 1991 Strain no. NCPF 2140, CBVS 143.89, Mycelium, Water soluble material from ethanol ppt Culture filtrate	18, 28, 35, 38, 45, 94, 110, 125 kDa	35 kDa reacted with specific IgG of 100% of aspergilloma patients 18 kDa reacted with specific IgG of 80% of the 75 aspergilloma patients
Fratamico and Buckley 1991 Strain no. F 92 (isolated from sputum of an aspergilloma), Mycelia	58 kDa	100% of the 38 invasive aspergillosis patients showed IgG to 58 kDa, 90% showed only 58 kDa band, MoAb recognised both 58 and 45 kDa
Burnie and Matthews 1991 Strain no. NCPF 2109, Mycelium	88 kDa, heat shock protein, reacts with MAb against hsp of *C. albicans*	Reacted to specific IgG of 50 patients with proven invasive aspergillosis, 35 ABPA patients, 28 patients with suspected invasive aspergillosis, 10 patients with aspergilloma
Arruda et al. 1992a Strain no. 5167, Mycelium	18 kDa, Asp f 1, allergen, homology to cytotoxins	85% of the 14 ABPA patients showed IgE antibodies to 18 kDa
Arruda et al. 1992b Strain no. 5167, Mycelium	18 kDa, Asp f 1	11of the 13 aspergillosis patients showed IgE antibodies to 18 kDa
Arruda et al. 1992c Strain from Greer Labs Mycelia	16, 18, and 45 kDa	12/12 ABPA patients showed IgG and IgE antibodies to all 3 Ag, murine MoAb raised against Asp f 1 recognised both Asp f 1 and 16 kDa
Kumar et al. 1993 Strain no. AF-102, ATCC 42202, Mycelium	65 kDa, heat shock protein	Reacted with IgE and IgG antibodies of ABPA patients (pooled sera)
Kobayashi et al. 1993 Strain no. CVS 144.89 Water soluble ethanol ppt	88 kDa, glycoprotein	88 kDa antigen showed binding with all 72 sera of culture filtrate aspergilloma patients
Teshima et al. 1993 Strain NHL-5759	55 kDa, glycoprotein	Bound to specific IgE antibodies in sera of ABPA patients by western blot
Markaryan et al. 1994 Strain no. CBS-144.89 Culture filtrate (48 h)	40 kDa, metalloprotease	Specific IgG antibodies against 40 kDa were observed in all 10 sera of aspergilloma patients
Tomee et al. 1994 Strain isolated from a patient of aspergilloma Culture filtrate	32 kDa glycoprotein	Reacted with sera of patients with aspergilloma (n=10) and ABPA (n=10) showed strong binding with glycoprotein antigens
Reichard et al. 1994 Strain no. DF 41 Culture filtrate	38 kDa, extracellular aspartic proteinase	Sera of 1 aspergilloma and 1 *Aspergillus* pneumonia reacted on dot blot with proteinase
Latge et al. 1994 Strain no. CBS-143.89 Mycelium	galactomannan	Sera of only 26% of aspergilloma patients showed positive response
Lopez Medrano et al. 1995 Strain ATCC 9197 fraction	p90, p60, p40, p37, N-glycosylated	Sera of 76% of the Cytosolic 80 aspergilloma patients demon strated specific binding with glycoprotein antigens
Banerjee et al. 1995 Strain ITCC 2605	45 kDa glycoprotein with elastase and protease activity	Sera of 5 ABPA and 2 Aspergilloma patients showed specific IgG and IgE reactivity by ELISA and Western blot
Haynes et al. 1996 Strain NCPF 2109	93 kDa	Sera of 4 ABPA patients recognise *93 kDa*
Beauvais et al. 1997 Culture filtrate Strain CBS 144.89	97 kDa (Dipeptidyl peptidase)	Reacted with IgG antibodies in pooled sera of aspergilloma patients
Banerjee et al. 1997 Culture filtrate	37 kDa	Reacted with IgE antibodies from 24 ABPA patients
Chow et al. 2000 Strain (isolated from Tainan area from Taiwan)	Asp f 13	Reacted with IgE antibodies in pooled sera of subjects allergic to *Aspergillus fumigatus*

factors (Sarma et al. 1998). Various biological activities possessed by *Aspergillus* proteins include elastase, protease, ribonuclease, cytotoxic and immunosuppressant activities (Markaryan et al. 1994, Reichard et al. 1994, Banerjee et al. 1995, Madan et al. 1997a, b, Purkayastha et al. 2000). These activities contribute to the disease process by causing extensive tissue damage. However, their exact role 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 (Tran-van Ky et al. 1969). A high molecular weight antigen fraction of 250 kilo Dalton (kDa) with catalase activities (a tetramer of catalase) was observed to be diagnostically relevant (binding to specific IgG antibodies in all 3 patients of aspergillosis and none of 3 controls) (SchonHeyder et al. 1984a). Another immunodominant antigen with molecular weight 90 kDa from *A. fumigatus* belongs to a catalase subclass that reacted with more than 90% of serum samples from patients with aspergilloma (Lopez-Medrano et al. 1995). Two partially purified proteases of molecular weights 25 and 50 kDa reacted significantly with sera from patients with aspergillosis (96% of 26 patients with definite pulmonary aspergillosis, 65% of 23 patients with suspected pulmonary aspergillosis, 13% of 8 patients with precipitating antibodies to *A. fumigatus* of undetermined clinical significance) (SchonHeyder et al. 1984b). Asp f 1, an 18 kDa allergen/ antigen showed cytotoxicity towards four eukaryotic cell lines with an IC_{50} of 80 ng/ml and the cytotoxicity was dependent on the ribonuclease activity of the protein (figure 2) (Madan et al. 1997a, b).

Immunological activities of allergenic/ antigenic proteins secreted by *Aspergillus*, are mediated by type I (specific IgE antibodies mediated) and type III (specific IgG antibodies mediated) hypersensitivity reactions in animals and human beings which has been used as one of the diagnostic criteria for allergic aspergillosis (table 1). The wide clinical spectrum of the diseases caused by *Aspergillus fumigatus* and other species can be attributed to the complex, multifunctional allergens and antigens with varied immunological and biological properties (Purkayastha et al. 2000).

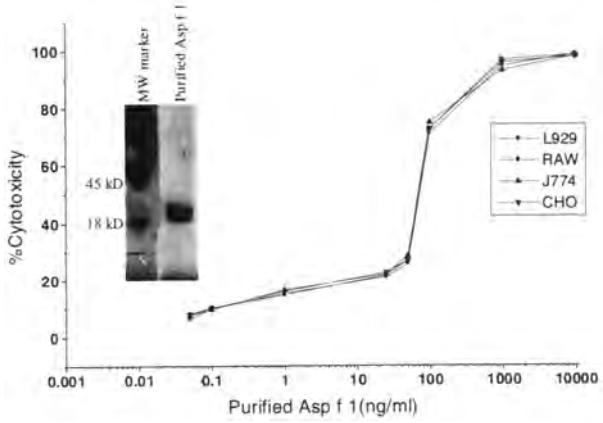

Figure 2 Cytotoxicity of purified Aspf1, allergen/ antigen of *A. fumigatus* towards four eukaryotic cell lines. The purity of Aspf1 was analysed by the SDS-PAGE and High Performance Liquid Chromatography (Profiles inserted in the figure).

Host Factors

Aspergilli are ubiquitous in nature and universal in distribution. Hence, all individuals are exposed to the atmospheric fungal spores, but the normal defense mechanisms act as a barrier to infection from inhaled *Aspergillus* conidia. Understanding of the nature of the pathogen and the host factors is important to develop specific strategies for prevention, diagnosis and therapy of various infectious diseases.

Innate Immune Response

The majority of the conidia of *A. fumigatus* are probably excluded from the lungs through the ciliary action of the mucous epithelium. The conidia, which overcome this barrier by secreting inhibitory factors such as toxins and proteases, encounter alveolar macrophages which function as the first line of defense (Schaffner et al. 1982). Germinated conidia or the hyphal form of fungus is attacked by the granulocytes of the host (Schaffner et al. 1982). The innate immune defense by macrophages and neutrophils is supported by several secretory molecules such as cytokines (TNF-α, IFN-γ, GM-CSF, G-CSF), chemokines, complement molecules (C3), collectins (lung surfactant protein A (SP-A), lung surfactant protein D (SP-D), Mannan binding protein (MBP)) etc. Recently, it was shown that SP-A and SP-D enhanced agglutination, phagocytosis and killing of conidia of *A. fumigatus* by alveolar

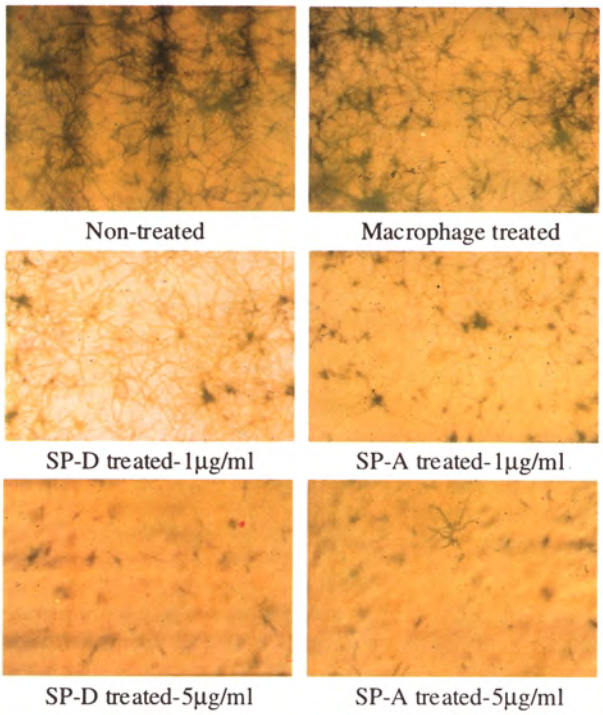

Figure 3 Killing of *A. fumigatus* conidia (as observed by light microscopy) by alveolar macrophages isolated from the bronchoalveolar lavage of a healthy control in presence and absence of various concentrations of purified human lung surfactant proteins A (SP-A) and D (SP-D). (1 or 5 mg /ml of SP-A/ SP-D in sterile Phosphate buffered saline with 5 mM $CaCl_2$ incubated for 60 min with alveolar macrophages and conidia)

macrophages and neutrophils (figure 3)(Madan et al. 1997c). Administration of SP-A and D SP-D to the murine models of ABPA and invasive aspergillosis was effective in alleviating the disease symptoms (such as reduction in levels of specific IgE, IgG antibodies, pulmonary and peripheral eosinophilia, and colony forming units) and mortality (Madan et al. 2001a, Madan et al. 2001b). Macrophage inflammatory protein- 1 a (MIP-1 a) is a chemokine with potent chemotactic activity. This chemokine was observed to be a critical mediator of host defense against *A. fumigatus* in neutropenic condition. Antibody-mediated depletion of MIP-1 a resulted in increase in mortality in neutropenic mice suggesting that MIP-1 a may be an important target in devising future therapeutic strategies against invasive aspergillosis (Mehrad et al. 2000). Platelets have also been reported to play a role in host defense against aspergillosis (Christin et al. 1998). They attach to the cell walls of the invasive hyphal form of *A. fumigatus* and enhance several anti-*Aspergillus* functions.

Humoral Immune Response

Aspergillus antigens induce type I, IgE antibody mediated and type III, IgG antibody mediated hypersensitivity reactions in humans. The atopic population with increased levels of total IgE antibody levels are susceptible to *Aspergillus* induced disorders. Allergens crosslink the IgE antibodies present on the receptors of mast cells resulting in degranulation and release of histamine, leukotrienes mediating bronchial hyper-responsiveness. The eosinophilic infiltration in response to *A. fumigatus* allergens and IgE antibody interaction results in the release of additional proinflammatory mediators. The mast cell and eosinophil degranulation induce further chemotaxis of inflammatory cells including $CD4^+$ lymphocytes and eosinophils (Kurup et al. 1994). These reactions lead to generation of significant amounts of IgG and IgE antibodies in patients suffering from ABPA and aspergilloma. Polyclonal activation of allergens/ antigens of *A. fumigatus* has been reported by various groups resulting in increase of IgE, IgG, IgM, IgA and IgD antibodies (Brummund et al. 1987, Kaufmann et al. 1989, Leung et al. 1988). IgG antibodies participate in the formation of immune complexes resulting in extensive tissue damage (Bhatnagar et al. 1990). A glycoprotein allergen/ antigen with molecular weight 45 kDa has been found to be predominant *A. fumigatus* allergen/ antigen in formation of immune complexes isolated from ABPA patients (Madan et al. 1997f). These immune complexes have been implicated in the pathogenesis of ABPA. The role of antigen presenting cells such as dendritic cells and mechanisms of eosinophil apoptosis contributing to the pathogenesis of allergic disease need to be investigated.

Acquired Cellular Immunity

Cell-mediated immune responses and Th1/Th2 cytokine (Interleukins (IL)) dysregulation has been observed in human mycoses (Lucey et al. 1996, Murphy et al. 1998). The studies on acquired immunity to *Aspergillus* infection are primarily on experimental murine models of aspergillosis: ABPA model induced by inhalation of *A. fumigatus* antigens and invasive aspergillosis model induced by spores in immunosuppressed animals.

In mice, as in humans, ABPA is associated with pulmonary eosinophilia, Th2 cytokine profile manifested by IL-4 (responsible for class switch of B cells from IgG to IgE), IL-5 (responsible for eosinophil maturation), and IL-10 to *A. fumigatus* antigens (Knutsen et al. 1994, Kurup et al. 1994). An increase in the production of the proinflammatory cytokines IL-1α and TNF-α, associated with an upregulation of intercellular cell adhesion molecule 1 (ICAM-1) was reported in the early stages of ABPA patients (Chu et al. 1995). *Aspergillus* specific CD4+ T cell clones isolated from ABPA patients have a Th2 phenotype (Knutsen et al. 1994, Chauhan et al. 1996). Studies of cell-mediated immunity in invasive aspergillosis have been few. Challenging the immunocompetent as well as immunosuppressed (cyclophosphamide-treated) mice intravenously with spores, showed that progression of the invasive disease is also associated with a Th2 response, correlating with an increase in IL-4 and IL-10 levels and lower levels of IFN-γ (Cenci et al. 1997).

Diagnostic Antigens of *A. fumigatus*

The criteria of diagnosis for ABPA include the demonstration of presence of specific IgG and IgE antibodies to *Aspergillus* antigens. Currently the antigens used to detect circulating antibodies to *A. fumigatus* are based on crude extracts or partially purified extracts containing allergens/ antigens obtained from mycelial extracts or culture filtrate. A typical sodium dodecyl polyacrylamide gel elctrophoresis (SDS-PAGE) profile of three week culture filtrate allergens/ antigens of *A. fumigatus* (both clinical and soil isolates) with molecular weights ranging from 12 to 80 kDa, is shown in figure 4. Immunoblot analysis with pooled sera of ABPA patients showed both IgG and IgE antibodies reacting predominantly with 66, 55, 45, 34 and 18kDa antigens (figure 5). An acidic glycoprotein with 12.5% neutral hexose and with an isoelectric point (pI) of 5.2-5.6 and 4 polypeptide chains of molecular weight 45 kDa, was isolated from cell sap (Calvanico et al. 1981). This protein detected specific antibodies in 75% of sera of patients of ABPA and did not react with normal sera. Using a combination of protein purification techniques Ag3, Ag5, Ag7 and Ag 13 antigens were purified which reacted with sera of patients of ABPA and aspergilloma (Ag 3 reacted

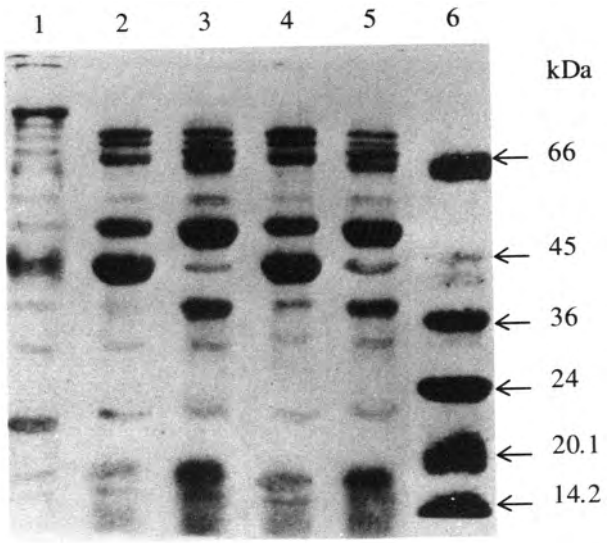

Figure 4 SDS-PAGE analysis of three week culture filtrate allergens/ antigens of various soil and clinical strains of *A. fumigatus*.

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

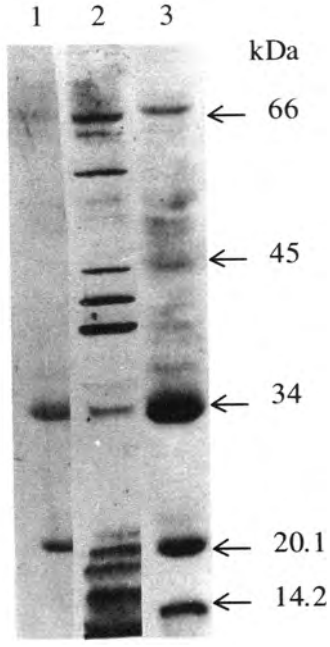

Figure 5 Western blots of three week culture filtrate allergens/antigens of *A. fumigatus* with sera of ABPA patients probed for specific IgG and IgE binding.

Lane 1: Specific IgE binding activity; *Lane 2*: Specific IgG binding activity; *Lane 3*: Molecular weight markers

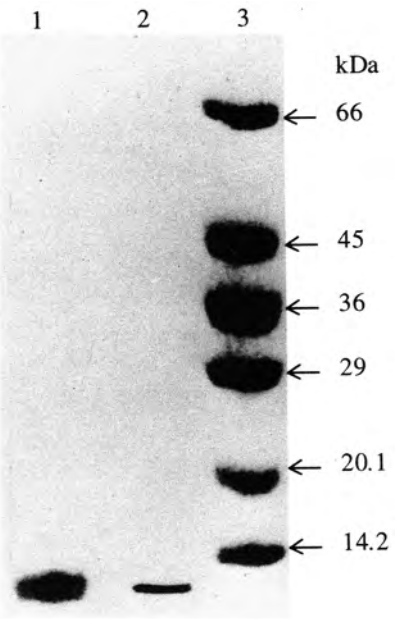

Figure 6 SDS-PAGE profile of purified Aspf1 allergen/ antigen of *A. fumigatus*.

Lane 1& 2: Different concentrations of purified Aspf1 (18 kDa) protein loaded; *Lane 3*: Molecular weight markers

with pool of ABPA sera, Ag 13 reacted with 25/25 ABPA sera, 2/5 aspergilloma sera, did not react with 10/10 control sera, 10/10 fungal atopics, 5/12 farmer's lung sera had low but positive values of Ag 13 specific IgG, Ag 13 has a sensitivity of 100% and specificity of 80% for diagnosis of aspergillosis) (Longbottom 1986, Longbottom & Austwick 1986, Taylor & Longbottom 1988, Longbottom et al. 1989). Antigens with molecular weights 20, 40 and 80 kDa showed significant antibody reactivity with sera of patients of allergic aspergillosis (Kurup et al. 1986). An IgE antibody mediated response to Asp f 1, a major allergen/ antigen/ cytotoxin, has been reported in 85% (11 out of 13) of the patients with *A. fumigatus* induced allergic disorders (Arruda et al. 1992a). Moreover, the Asp f 1 has also been reported to be one of the major antigens secreted *in vivo*, and excreted in the urine of patients of invasive aspergillosis (Latge et al. 1991). Figure 6 shows an SDS-PAGE profile of 18 kDa antigen purified by a combination of conA sepharose, phenyl sepharose CL-4B chromatography and reverse phase- HPLC (Bir et al. 1999). Figure 7 shows SDS-PAGE profile of immunodominant antigens such as 66 kDa, 45 kDa

Table 2 *ELISA absorbance values for specific IgE and IgG antibodies against 45 kDa antigen in sera of patients with aspergillosis*

Study group	Patient No.	ELISA Abs for IgE	ELISA Abs for IgG
ABPA	1	0.685	0.831
	2	0.165	0.750
	3	0.435	0.986
	4	0.109	0.583
	5	0.434	0.745
Aspergilloma	1	0.050	0.460
	2	0.110	0.480
Normal	1	0.045	0.091
	2	0.065	0.175
	3	0.034	0.161

and 34 kDa (figure 7a) and 56 kDa and 27 kDa (figure 7b), in a purified form. Immunoreactivity of the purified 45 kDa glycoprotein antigen with the sera of ABPA and aspergilloma patients was observed by immunodiffusion, Western blot and ELISA (table 2) (Banerjee et al. 1995). Teshima et al. reported another diagnostically relevant glycoprotein allergen/antigen, gp 55, from *A. fumigatus* (Teshima et al. 1993). Diagnostic relevance of Asp f 2, a glycoprotein of molecular weight 37 kDa with N-terminal homology to gp 55, has been recently demonstrated (Banerjee et al. 1998). Polysaccharide fractions containing galactomannans 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 and microbes which create a major limitation in interpreting test results (Azuma et al. 1969, DuPont et al. 1987). The carbohydrate antigens are reported to be useful in antigen-based immunodiagnosis of invasive aspergillosis.

The array of *A. fumigatus* antigens identified for diagnosis depends on various factors like culture conditions (culture filtrate or mycelial intracellular extract), the strain used, the method of extraction etc. Moreover, the cross-reactivities among different species of *Aspergillus* as well as between *Aspergilli* and humans, other fungi, bacteria etc. also affect the sensitivity of various serodiagnostic methods. Hence, characterisation of diagnostic

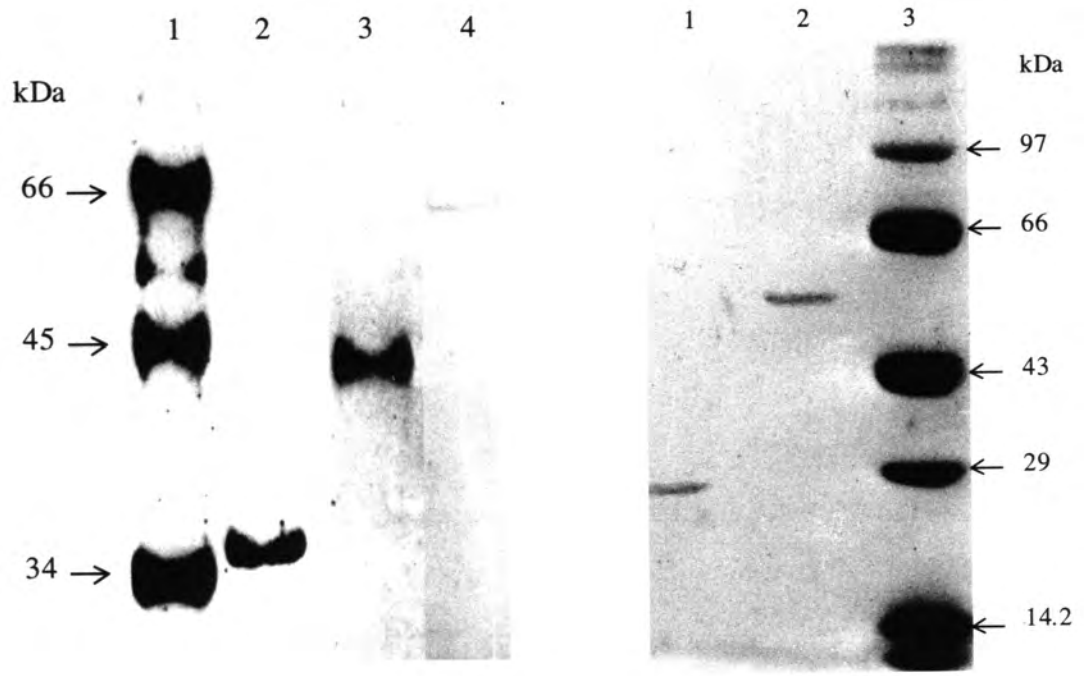

Figure 7 SDS-PAGE profile of a. purified 66 kDa (*Lane 4*), 45 kDa (*Lane 3*) and 34 kDa (*Lane 2*) allergens/ antigens of *A. fumigatus*. Molecular weight markers (*Lane 1*). b. purified 56 kDa (*Lane 2*) and 27 kDa (*Lane 1*) allergens/ antigens of *A. fumigatus*. Molecular weight markers (*Lane 3*).

antigens is necessary for giving a definition to antigens for universal diagnostic applications.

Recombinant Allergens/Antigens

The allergenic/ antigenic proteins relevant to pathogenesis and diagnosis are highly complex macromolecules and are difficult to purify by single step purification methods. Two major difficulties encountered by immunologists in using fungal antigens for immunodiagnosis are non-availability of standardised antigens and insufficient knowledge of the molecular nature of fungal allergens. Total allergen extracts prepared from natural sources may sometimes lack certain components due to either poor extraction conditions or enzymatic degradation during isolation and purification of antigen preparations. Hence, the currently used *in vitro* and *in vivo* diagnostic methods could be improved by using recombinant allergens. Use of recombinant DNA and synthetic peptide approach has also resulted in dissection of various functional domains of some of these macromolecules and understanding of structure-function relationships. Several of these proteins have been individually cloned and expressed in prokaryotic and eukaryotic vectors (table 3a). One of the major allergens and a proposed virulent factor, Asp f 1, was cloned and studied in greater detail with respect to its structure and function (Moser et al. 1992, Madan et al. 1997a,b). Some of the recombinant proteins showed specific binding to IgE antibodies from asthmatic and allergic bronchopulmonary aspergillosis (ABPA) patients (table 3b). Asp f 2, a 268 amino acid protein (molecular weight 29 kDa), exhibited N-terminal sequence homology to a 37 kDa *A. fumigatus* culture filtrate protein and gp 55, a 55 kDa glycoprotein allergen/ antigen secreted by *A. fumigatus* (Banerjee et al. 1998). The immunochemical characterisation of various *Aspergillus fumigatus* allergens/antigens and the amino acid sequence data suggest that various allergens/ antigens may have some common epitopes. Differential glycosylation and proteolytic processing of the antigens and the strain variability in antigen preparations may contribute to the variations in the performance of antigens in immunodiagnosis. In an enzyme-linked immunosorbent assay (ELISA), serum IgE antibody

Table 3 a *Recombinant A. fumigatus Allergens*

Aspergillus Allergens	Properties	Size (bp)	Mol.Wt. kDa	IgE Reactivity	Gen Bank Accession No.
rAsp f 1	Ribonuclease	678	18	++++	M83781
rAsp f 2	-	1572	37	++++	U56938
rAsp f 3	Peroxisomal protein	630	21	++++	U58050
rAsp f 4	-	1080	40	++++	AJ001732
rAsp f 5	Metalloprotease Manganese	1270	42	++	Z30424
rAsp f 6	Superoxide dismutase (MnSOD)	666	26.7	++++	U53561
rAsp f 7	-	860	12	++	AJ223315
rAsp f 8	-	870	11	++	AJ223333
rAsp f 9	-	920	43	++++	AJ223327
rAsp f 10	Aspertic protease	1000	34	++	X85092
rAsp f 11	Peptidyl-prolyl isomerase	684	20	++	AJ006689
rAsp f 12	Heat shock protein	1649	47	++	U92465
rAsp f 13	Alkaline protease	-	34	++	
rAsp f 15	Serine protease	-	16	++	AJ002026
rAsp f 16	-	-	43	++++	G3643813
rAsp f 17	-	-	-	++	AJ224865
rAsp f 18	Vacuolar serine proteinase	-	34	++	
Asp f X*	-	758	19	++++	AJ006688

reactivity to rAsp f 3 could be detected in 72% of 89 individuals sensitized to *A. fumigatus*, demonstrating that the protein represents a major allergen of the mold (Hemmann et al. 1997). A secreted ribotoxin (rAsp f 1) and a peroxisomal protein (rAsp f 3) were recognized by sera from *A. fumigatus*-sensitized CF-patients with or without ABPA, while an intracellular manganese superoxide dismutase (rAsp f 6) and rAsp f 4, a protein with unknown function, were recognized exclusively by IgE antibodies from sera of CF patients with ABPA. Therefore, rAsp f 4 and rAsp f 6 represent specific markers for ABPA and allow a sensitive, fully specific diagnosis of the disease (Hemmann et al. 1998). Analysis of IgE-antibody mediated immune responses to single recombinant *A. fumigatus* allergens in asthmatic patients facilitates discrimination between ABPA and *A. fumigatus* sensitization with high specificity (100%) and sensitivity (90%) (Crameri et al. 1998). Asp f 6 allergen, a 26.7 kDa protein identified as the enzyme MnSOD (superoxide dismutase) shows a 51.1% identity and 67.2% homology to the corresponding human enzyme. Both recombinant Asp f 6 and human MnSOD reacted with IgG and IgE antibodies in the sera and could cause proliferation of periphera! blood mononuclear cells (PBMC's) isolated from an *A. fumigatus*-allergic patients who were positive for skin-prick test. This implies the existence of an autoimmune reactivity to human MnSOD in patients allergic to *A. fumigatus*. In contrast to fungal extracts, rAsp f 4 and rAsp f 6 allergens are suitable for an automated serologic diagnosis of ABPA, facilitating their introduction in clinical practice (Banerjee et al. 1999). Recombinant *Aspergillus* allergens Asp f 1, f 2, f 3, f 4, and f 6 were studied for their specific binding to IgE antibodies in the sera of ABPA patients, *A. fumigatus* skin prick test

Table 3 b *The Reactivity of Various Recombinant Allergens to IgE Antibodies in Sera of Patients with Allergic Aspergillosis (%)*

Allergens	Subjects	Numbers	ELISA	Western blot	Immediate skin test reactivity	Reference
Asp f 1	ABPA	15	10/15 (67%)	+	8/15(53%)	Moser et al. 1994
	Allergic asthma	10	5/10 (50%)		5/10(50%)	
	Atopics non *A. fumigatus*	6	0		0/6	
	Non-atopic, normals	7	0	-	0/7	
	ABPA	9	+	-	5/9(56%)	Moser et al. 1992
	Allergic asthma	5	2 (40%)		2/5(40%)	
	Atopics non *A. fumigatus*	6	0		0/6	
	Non-atopic, normals	7	-		0/7	
	ABPA	30	24(80%)		24/30(80%)	Crameri et al.1996
	Allergic asthma	5	2(40%)		2/5(40%)	
	Atopics non *A. fumigatus*	9	0		0/9	
	Non-atopic, normals	10	0		0/10	
	ABPA	24	20(83%)			Kurup et al. 2000
	Allergic asthma	16	13(81%)			
	Non-atopic, normals	10	1(10%)			
	ABPA	24	16(67%)			
	Allergic asthma	16	0			
	Non-atopic, normals	10	0			
	ABPA	60	50(83%)			Crameri et al. 1998
	Allergic asthma	40	18(45%)			
	Non-atopic, normals	20	0			
Total		339	161		46	
Asp f 2	ABPA	20	15(75%)			Banerjee et al. 1998
	Allergic asthma	8	1(13%)			
	Non-atopic, normals	5	0			
	ABPA	24	21(88%)	+		
	Allergic asthma	16	0	-		
	Non-atopic, normals	10	0	-		
	ABPA	24	21(88%)	+		
	Allergic asthma	16	1(6%)	-		
	Non-atopic, normals	18	0	-		
Total		141	59			
Asp f 3	ABPA	11	11(100%)		11/11(100%)	Hemmann et al. 1998a
	Allergic asthma	8	5(63%)		5/8(63%)	
	Non-atopic, normals	4	0		0/4	

Allergens	Subjects	Numbers	ELISA	Western blot	Immediate skin test reactivity	Reference
	ABPA	24	20(83%)			Kurup et al. 2000
	Allergic asthma	16	6(38%)			
	Non-atopic, normals	10	1(10%)			
	ABPA	24	19(79%)			
	Allergic asthma	10	5(50%)			
	Non-atopic, normals	10	1(10%)			
	ABPA	60	58(97%)			Crameri et al. 1998
	Allergic asthma	40	21(53%)			
	Non-atopic, normals	20	0			
Total		237	147		16	
Asp f 4	ABPA	60	48(80%)			Crameri et al. 1998
	Allergic asthma + Ab	40	0			
	Non-atopic, normals	20	0			
	ABPA	24	22(92%)			Kurup et al. 2000
	Allergic asthma + Ab	16	0			
	Non-atopic, normals	10	0			
	ABPA	24	15(63%)			
	Allergic asthma + Ab	16	3(19%)			
	Non-atopic, normals	10	0			
	ABPA	12	10(83%)		10/12(83%)	Hemman et al. 1999
	Allergic asthma	12	0		0/12	
	Non-atopic, normals	5	0		0/5	
Total		249	98		10	
Asp f 6	ABPA	60	33			Crameri et al. 1998
	Allergic asthma + Ab	40	0			
	Non-atopic, normals	20	0			
	ABPA	24	14			Kurup et al. 2000
	Allergic asthma	16	0			
	Non-atopic, normals	10	0			
	ABPA	24	16			Kurup et al. 2000
	Allergic asthma + Ab	16	4			
	Non-atopic, normals	10	0			
	ABPA	12	5		5/12	Hemmann et al. 1999
	Allergic asthma	12	0		0/12	
	Non-atopic, normals	5	0		0/5	
Total		249	76		5	

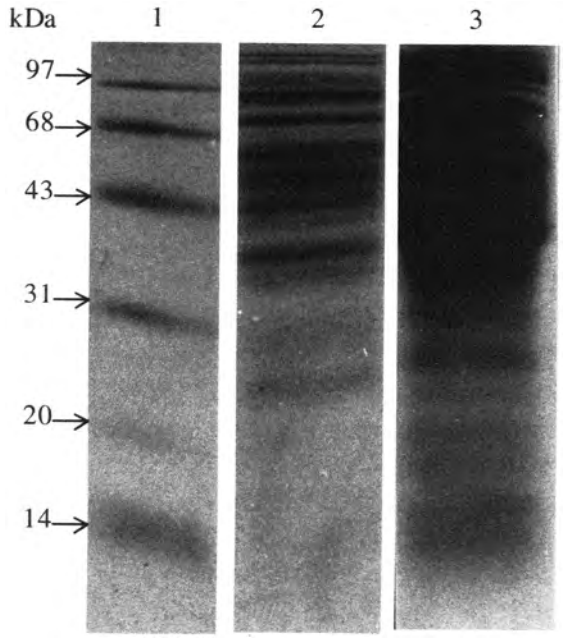

Figure 8 Overexpression of a novel PDI-family gene of *A. fumigatus* in *E. coli*.

Lane 1: Molecular weight markers; *Lane 2*: SDS-PAGE profile of supernatant of uninduced *E. coli* cells; *Lane 3*: SDS-PAGE profile of supernatant of induced *E.coli* cells overexpressing the PDI-family gene of *A. fumigatus* (40.2 kDa).

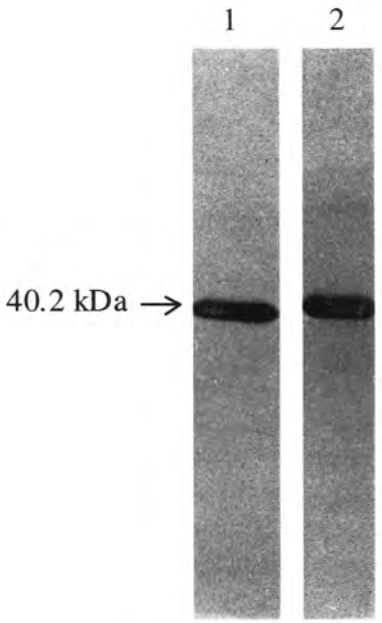

Figure 9 Western blots for specific IgG and IgE binding of purified recombinant protein of the PDI-family gene of *A. fumigatus*.

positive asthmatics, and normal controls from USA and Switzerland. The results demonstrate that Asp f 2, f 4, and f 6 can be used in the serodiagnosis of ABPA, while IgE antibody binding to Asp f 1 and f 3 was not specific (Kurup et al. 2000). On the other hand, Asp f 1 was found to be an important allergen with respect to immunoreactivity and skin testing with patients of allergic asthma sensitised to *A. fumigatus* (Disch et al. 1995)

Few of these *A. fumigatus* allergens also exhibited high sequence homologies with the known proteins and enzymes derived from other sources. Alkaline serine proteinases from *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus oryzae* (Asp f 13, Asp f 1 13, and Asp o 13 respectively) have been reported to have allergenic properties (Chow et al. 2000). Another group of homologous vacuolar serine proteinases, Asp f 18, with conserved sequence have been reported from *Aspergillus* and *Penicillium*. Another allergenic protein of *A. fumigatus*, Asp f 12 (65 kDa fusion protein) belongs to heat shock protein (Hsp 90) family. Recently, one of the cDNA's sequenced from *A. fumigatus* showed sequence homology with protein disulphide isomerase (PDI) gene family (Nigam et al. 2001, GenBank Acc. No. Nigam BF 112419). A protein from PDI family is being reported for the first time from *A. fumigatus* which may play an important role in protein folding and conformation. The gene has been cloned and expressed in *E. coli* (figure 8). Immunological characterisation of the expressed protein showed reactivity to specific IgG and IgE antibodies in the sera of ABPA patients (figure 9).

Epitopes of Allergens/Antigens

Epitopes are the sites on an allergen molecule that interact with the cells of the immune system or antibodies. The allergen usually interacts with the immunoglobulin by its three dimensional structure (B-cell epitope). After ingestion and processing of the allergen by antigen-presenting cell (APC), peptide fragments are formed. These peptide fragments (T-cell epitope) are presented to T cells by major histo-compatibility (MHC II) molecules on the antigen presenting cells. The knowledge of IgE-binding epitopes of the major *A. fumigatus* allergens may be of importance for increasing the specificity and sensitivity of diagnostic tests for designing

model allergens representing such epitopes for *in vitro* tests. Advances made in epitope mapping of major antigens of pathogenic microbes resulted in the rapid development of diagnostic technologies and products. Availability of the deduced amino acid sequences of a few major *A. fumigatus* allergens/antigens and partial sequencing of several other allergens/antigens have now provided opportunity to analyse regions both by algorithms and by experiment (by chemical/enzymatic cleavage of allergenic/antigenic proteins). Identified peptide fragments can be synthesised and evaluated for their potential in immunodiagnosis. Synthesis of overlapping peptides spanning the whole protein is one of the approaches for development of peptide based diagnostics for infectious diseases. Another approach is based on the recombinant expression of the epitopic sequences. Availability of information on gene sequence of Asp f 1 facilitated investigations on the immunological and biochemical functionalities of the allergen with respect to important domains. Evaluation of synthetic peptides from the N-terminal of Asp f1 suggested their diagnostic relevance by ELISA, dot-blot, lymphoproliferation, cytokine analysis and histamine release assays (Sarma et al. 1995) (figure 10). One of the promising peptides for immunodiagnosis was characterised with respect to structure-function relationship. Tryptophan residue plays an important role in the immunoreactivity of the peptide (figure 11). The N-terminal region of Asp f 1 comprising the immunodominant domain was expressed in *E. coli* and expressed recombinant protein was observed to react with specific IgE and IgG antibodies of patients of ABPA (Sarma et al. 1999). Various epitopes of Asp f 1 differentially induced Th1 and Th2 responses (Kurup et al. 1996). Of the five peptides studied for their cytokine profile, one showed a clear Th1, whereas another showed a Th2 response. Hence, for the first time an epitope of Asp f 1 was reported to induce protective Th1 response in mice. While the protective nature of *Aspergillus* allergens has not been observed so far, the Th2 responses due to mixture of allergens have been implicated in the pathogenesis of ABPA. Further, synthetic peptide epitopes of Asp f 1, based on their possible T-cell

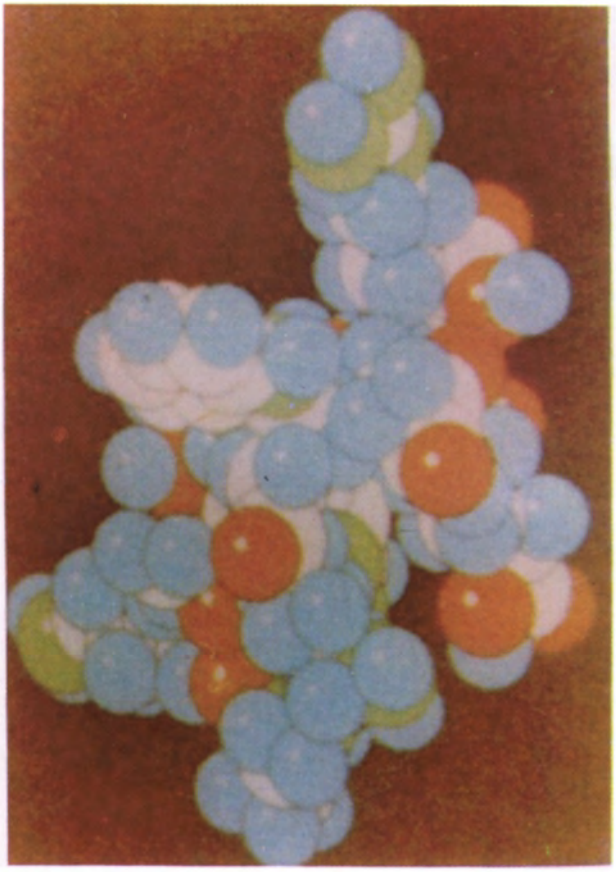

Figure 10 Three dimensional structure of a diagnostically relevant (eleven amino acid) epitope located in the N-terminal region of Asp f 1, an allergen/antigen of *A. fumigatus* (structure of the epitope predicted by a computer programme MODEL).

stimulatory properties were observed to stimulate the peripheral blood mononuclear cells from ABPA patients and normal controls (Kurup et al. 1998). Several peptide epitopes of Asp f 1 also demonstrated distinct IgE antibody binding with sera of ABPA patients. The carboxy-terminal region of Asp f 1, representing amino acid residues 115-149, as an epitope was involved in both humoral and cell mediated immune responses in ABPA was patients (Kurup et al. 1998).

Evaluation of synthetic decamer peptides of Asp f 2 on derivatized cellulose membranes showed that either the N- or C-terminal region of Asp f 2 is essential for its correct folding and conformation, needed for binding to specific IgE antibodies (Banerjee et al. 1999). A recent study showed protective nature of some of the epitopes of Asp f 2 against crude antigenic extract *in vivo* (Svirshchevskaya et al. 2000).

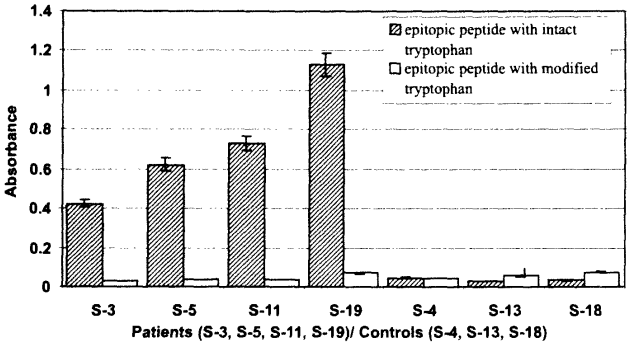

Figure 11 Absorbance values of the eleven amino acid synthetic epitope of Asp f 1 with intact and modified tryptophan binding the specific IgG antibodies in the sera of ABPA patients by ELISA.

Several epitopes of Asp f 13 were found to react with rabbit anti-Asp f 13 antiserum. Three of these immunodominant epitopes, near the C-terminal region of the protein, bound to IgE antibodies from *A. fumigatus*-sensitive patients (Chow et al. 2000).

Indigenous Efforts

The ELISA based test developed using well defined diagnostic antigens, is sensitive and specific and is being routinely used for the screening of patients from various parts of the country at Molecular Biochemistry and Diagnostics Division, Centre for Biochemical Technology, Delhi. Using indigenous diagnostic reagents, high levels of *Aspergillus* specific IgE and IgG antibodies were detected in paediatric cases of ABPA (Banerjee et al. 1992). Diagnostic relevance of *Asperglus* specific antibodies in multitransfused children with HIV infection was also observed (Bhatnagar et al. 1996). Currently diagnostic antigens are being prepared based on the culture technology which has several limitations. In the light of availability of information on epitopic regions of diagnostically relevant antigens of *A. fumigatus* , efforts are being made to replace culture technology with that of synthetic peptide based epitopes for immunodiagnosis. A PCR based detection test for *A. fumigatus* in clinical samples (sputum, bronchial aspirate and bronchoalveolar lavage) has been developed using Asp f 1 gene of *A. fumigatus* (Sarma et al. 1997).

Laboratory Diagnosis of Aspergillosis

Despite the recent progress achieved in understanding the pathophysiological mechanisms, problems relating to a reliable diagnosis of aspergillosis remain to be solved. This needs development of international standards to obtain universal diagnostic antigens of *A. fumigatus*. Cost-effective, simple and sensitive diagnostic kits for laboratory diagnosis can then be developed. The recombinant and synthetic peptide technology have paved way for such an approach towards preparation of standardised diagnostic reagents and international reference standards useful for immunodiagnosis.

The methods used currently are based on detection of the pathogen on i) direct microscopic examination or in histopathologic sections, ii) isolation and identification of the pathogen in culture, and iii) detection of an immunological response to the pathogen or some marker of its presence, such as a metabolic product by using various serodiagnostic methods. These serodiagnostic methods include immunodiffusion, counter-immuno-electrophoresis, Enzyme linked immunosorbent assay (ELISA), Biotin avidin linked immunosorbent assay (BALISA), immunogold assay, immunoblotting, Radio immuno assay (RIA), etc. Each approach has its advantages as well as limitations. In patients with severely compromised immunity, histological and cultural confirmation of tissue invasion is very difficult to obtain during the life of the patient (Cohen 1991). Moreover, fungal elements often demonstrate atypical morphology depending on a number of factors including steric orientation, age of fungal lesion, effects of antifungal therapy, type of infected tissue and host responses (Miyake et al. 1968, Schwarz 1982, Jensen & Schonheyder 1989). Further, positive cultures from sputum represent inconclusive evidence of disease in that, they may constitute aerial contamination of the mucoid secretions. The isolation of *Aspergillus* from BAL fluid is positive in only 50-57% of cases of aspergillosis (Albeda et al. 1984, Kahn et al. 1986, Levy et al. 1992). On the other hand, in severely immunocompromised patients due to the occurrence of rapid systemic disease, the occurrence of specific antibodies is very low or absent. Detection of circulating antigens in the serum and urine of patients with invasive aspergillosis is a more promising approach (Hopwood et al. 1991).

However, as antigenemia and antigenuria are transient in nature, detection of antigen may not always serve the purpose. With development of sensitive immunodiagnostic methods such as ELISA, some groups have made efforts to detect low levels of antibodies in serum samples of patients of invasive aspergillosis (Cohen et al. 1981, Lamy et al. 1991, a, b).

Skin Test: Skin test reactivity to *A. fumigatus* antigens is widely used to identify patients with suspected 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 developed within 4 to 8 hrs after skin test. Patients are regarded as being sensitive to a particular antigen preparation if the wheal diameter is greater than 9 mm^2.

Direct Microscopy and Culture: In standard mycology laboratories, the most reliable method for diagnosis of aspergillosis remains the recovery of the organism from an infected patient together with the demonstration of the organism in the tissue. The direct examination of biopsy material or other clinical specimens is helpful because the narrow angle branching hyphae of *Aspergillus* are easily observed using the potassium hydroxide (KOH) preparation. The hyphae of *Aspergillus* can be easily visualised using the periodic acid schiff (PAS) and haematoxylin & eosin (H & E) stains. As respiratory mucosal colonisation with *Aspergillus* is common, a positive respiratory tract culture for *Aspergillus* is difficult to assess. Repeated recovery of the organism from multiple specimens from the same source would be required for evaluation of the etiologic significance.

Immunodiagnosis of Aspergillosis: Presence of circulating antibodies against *A. fumigatus* in the sera of patients is a valuable criterion in the diagnosis of various forms of aspergillosis (Kiran et al. 1998). A variety of tests with varied sensitivities have been developed to detect circulating antibodies to *Aspergillus* antigens, such as, the Radio-allergosorbent test (RAST), Enzyme linked immunosorbent assay (ELISA), and Western blot. These tests show diverse sensitivities depending on the quality of antigen and the type of antibodies being investigated (Kurup & Kumar 1991).

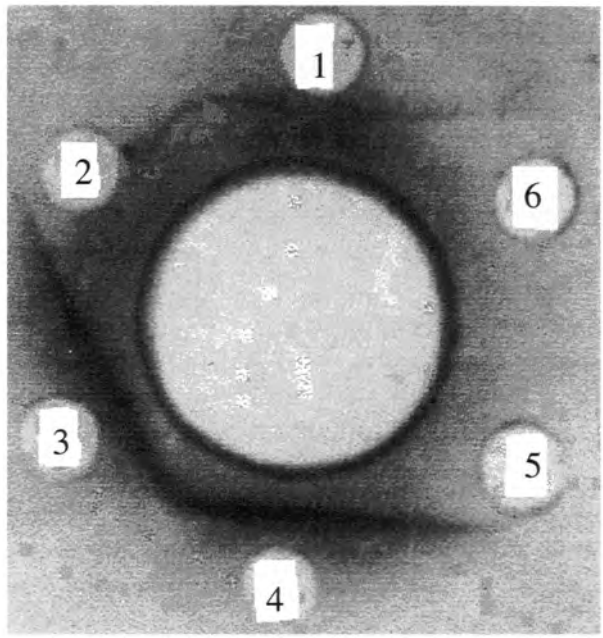

Figure 12 Immunodiffusion of culture filtrate antigens of *A. fumigatus* (central well) with sera of ABPA patients (wells 1, 2, 3 and 4) and healthy controls (wells 5 and 6).

Immunodiffusion Test: The diagnostic utility of the immunodiffusion test (ID), the most common technique used for the serodiagnosis of aspergillosis, has been reported by many investigators (Khan et al. 1976, Chaparas et al. 1980, Malo et al. 1981, Kurup et al. 1983). A precipitin reaction seen with the culture filtrate allergens / antigens of *A. fumigatus* and sera of ABPA patients is shown in figure 12. Although the ID test lacks sensitivity and conveys no quantitative information about antibody concentrations, a number of clinical laboratories still use this test because of its simplicity and ease of performance. This test does not require skilled personnel.

Immunogold Assay: An immunogold assay was developed by Gugnani et al. 1990, for detection of *A. fumigatus* specific IgG and IgE antibodies. Slide cultures of *A. fumigatus*, fixed in methanol, are treated with different dilutions of sera of patients, then with goat anti-human IgG or IgE antibodies and finally with rabbit anti-goat IgG conjugated with gold. This method is more sensitive and does not require any specialised equipment but is only semi-quantitative.

Immunoelectrophoresis: Several types of immunoelectrophoretic techniques have been used in diagnosing aspergillosis. Among these counter

immunoelectrophoresis (CIE) has been widely used, mainly because of the short time required to obtain the results. By using CIE, Longbottom detected precipitins in sera of 90% (29 out of 32) of ABPA patients whereas the immunodiffusion test was positive in only 64% of same cases (Longbottom 1983). A positive correlation was reported between number of precipitation lines and the titres of complement fixing antibodies (Gerber & Jones 1973, Wang et al. 1979, Sorenson & Shonheyder 1983). However, the nonprecipitating nature of antigen-antibody complexes proves disadvantageous in performing these techniques (Warren et al. 1975, Kauffman et al. 1980).

Assays for A. fumigatus Specific IgE and IgG Antibodies

A variety of immunoassays have been developed to achieve high degree of sensitivity and specificity of the diagnostic test. These include Radioimmunoassay (RIA), Enzyme linked immunosorbent assay (ELISA), Biotin avidin linked immunosorbent assay (BALISA), Luminiscence Immunoassay (LIA) and Pharmacia ImmunoCAP system.

Radioimmunoassay (RIA)

RIA was first developed to measure antibodies to radiolabelled acid-soluble antigenic fractions of *A. fumigatus* (Bardana 1974). The most commonly used RIA, however, is the radio allergosorbent test (RAST), used extensively for the detection of specific IgE antibodies. RAST has several disadvantages for use in a routine diagnostic laboratory such as radioactive hazard, disposal of wastes and need for expensive radioactive detectors.

Indirect ELISA

ELISA has been demonstrated to be a sensitive, reliable, and quantitative diagnostic tool for allergic disorders. Although the sensitivity of the conventional ELISA system depends on many variables, the nature and type of antigens used and their ability to bind to polystyrene plates are the most important decisive factors. Currently, a mixture of diagnostically relevant antigens of *A. fumigatus*, is being used in ELISA based detection for specific IgG and IgE antibodies (Banerjee et al. 1990a, Banerjee et al. 1990b, Sharma & Sarma 1997b).

Biotin avidin linked immunosorbent assay [BALISA]

BALISA, a more sensitive version of ELISA is based on biotin-streptavidin system (biotin linked secondary antibody probed with streptavidin-conjugated enzyme), and is useful to detect relatively low levels of antibodies in some of the ABPA patients (Brummund et al. 1987).

Luminiscent Immunoassay (LIA)

Luminiscent immunoassay, a modified and sensitive ELISA for detection of antibodies, is based on the substrate luminol for the peroxidase conjugated secondary antibody, which produces luminiscence through emission of photons, measured in specialised microtiter plates in a luminiscence analyser (Sharma & Sarma 1993, Sharma et al. 1997a).

Automated specific IgE assay [Pharmacia immunoCAP system]

The Pharmacia company developed an automated system called immunoCAP system for detection of circulating IgE antibodies (Crameri et al. 1996). This immunofluoro assay was 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_A/l]. The antigens are immobilised on cap like structures made of spongy matrix which capture the specific IgE antibodies from the patient sera.

Antigen Detection in Patients of Invasive Aspergillosis

Aspergillus galactomannan can be detected by monoclonal antibody based ELISA in serum, urine and bronchoalveolar lavage (BAL) samples from patients of invasive aspergillosis. Higher sensitivity of the test has been observed for serum samples. Disappearance of the antigen during antifungal therapy seems to correlate with good clinical outcome. A commercial enzyme-linked immunosorbent assay (ELISA, Platelia *Aspergillus*) is now available to detect *Aspergillus* antigen in serum, urine and bronchoalveolar lavage (BAL). Salonen et al. 2000, showed utility of this test, by screening various clinical samples obtained from 105 haematological patients on empirical amphotericin B treatment.

Immunoblotting

Reactivity of the particular protein to the antibody in the sera of patients of aspergillosis can be visualized by this assay which was shown to be specific and sensitive. Immunoblotting involves (i) separation of antigens in polyacrylamide gel, and (i) transfer of separated proteins onto nitrocellulose membranes and iii) detection of specific antigen-antibody binding. 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 (Banerjee et al. 1995, Teshima et al. 1993).

Lymphocyte Transformation

The correlation between proliferative response of lymphocytes from patients of ABPA with purified antigens and the disease activity in these patients is well known (Murali et al. 1994, Walker et al. 1989).

The immunologic evaluation of recently purified and characterised native and recombinant allergens of *A. fumigatus*, using various diagnostic methods mentioned above, is shown in table 1 and table 3b respectively.

Polymerase Chain Reaction

Molecular techniques, based on the detection of pathogen, have been applied to the diagnosis of invasive aspergillosis. One difficult step in application of molecular techniques for detection of *Aspergillus fumigatus* is isolation of DNA which requires disruption of tough chitin cell wall without causing damage to the DNA. Bir et al. 1995, had reported a method for isolation of genomic DNA of *Aspergillus fumigatus* using chitinase enzyme and microwave treatment. Recent advances in diagnosis using molecular techniques include the development of PCRs targeting either panfungal or *Aspergillus*-specific sequences. When a sensitive PCR is used, invasive aspergillosis in bone marrow transplant patients can be detected in serum samples, several weeks before antigen tests become positive, and a positive PCR often pre-dates the initiation of antifungal therapy (Williamson et al. 2000). PCR amplification of various genes of *A. fumigatus* namely, 18S rRNA, 26S region of rDNA complex and Asp f 1, have been standardised to develop specific diagnostic tests for the pathogen in the clinical samples from patients of allergic and invasive aspergillosis [(serum samples) Golbang et al. 1999, (serum samples) Yamakami et al. 1996, (bronchoalveolar lavage) Melchers et al. 1994, (bronchial samples) Spreadbury et al. 1993, (blood and urine samples) Reddy et al. 1993). Based on Asp f 1 gene, a colorimetric PCR for detection of the pathogen in clinical samples (sputum, bronchial aspirate and bronchoalveolar lavage) was also developed and the results were compared with various diagnostic methods such as microscopy, culture, immunodiffusion and ELISA (Sarma et al. 1997) (table 4, figure 13).

Advances made during past decade on understanding the biology, molecular nature of *Aspergillus* allergens and antigens and their diagnostic relevance, has improved our ability to diagnose allergic bronchopulmonary aspergillosis and *Aspergillus* induced infections over the years. However, continued efforts are needed to improve the quality control aspects of the present tests and their accessibility to various clinical and pathological laboratories at an affordable cost.

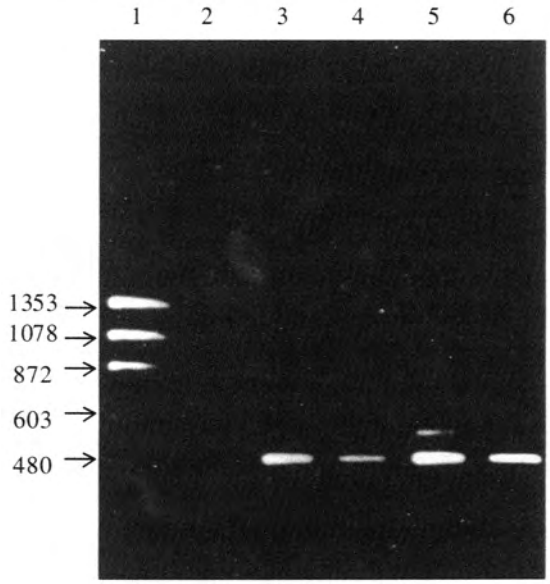

Figure 13 Analysis of PCR amplified products using primers of Asp f 1, an allergen antigen of *A. fumigatus*, in clinical samples on agarose gel.

Lane 1 : ϕ 174/ Hae III digest as base-pair marker; Lane 2 : PCR using a sputum sample negative for culture and microscopy; Lane 3 & 5: PCR product amplified from processed sputa of ABPA patients; Lane 4 & 6 : PCR product amplified from processed sputum and bronchial aspirate of aspergilloma patients.

Table 4 *Results of different diagnostic methods to identify A. fumigatus infection.*

S. No.	Clinical Microscopy Sample	Culture	ELISA abs at 490 nm for Specific IgG and IgE antibodies (average of duplicates)	Immuno diffusion test on agarose	Detection of PCR product	Immuno PCR abs. at 405nm (average of duplicates)
ABPA						
1.	Sputum +ve	+ve	1.530(+) 0.604(+)	+ve	+ve	1.410
2.	Sputum +ve	+ve	1.864(+) 0.539(+)	+ve	+ve	1.420
3.	Sputum +ve	+ve	1.339(+) 0.620(+)	+ve	+ve	1.432
4.	Sputum +ve	+ve	1.353(+) 0.539(+)	NA	+ve	1.336
5.	Sputum -ve	-ve	0.996(+) 0.535(+)	+ve	+ve	1.439
6.	B.A. -ve	+ve	0.637(+) 0.630(+)	+ve	+ve	1.502
7.	Sputum +ve	+ve	1.928(+) 0.499(+)	+ve	+ve	1.509
8.	B.A. -ve	+ve	1.895(+) 0.617(+)	+ve	+ve	0.909
9.	Sputum +ve	+ve	1.038(+) 0.536(+)	+ve	+ve	0.932
Aspergilloma						
10.	B.A. +ve	+ve	0.922(+) 0.464(+)	NA	+ve	1.309
11.	Sputum +ve	+ve	1.607(+) 0.406(+)	+ve	+ve	1.535
12.	Sputum +ve	+ve	0.928(+) 0.450(+)	NA	+ve	1.504
13.	Sputum +ve	+ve	1.445(+) 0.399(+)	+ve	+ve	1.525
14.	Sputum +ve	+ve	0.985(+) 0.435(+)	+ve	+ve	0.869
15.	Sputum -ve	-ve	1.513(+) 0.419(+)	+ve	+ve	0.888
Immunocompromised patients						
16.	BAL -ve	+ve	0.252(-) 0.110(-)	-ve	+ve	1.232
17.	BAL -ve	-ve	0.111(-) 0.080(-)	NA	+ve	0.978
18.	BAL -ve	+ve	0.222(-) 0.151(-)	NA	+ve	1.839
19.	BAL -ve	-ve	0.188(-) 0.103(-)	NA	+ve	0.910
Other allergic disorders						
20.	Sputum -ve	-ve	0.232(-) 0.090(-)	NA	-ve	0.315
21.	Sputum -ve	-ve	0.202(-) 0.050(-)	-ve	-ve	0.213
Normal Controls						
1.	Sputum -ve	-ve	0.090(-) 0.035(-)	-ve	-ve	0.242
2.	Sputum -ve	-ve	0.151(-) 0.056(-)	-ve	-ve	0.251
3.	Sputum -ve	-ve	0.211(-) 0.031(-)	-ve	-ve	0.212
4.	Sputum -ve	-ve	0.099(-) 0.042(-)	-ve	-ve	0.222
5.	Sputum -ve	-ve	0.165(-) 0.039(-)	-ve	-ve	0.205
6.	Sputum -ve	-ve	0.142(-) 0.091(-)	-ve	-ve	0.255
7.	Sputum -ve	-ve	0.210(-) 0.073(-)	NA	-ve	0.210
8.	Sputum -ve	-ve	0.102(-) 0.081(-)	NA	-ve	0.203
9.	Sputum -ve	-ve	0.198(-) 0.065(-)	NA	-ve	0.238
10.	Sputum -ve	-ve	0.221(-) 0.092(-)	NA	-ve	0.258

Cut off Absorbance value : IgG<0.350; IgE<0.200; NA : not applicable

New Therapeutic Strategies for Fungal Diseases

In view of high mortality rates observed in the patients of invasive aspergillosis and the side effects observed due to corticosteroid therapy in allergic disorders, there is a need to examine new therapeutic strategies for fungal diseases. Adequate information on the mechanisms of host-pathogen interactions and the pathogenesis of aspergillosis would facilitate design and development of safer and effective therapeutic agents.

Vaccination approaches in *Aspergillus* induced infections have been limited due to lack of suitable agents providing protection. Administration of antigenic preparations in animals has resulted in development of disease rather than protection (Slavin et al. 1978, Murali et al. 1992, Kurup et al. 1994). There are reports indicating existence of protective acquired immunity against *A. fumigatus* in animals (Smith 1972, Lehmann & White 1976, Richard et al. 1982). Acquired immunity could be transferred to naïve animals through sensitised macrophages and lymphocytes of challenged mice [de Repentginy et al. 1993). Th1-mediated resistance observed upon vaccination of mice with *Aspergillus* crude culture filtrate antigens, suggested the existence of fungal antigens useful as a candidate vaccine against invasive pulmonary aspergillosis [Cenci et al. 2000).

Cytokine therapy, yet in experimental stage for aspergillosis, seems promising. Phagocytosis of conidia from 15 to 35% was observed to be enhanced when neutrophil monolayers were exposed to IL-8 (Richardson & Patel 1995). The oxidative response and hyphal damage caused by normal and cortisone treated neutrophils was enhanced by granulocyte colony stimulating factor (G-CSF) and gamma-interferon (γ-IFN) (Roilides et al. 1993a,d). Neutrophils and macrophages of HIV patients were less efficient in killing the *Aspergillus* and their killing capacity was also enhanced in presence of G-CSF and γ-IFN (Roilides et al. 1993 b,c). Elevated pulmonary eosinophilia could be abrogated by administration of IL-5 in a murine model of allergic aspergillosis, indicating probable use of cytokines for therapy (Murali et al. 1993). Treatment of a chronic granulomatous disease (CGD) patient (with multiple brain abcesses due to *A. fumigatus*) with IFN-γ resulted in survival of the patient (Touza et al. 2000).

An initiative on genome sequencing of *A. fumigatus* has been made by a group of scientists along with the Wellcome Trust-funded Sanger Centre (U.K.). Full genome sequencing of *A. fumigatus* would provide the information of genes coding various proteins involved in development, metabolism and virulence of the pathogenic fungus. Further, it is anticipated to lead to identification of specific motifs for *in silico* designing and modelling to synthesise newer drug molecules for specific targetting. This approach undoubtedly will result in a wide variety of new drug molecules with higher specificity and efficacy (Sarma et al. 2000).

Genetic Predisposition to Allergic Bronchopulmonary Aspergillosis : Predictive Diagnosis?

Aspergillus fumigatus is ubiquitous and universal in distribution. However, ABPA occurs in atopic individuals with asthma, allergic sinusitis, rhinitis, extrinsic allergic alveolitis and in patients of cystic fibrosis. Genotype analysis of the T-cell clones (specific for the Asp f 1 antigen), isolated from ABPA patients, showed that most of them are restricted by HLA-DR alleles (90% of the ABPA patients showed either HLA-DR2, HLA-DR5 or both the alleles) (Knutsen et al. 1994, Chauhan et al. 1996, 1997, 2000). HLA-DR alleles DR2, DR5, and possibly DR4 or DR7 contribute to susceptibility of the subjects to ABPA while individuals with HLA-DQ2 allele are resistant to develop ABPA. Further, a combination of these genetic elements may determine the outcome of ABPA in the patients of cystic fibrosis and asthma.

Mutations in cystic fibrosis transmembrane protein (CFTR) encoding gene of cystic fiborsis patients lead to defectiye synthesis and regulation of cystic fibrosis transmembrane protein. This protein is directly involved in the transportation of chloride ions. Such a defect in chloride transportation results in thick mucus secretion in these patients facilitating colonisation of pulmonary tract by other microbes. Presence of mutations in CFTR gene in ABPA patients suggest that there is some association of ABPA with cystic fibrosis (Miller et al. 1996). In a recent study, the frequency of CFTR mutation carriers was observed to be significantly higher in ABPA patients (6 of 21 patients; 28.5%) than in control asthmatic subjects (2 of 43 subjects; 4.6%; $p = 0.01$)(Marchand et al. 2001). Occurrence of

Aspergillus induced infections in tuberculosis patients necessitates investigation for identification of common host genetic factors. A series of studies have established that lung surfactant proteins A (SP-A) and D (SP-D) have an important role in strengthening the host defense against both allergic and invasive aspergillosis (Madan et al. 1997c, d, 2001a, 2001b). SP-A and SP-D genes are being explored as candidate markers for genetic predisposition to aspergillosis and other respiratory diseases. In view of these developments predictive diagnostics based on molecular probes for genetic predisposition to respiratory diseases are possible in near future.

Epilogue

Need for diagnosis of *Aspergillus* induced allergic and infectious forms of disease is being emphasized in view of their increasing incidence. Identification of diagnostic antigens of *A. fumigatus* has marked the beginning of immunodiagnostic tests for aspergillosis. DNA technology facilitated homogeneous preparation of recombinant allergens and provided sequence information. This along with identification of diagnostically relevant synthetic peptide epitopes has provided scope for preparation of International reference standards. Availability of gene information has paved the way for development of molecular probes for such fungal diseases.

This article covered some of these recent developments towards the above mentioned aspects. An insight into the innate immune mechanisms and associaton of genetic factors have brought scope for identification of relevant genes useful for predictive diagnosis of fungal diseases, to be effectively used for health care in various clinical and pathological laboratories. In this scenario, novel therapeutic approaches for allergic and respiratory infectious diseases are also possible today.

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