CHALLENGES IN PREVENTION, DIAGNOSIS AND THERAPY OF EMERGING FUNGAL DISEASES. ASPERGILLOSIS : A CASE STUDY

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ABSTRACT

Diseases caused by pathogenic filamentous fungi, are an emerging threat to public health in the wake of increasing incidence of HIV and tuberculosis. At this point, discovery and development of fungal therapeutics and diagnostics are serious challenges for biomedical researchers. Recent technological advances in genomics and proteomics offer great scope for development of preventive and therapeutic measures for fungal diseases. *Aspergillus*, one of the medically important filamentous pathogenic fungi causes a wide spectrum of clinical disorders ranging from allergic aspergillosis to systemic invasive aspergillosis. Increase in incidence of drug resistance and the cytotoxic effects are two serious limitations of the antifungal drugs presently in use. This is primarily due to lack of understanding of biological mechanisms operative in these fungi. Today, it is possible to understand the biological mechanisms of the fungus for its colonisation, survival and invasion of the host. Future developments based on such leads can result in development of precise and specific diagnostic, therapeutic and preventive measures for a wide clinical spectrum of fungal diseases.

KEY WORDS : Fungal diseases, aspergillosis, pathogenesis, genomics, proteomics, host's susceptibility.

INTRODUCTION

Fungal diseases have emerged as the leading group of infectious diseases affecting animals, plants and humans. The estimated cost of combating fungal diseases is 30 billion US\$ annually on a world wide basis. Three major forms of fungal diseases are often encountered in humans. 1. infections with fungal agents are the most important and largest form, 2. allergies due to fungal agents constitute a second form, and 3. mycotoxicosis due to ingestion of toxic metabolites comes under the third form. These forms of the fungal diseases have been recognised for centuries. However, they have been posing greater problems during the last decade. Emergence of AIDS, and multidrug resistant tuberculosis, increasing usage of immunosuppressive and cytotoxic drugs have

contributed to a great extent to the proliferation of opportunistic fungal infections (1).

Aspergillus fumigatus along with other less frequently reported *Aspergillus* species such as *A. flavus* and *A. niger*, cause a large spectrum of diseases ranging from allergic disorders in immunocompetent individuals to invasive form in immunosuppressed hosts. Majority of the fungal allergies are due to *Aspergillus* species. Among the various medically important fungi, filamentous pathogenic fungi such as *Aspergilli* 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 prevention of the disease and development of antifungal drugs and molecules.

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BIOLOGY OF *A. FUMIGATUS*

Understanding the molecular mechanism of colonisation of the pathogen in the host helps in

overcoming the limitations of current therapeutic approaches and could lead to the design and development of precise and efficient antifungal drug molecules. The mechanism of pathogenesis is based on the interplay of a large number of the proteins of human host and the *Aspergillus*. Hence, it is important to know salient pathogen and host factors relevant for the disease process to develop specific strategies for prevention, diagnosis and therapy of allergic and invasive forms of fungal diseases.

Aspergillus secretes a plethora of somatic and metabolic proteins which are complex and biologically and immunologically active (Table 1) (2, 3). Some of them have relevance in immunodiagnosis and have been proposed as potential virulent factors.

Various biological activities possessed by *Aspergillus* proteins include elastase activity, protease

activity, ribonuclease activity, cytotoxicity and immunosuppressant activity. Allergenic/ antigenic proteins secreted by *Aspergillus*, cause type I and type III hypersensitivity reactions in animals and human beings. The wide clinical spectrum of the diseases caused by *Aspergillus fumigatus* and other species can be attributed to their complex, multifunctional allergens and antigens. This necessitates understanding on the molecular and biological operations of the fungus for its survival over a broad range of conditions such as pH, temperature, salt concentrations etc.

The pathogenic factors of *Aspergillus* are highly complex macromolecules and are difficult to purify. Use of recombinant DNA approach and synthetic peptide approach has resulted in dissection of various functional domains of these macromolecules and understanding of structure-function relationships.

Table 1. Pathogen factors secreted by *Aspergillus fumigatus* and their biological activities

Source of the pathogenic factor	Characterisation	Clinical importance
Pathogenic strain of Cytosolic proteins	CS2 composed of 81, 53, and 41 kD on SDS-PAGE	reacted with specific IgG 75% of aspergilloma patients
Soil strain no. 2605, & strain isolated from sputum of ABPA patient Three week culture filtrate	70, 55, 34, 28, 18 and 14 kD.	Reacted with specific IgG and IgE antibodies in ABPA patients.
Strain no. NCPF 2140, CBVS 143.89, Mycelium, Water soluble from ethanol ppt filtrate	18, 28, 35, 38, 45, 94, 110, 125 kDa.	35 kD reacted with specific IgG of 100% of aspergilloma patients 18 kD reacted with specific IgG of 80% of aspergilloma patients
Strain no. F 92 (isolated from sputum of an aspergilloma), Mycelia only 58 kD band MoAb	58 kDa	100% of the invasive aspergillosis patients showed IgG to 58 kD 90% showed both 58 and 45 kD
Strain no. NCPF 2109, Mycelium	88 kDa, <u>heat shock protein</u> , reacts with MAb against hsp of <i>C. albicans</i> .	reacted to specific IgG of 50 patients with proven IA, 35 ABPA patients, 28 patients with suspected IA, & 10 patients with aspergilloma.
Strain no. 5167, Mycelium	18 kDa, Asp fl, allergen, <u>homology to cytotoxins</u> .	85% of the ABPA patients showed IgE antibodies to 18 kD (by RAST)

Table 1 contd.

Strain no. 5167, Mycelium	18 kDa, Asp fl.	11 of the 13 ABPA patients showed IgE antibodies to 18 kD
Strain from Greer Labs Mycelia	16, 18, and 45 kD	12/12 ABPA patients showed IgG and IgE Ab to all 3 Ag murine MoAb raised against Asp fl recognised both Asp fl and 16 kD.
Strain no. AF-102, ATCC 42202, Mycelium	65 kDa, <u>heat shock protein</u>	reacted with IgE and IgG
Strain no. CVS 144.89, Water soluble ethanol ppt filtrate	88 kD, glycoprotein.	88 kD antigen showed binding in all 72 sera of aspergilloma patients.
Strain no. CBS-144.89, Culture filtrate (48 h).	40 kD, Metalloprotease.	Specific IgG antibodies against 40 kD were observed in all 10 sera of aspergilloma patients.
Strain isolated from patient of PA, Culture filtrate	32 kD glycoprotein, 67 and 94 kD glycoproteins	Sera of all patients with PA (n=10) and ABPA (n=10) showed strong binding with glycoprotein antigens.
Strain no. DF 41, Culture filtrate	38 kD, extracellular <u>aspartic proteinase</u>	Sera of 1 PA and 1 aspergillus pneumonia reacted on dot blot with proteinase.
Strain no. CBS-143.89, patients Mycelium	galactomannan.	Sera of only 26% of PA showed positive response
Strain ATCC 9197 fraction	p90, p60, p40, p37, N-glycosylated	Sera of 76% of the Cytosolic aspergilloma patients demonstrated specific binding with glycoprotein antigens.

Several of these factors have been individually cloned and expressed in prokaryotic and eukaryotic vectors (2). One of the major allergens and proposed virulent factor, Asp fl, has been studied in greater detail with respect to its structure and function. Asp fl, a conA non-binding major cytotoxic allergen/antigen, was observed to be cytotoxic to mammalian cell lines and its cytotoxicity was dependent on protein synthesis inhibition and ribonuclease activity (4, 5, 6). Based on Asp fl gene, a colorimetric PCR for detection of the pathogen in clinical samples was developed (7).

A glycoprotein allergen/antigen with a molecular weight of approximately 45 kD, showed elastase activity and was the predominant antigen observed

in the circulating immune complexes isolated from ABPA patients (8, 9). Various kinds of proteases have been isolated and characterised from both *Aspergillus fumigatus* and *Aspergillus flavus* and have been proposed to contribute to the tissue damage observed in patients of aspergillosis (10, 11, 12, 13).

Aspergillus strains lacking one or more of these proposed virulent factors (knockouts for Asp fl and alkaline protease) showed pathogenicity similar to that of intact strains suggesting the presence of some more factors, not identified yet, may facilitate the organism to survive and multiply (10, 11, 12). These observations indicate that possibly virulence of filamentous fungi is contributed by a set of factors

unlike the lower pathogenic microbes such as bacteria and viruses. Hence, a comprehensive study on the genome organisation and functions of various genes of the pathogenic filamentous fungi is necessary for applications.

A comparative analysis of genome sequence of nonpathogenic filamentous fungi such as *Aspergillus nidulans* and *Neurospora crassa* with pathogenic filamentous fungus such as *Aspergillus fumigatus* would probably indicate the group of genes accounting for its pathogenicity. Genome sequencing would also aid in selection of new antifungal drugs by comparison of human and fungal genomes. Efficacy of an antifungal drug is basically dependent on a few physiological differences between the fungal and the host cell. *A. fumigatus* and many other fungi, contain ergosterol in its plasma membrane whereas, plasma membrane of human cells have cholesterol. Most of the drugs available for treatment of systemic aspergillosis either affect the synthesis of ergosterol or bind irreversibly to it. Allergic forms of aspergillosis are treated with steroids (a symptomatic treatment) and there are reports stating that steroid treatment makes the host more prone to the invasive form of the disease (14). Needless to say that the number of antifungal drugs available today in the market is limited. This necessitates application of functional genomics and rational drug designing for development of efficient and safe antifungal drugs.

HOMOLOGIES, MOLECULAR MIMICRY AND AUTOIMMUNITY

Structural and sequential similarity of some of the *A. fumigatus* proteins with human proteins has been proposed to contribute to the autoimmune nature of allergic bronchopulmonary aspergillosis (15). Such homologous proteins could also be identified by comparative analysis and may be useful for better understanding of this important aspect of pathogenesis.

ASSOCIATION OF ASPERGILLUS INFECTIONS WITH TUBERCULOSIS

There are a number of reports indicating co-existence of aspergillosis and tuberculosis and tuberculous cavities being occupied by *Aspergillus* species in the aspergilloma patients, but how such coexistence affects the host resistance/

susceptibility to any of the diseases, is not known (16). So far, homologies of both pulmonary pathogens at protein level have not yet been investigated. The genomic sequencing of *A. fumigatus* may provide an opportunity to unravel the nature of coexistence of *A. fumigatus* and mycobacterium.

The sequencing of the microbial genomes is anticipated to answer some of these intricate problems and better understanding on the mechanism of host-pathogen interactions for a wide variety of infectious diseases.

GENOME SEQUENCING

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. Hence, an initiative on genome sequencing of *A. fumigatus* has already been taken by a group of scientists along with the Wellcome Trust-funded Sanger Centre (U.K.). The project has been funded by the Wellcome Trust's Beowulf Genomics Initiative to investigate the biology of this deadly fungus.

GENOME SEQUENCING STRATEGIES

Fungal genomes are small and well within the reach of current DNA sequencing technologies. Filamentous fungal genomes range in size from two to five times that of yeast and 30% of the genes do not match with any current data base even that of yeast. Work on filamentous fungal genomics was initiated at International level with two filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, whose classical genetics with a detailed genetic map have been worked out and physical maps for these are also being completed.

The first step to genome sequencing of any organism is through cataloguing functionally important genes and characterising their biological functions and one of the approaches for such study is the establishment of genomic structure through cDNA sequencing. ESTs or 'expressed sequence tags' are DNA sequences read from both ends of expressed gene fragments. These ESTs are widely used to discover novel members of gene families, to map genes to chromosomes as 'sequence-tagged

sites' (STSs), and to identify virulent factors and their possible interacting targets in the human host. A feasible approach may be determination of 300-600 bp of nucleotide sequence from random cDNA clones of *Aspergillus fumigatus*. The EST's thus generated would constitute a broad range of transcripts distributed among cytoplasmic structural and regulatory proteins, enzymes, membrane, nuclear and secretory proteins, and proteins with other functions.

Nucleotides present in open reading frames from ESTs that would align with known sequences would also be useful to determine codon usage for *A. fumigatus*. This straight-forward EST-based approach to gene discovery will expedite the identification and isolation of *A. fumigatus* genes encoding potential metabolic and antigenic proteins. Also, it would facilitate the tagging of most *Aspergillus fumigatus* genes at a fraction of the cost of complete genomic sequencing, and would be providing valuable data for understanding the mechanisms of pathogenesis for development of precise and specific therapeutic measures. The generation of an EST database of filamentous pathogenic fungi and industrially important *Aspergillus* is now an important and achievable goal and various groups are making consistent efforts to link these new genes with their putative functions thus making the data meaningful for useful medical and industrial applications (Table 2).

Table 2. EST information on other fungi (from EST databases at GenBank)

Name of the fungi	No. of EST's submitted
<i>Aspergillus niger</i>	335
<i>Neurospora Crassa</i>	10,229
<i>Saccharomyces cerevisiae</i>	3,041

To sequence whole genome of an organism, a combined approach is used which involves the systematic sequence analysis of selected large insert clones (cosmids and BACs) as well as random small-insert clones from a whole-genome shotgun library. However, the technological advancement in the genome sequencing has led to the development of faster and effective methods of DNA sequencing and analysis. One such advancement is the high

throughput DNA sequencing combined with high throughput PCR.

High throughput DNA sequencing, has made full genome sequencing a cost effective approach. It may involve use of either the conventional radiation hybrid mapping or chromosomal libraries or the novel shearing DNA approach (shotgun) and can result in progress from gene to genome oriented science, which explains the functions encoded by the pathogenic fungi and their capability. Complete genomic DNA sequences for several viruses, three bacteria and one yeast are already available today. Information on several other bacteria, worms, the fly, malarial protozoans, the mouse, a mustard plant and homo sapiens is now available.

APPLICATIONS OF GENOME INFORMATION

1. DNA chip based diagnostics

This is a highly promising and powerful diagnostic technology, which can provide identification of a large number of disease genes within a very short spell and at a reasonable unit cost. A number of reports have already appeared on detection of mutations using very high density DNA chips containing the complete coding sequence for the cystic fibrosis gene, the human mitochondrial gene sequence, a segment of BRCA 1, the p53 gene and few others (17). The availability of information on human genome and important microbial genomes and DNA chip based diagnostic technologies has made it feasible to identify susceptibility of individuals for a given range of genetic disorders and diagnosis of more than 100 infectious diseases, for which precise and effective treatment can be given.

2. Proteomics of pathogenic filamentous fungus

Information of genomic sequence would make more sense when functions of these genes would be unravelled. One of the common approaches of mapping function to genes is by characterisation of their proteome (18). Proteomics of *Aspergillus fumigatus* would provide separation, visualisation and examination of the proteins that are produced and are differentially expressed. Two dimensional gel

electrophoresis is one of the ways to study the proteins that are displayed. A second approach to study the proteins is based on digestion with an endoproteinase which results in a mixture of hundreds to thousands of different peptides. After separation of such peptides on reversed phase columns, the peptides are fed to an on-line tandem mass spectrophotometer where the peptides are automatically fragmented. Finally, the sequence of the fragments are matched against databases to identify the proteins in a particular sample type. This approach facilitates detailed sample characterisation. The proteomic approach towards the pathogenic organism provides us with information about the entire range of expressed proteins by themselves as well as in concert with the host.

3. Bioinformatics : rational drug design

A complete genome information 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.

4. Preventive medicine

There is very little information on genetic susceptibility of infectious diseases. However, recent efforts have resulted in identification of some polymorphisms in the host genes responsible for variability in their response to various infectious pathogens. Host's resistance in malaria caused by *Plasmodium falciparum* has been associated with sickle cell mutations in its hemoglobin gene. Susceptibility to tuberculosis has been reported to have an association with the polymorphisms of specific HLA alleles and macrophage associated receptor gene polymorphisms (Nrampl) (19, 20).

It is interesting to notice that whole human population is exposed alike to the ubiquitously prevalent *Aspergillus fumigatus* conidia in the air but susceptibility of atopic individuals raises questions regarding the involvement of genetic factors in this group of patients. Allergic bronchopulmonary

aspergillosis, an allergic disorder caused by *A. fumigatus*, has shown prominent associations with HLA polymorphisms especially with HLA-DR5 (21). Frequent occurrence of ABPA has been reported in patients of cystic fibrosis (15-25%) (22). In one of the studies, ABPA patients were screened for genetic mutations in the CFTR gene, the locus directly linked with cystic fibrosis, a monogenic disorder. Five out of eleven ABPA patients showed presence of mutations in CFTR gene suggesting that there is some association of ABPA with cystic fibrosis (23). Simultaneous occurrence of aspergillosis and tuberculosis has also been reported by some groups (15). CFTR mutations in cystic fibrosis patients lead to defective 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. The pulmonary picture of ABPA and tuberculosis is similar and often poses diagnostic dilemmas. The association of ABPA with diseases such as cystic fibrosis and tuberculosis suggests that there are some specific pulmonary genetic factors responsible for host's susceptibility/ resistance to these diseases.

Several studies have shown that respiratory diseases are associated with disturbances in the composition of pulmonary surfactant leading to gradual collapse of the respiratory system. Administration of natural bovine surfactant and/or by synthetic sources restores the normal lung function in such diseased conditions. Pulmonary surfactant, secreted by respiratory epithelial cells and type II clara cells, is a unique system present in the respiratory tract meant for maintenance of surface tension during inspiration and expiration. Surfactant comprises of two components : phospholipids and surfactant proteins. Out of four surfactant proteins (namely A, B, C, and D) SP-A and SP-D are C-type collagenous lectins and are involved in host's innate immune defense and inflammatory processes of the lung, the two major components of pulmonary disease. SP-A, also, plays a role in the surface tension lowering abilities of pulmonary surfactant, which is essential for normal lung function.

A series of studies examining the role of SP-A and SP-D in host defense against an important respiratory fungal pathogen, *Aspergillus fumigatus* were carried out. Interaction studies of SP-A and SP-D with conidia/ antigens/ allergens of *A. fumigatus*, suggested that SP-A and SP-D enhance the killing of *aspergillus* conidia by increasing the phagocytosis and oxidative burst of macrophages and neutrophils (24). Further, they blocked the specific IgE binding of the *aspergillus* allergens and prevented the histamine release by sensitised mast cells (25). Administration of SP-A and SP-D to the murine models of allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis was effective in alleviating the disease symptoms such as pulmonary and peripheral eosinophilia, specific IgE & IgG antibodies and colony forming units leading to reduced mortality [unpublished data]. The studies established that SP-A and SP-D have an important role in strengthening the host defense against both allergic and invasive forms of the diseases induced by *A. fumigatus*.

In view of a multifunctional role in pulmonary system, the SP-A and SP-D genes are proposed as potential candidates for association with host's susceptibility/ resistance to respiratory infections such as aspergillosis. Efforts are being made to investigate the occurrence of polymorphisms of SP-A and SP-D and their association with prevalence of aspergillosis and tuberculosis. Such association

studies would facilitate development of novel diagnostic markers for predisposition to aspergillosis for the first time leading to development of strategies for preventive medicine and in designing of specific therapeutic approaches.

SCOPE

Fungal diseases are emerging as a public health problem, the most important being invasive and allergic forms. Colonisation and the host-pathogen interactions play a major role in the establishment of the disease.

The need of the day and worthy of pursuit are the following challenging tasks :

- i) Knowledge on genome and proteome of pathogenic filamentous fungi
- ii) Comparative genomic analysis with other microbes and human beings
- iii) Identification of the colonisation factors of the fungal pathogen
- iv) Identification of genetic markers for susceptibility to fungal diseases (Association of polymorphisms in lung surfactants and HLA genes with the disease)
- v) Rational drug designing and development of effective and precise antifungal therapeutics.
- vi) Molecular diagnostics for fungal diseases.

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